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SYNTHESIS AND EVALUATION OF CONFORMATIONALLY CONSTRAINED AMINOPIPECOLIC ACIDS FOR THE GENERATION OF NEW SMALL-MOLECULE INTEGRIN-TARGETED DRUGS

Settore Scientifico Disciplinare CHIM/06

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In Memory of Aldo Salimbeni

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Introduction

Chapter 1: Conformationally Constrained Peptidomimetics

1.1 Peptidomimetics

Peptidomimetic is a term of broad meaning, often invariably referred to modified peptides ("peptide-derived peptidomimetics") and to other molecules lacking a close structural relationship to natural peptides ("non-peptide peptidomimetics").[1] Generally speaking, peptidomimetics are compounds designed to mimic natural peptides or protein segments, retaining the capacity to interact with their biological targets, possibly with an improved activity and a better selectivity; depending on the agonistic or antagonistic behaviour of the specific peptidomimetic, the native peptide effects can be increased or inhibited. Moreover, peptidomimetics can prevent some of the problems usually associated with the pharmacological use of their natural correspondents: sensitivity to proteolysis and poor bioavailability. Therefore, this class of compounds can potentially express optimal drug candidates.[2]

One of the main issue concerning the design of peptide-derived peptidomimetics is the conformational behaviour, especially for those meant to mimic a small portion of a protein structure, lacking the intramolecular interactions characteristic of the protein. In fact, small peptides, especially those composed by less than twelve amino acids, are usually characterized by a certain flexibility due to the multiple conformations that are energetically accessible for each of their residues; in most cases they exist in numerous dynamically interconverting conformations. This flexibility tends to be a major problem in getting a good ligand-receptor interaction, since usually only one specific conformer is recognized by the binding site of the target. A mixture of interconverting conformers inevitably exhibits a lower concentration of the active one, not to mention the unfavourable entropic contribution associated with the forcing of a flexible molecule in a geometrically organized binding site; all this results in a reduction of the biological activity of the compound.

Therefore, the incorporation of conformational constraints into peptidomimetics is a well-established approach for enhancing their efficacy and selectivity. Obviously, such restrictions have to be designed to make the compound to assume a matching conformation for the binding site, otherwise the activity can be lost completely.

Chemists have available several methods to obtain a reduction of the conformational space in a molecule; in the next section we address the most widely adopted.

1.2 Global and Local Constrains

Cyclization, N-methylation and incorporation of conformationally restricted building blocks are all valid strategies to introduce conformational constrains into a peptide. While the first method leads to a global restriction in the molecule, the others can be used to constrain a portion of the peptide sequence, blocking the rotation of specific dihedral angles.[3] Obviously, it is also possible to effectively combine the two approaches in the design of a cyclic peptidomimetic.

Peptides can be cyclized in different fashions: head-to-tail, by connecting the N- with the C-terminus of the sequence (figure 1, **A**); backbone-to-side chain, by binding either the N- or the C-terminus with one of the side chains (figure 1, **B**); or side chain-to-side chain, by coupling

two side chains with each other (figure 1, **C**). In most of the cases, the side-chain-to-side chain cyclization is obtained introducing amide bonds or disulphide bridges between suitable side chains. In any case, the global conformational space of the peptide is drastically reduced, especially concerning the backbone angles. Independent rotations become strictly limited and a strong hydrogen-bond network is usually enabled to arise.[3]

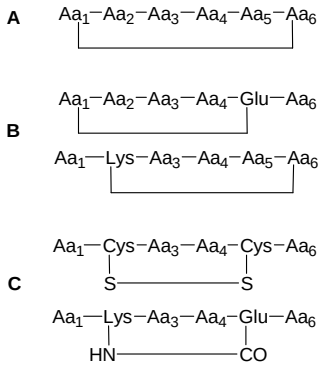

Figure 1. Examples of peptide cyclization: (**A**) head-to-tail, (**B**) backbone-to-side chain, (**C**) side chain-to-side chain.

On the other hand, local constrains can be obtained by introducing unnatural rigid amino acids, often synthesized by modifying natural amino acids to a certain extent. One of the simplest and most broadly adopted modifications is the replacement of the α-hydrogen with another residue, generating C_α-tetrasubstituted amino acids, characterized by a reduction of the rotational freedom of the peptide dihedral angles, due to the increased steric hindrance. One important representative of this class of compounds, the rare naturally occurring achiral α-aminoisobutyric acid (Aib), can be obtained by replacing the α-hydrogen on alanine with a methyl group (figure 2); this amino acid is a strong helix former and it is widely adopted in the synthesis of peptidomimetics.[4]

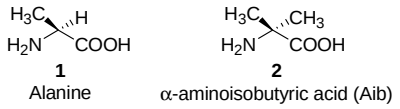

Figure 2.

Introducing extra cycles of various sizes is another efficient strategy to limit the possible conformations of a given amino acid, since this modification can constrain simultaneously the backbone dihedral angles (ψ and ϕ) and the side chain angles (χ_n) (figure 3).

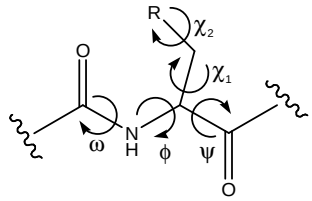

Figure 3. Peptide torsional angles.

In case extra cycles contain the amino group of the molecule, an entire category of azacycloalcane carboxylic acids can arise, comprehending aziridinecarboxylic acids, azetidinecarboxylic acids, pyrrolidinecarboxylic acids (proline derivatives), piperidinecarboxylic acids (pipecolic acid derivatives), indolinecarboxylic acids, polycyclic prolines, indolizidinonecarboxylic acids, octahydroindolecarboxylic acids, tetrahydroisoquinolinecarboxylic acids and many others (figure 4).[5-7]

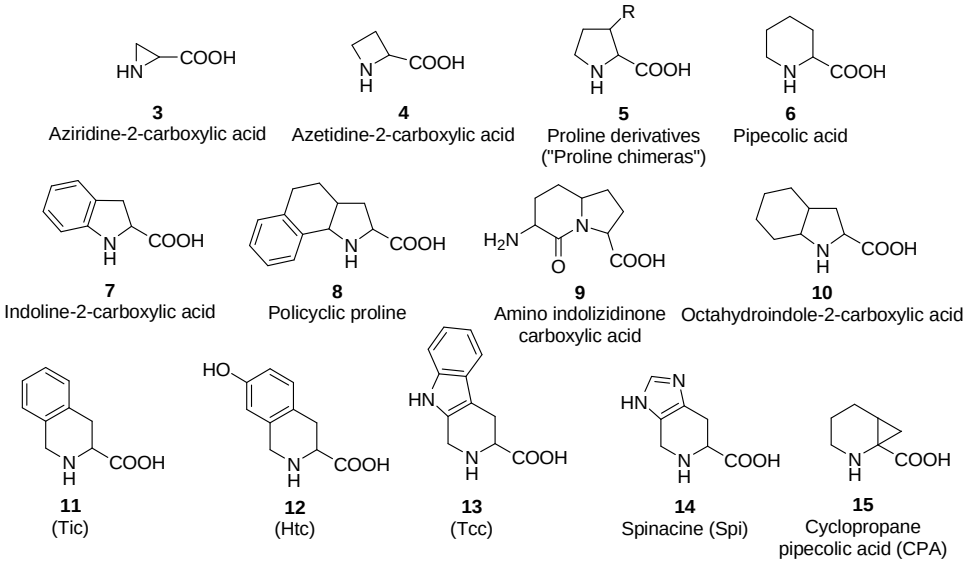

Figure 4. Azacycloalcane carboxylic acids.

The proline derivatives (also referred to as "proline chimeras"[8] when the side chain of a natural amino acid is incorporated in the pyrrolidine ring) doubtless represent the largest of these families (figure 4, **5**); they have been extensively synthesized and employed to prevent α-helix formation and to encourage the formation of β-turn in peptidomimetics, thanks to the strong constrain experienced by the rotation of the N-C_α bond. Moreover, proline analogues, exhibiting a low barrier to *cis*/*trans* isomerism about the peptide bond, can be exploited to orient this equilibrium towards a preferred geometry, due to its importance in peptide folding.[9]

On the other hand, pipecolic acid (figure 4, **6**), being a non-proteinogenic amino acid, can be used itself as a proline substitute in peptides or proteins, especially to investigate the role of ring size in structure and function.[10-12] Moreover, many functionalized pipecolic acids have

been prepared and used as constrained analogues of other amino acids, in a similar fashion to proline.[5,13] The most relevant examples are the constrained aromatic amino acids 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) (figure 4, **11**), 1,2,3,4-tetrahydro-7-hydroxyisoquinoline-3-carboxylic acid (Htc) (figure 4, **12**), 3-carboxy-1,2,3,4-tetrahydro-2-carboline (Tcc) (figure 4, **13**) and spinacine (Spi) (figure 4, **14**), where the aromatic rings of phenylalanine, tyrosine, tryptophan and histidine are fused with the piperidine moiety, resulting in a simultaneous restriction of backbone and side chain angles.[14] Another still poorly explored class of further constrained pipecolic acid derivatives is constituted by the cyclopropane pipecolic acids (or methanopipecolic acids), where two contiguous carbon atoms of the piperidine ring are tethered together by a methylene bridge (figure 4, **15**). Such molecules are part of the central topic of this work and will be addressed in a dedicated chapter (see section 2.3).

Regarding the cycles not involving the amino group, there are again examples of rigid amino acids incorporating all-carbon rings of any size.[5] However, the most relevant place is by far occupied by the just mentioned cyclopropane moiety, since the three-membered ring is one of the most appealing structural unit for the preparation of restricted building blocks, due to its rigidity and its partial unsaturated character.[5,15] Therefore, aside from being widely incorporated in constrained amino acids (cyclopropane amino acids will be addressed in chapter 2), this moiety has been also selected to be embedded in the target molecules of this work.

1.3 References

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Chapter 2: Cyclopropane Amino Acids

2.1 Monocyclic Cyclopropane Amino Acids

The simplest cyclopropane α-amino acid is 1-Aminocyclopropane-1-carboxylic acid (ACC, 2,3-methanoalanine) (figure 1, **1**), a natural intermediate in the conversion of methionine to ethylene, compound extensively utilized as hormone by plants. ACC was also the first cyclopropane amino acid incorporated in a peptide sequence, specifically an analogue of Leu^5-enkephalin, in which ACC replaced a glycine residue (figure 1, **4**).[1] This amino acid, together with other two natural secondary metabolites, coronamic acid (figure 1, **2**) and norcoronamic acid (figure 1, **3**), constituted the paradigm for a large number of artificial monocyclic quaternary α-amino acids, where the C_α and the C_β are tethered in a cyclopropane ring. Following this model, many "cyclopropylogues" of natural amino acids have been synthesized, not to mention numerous other molecules bearing functionalities not strictly connected with natural correspondents (figure 1, **5**).[2-4]

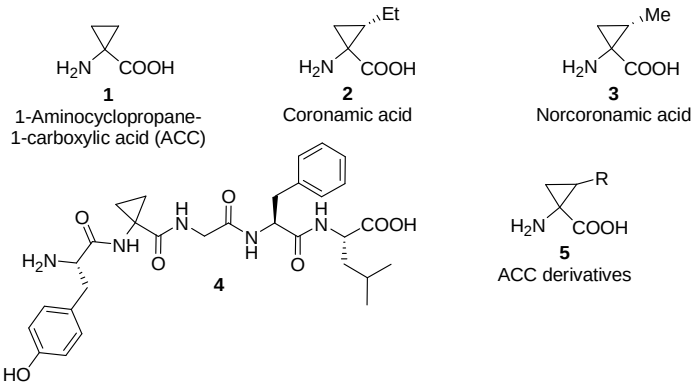


Figure 1.

These amino acids have been employed for the synthesis of either model and biologically active peptidomimetics, showing interesting structural features.[5-11] Combined NMR and computational studies have demonstrated how the presence of the cyclopropane moiety may deeply reduce the conformational space accessible to the peptidomimetics incorporating cyclopropylogues. On one side, the χ_1 angle results locked by tethering C_α to C_β, leading to a rigid control on the orientation of the side chain of the single cyclopropylogue; on the other hand, the large steric hindrance of the cyclopropane ring restricts also the proximal backbone torsion angles, influencing the conformation of the vicinal amino acids. Accordingly, the presence of cyclopropylogues has been seen promoting the formation or augmenting the stability of different secondary structure elements, especially γ-turn, even in very short peptides.[5-7]

Other important and numerous monocyclic cyclopropane α-amino acids are those bearing the cyclopropane ring on the side chain, not involving any backbone atom (figure 2). The prototype of this class is the cyclopropylglycine (figure 2, **6**), but many other differently functionalized derivatives have been synthesized and studied; the cyclopropane moiety can either involve only one carbon atom of the side chain or comprise two contiguous positions tethering them together by a methylene bridge.[12] Among the second type, the most important

derivatives are the various stereoisomers of 2-(carboxycyclopropyl)glycine (CCG) (figure 2, **8**), in which the cyclopropane ring tethers together C_β and C_γ of a glutamic acid side chain. These constrained analogues of glutamic acid, found as secondary metabolites in plants,[13] are not so relevant for peptide synthesis, but they are extremely active agonists of the glutamic acid receptors in the mammalian central nervous system (CNS).[14] Since their considerable pharmacological significance, many variously substituted versions of these amino acids have been synthesized, yielding also to a new class of selective and potent mGLUr receptors antagonists (figure 2, **9**).[15,16]

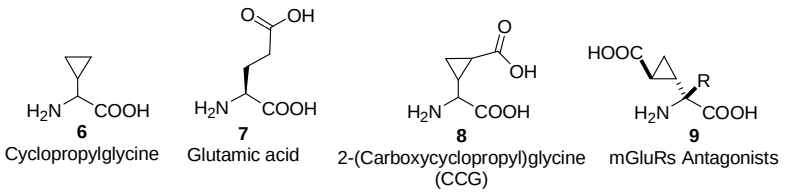


Figure 2.

More recently, also cyclopropane β-amino acids have attracted attentions as constituents of peptidomimetics. Whereas there is an univocal way to build a cyclopropane ring on the backbone of an α-amino acid, in the case of β-amino acids there are three different possibilities. Starting from the simple linear structure of β-alanine, the cyclopropyl group can be placed like two geminal substituents in the α- or β-position (figure 3, **11** and **12**), as similarly illustrated for α-cyclopropylogues; otherwise, the cyclopropane ring can incorporate both the positions, linking theme by a methylene bridge (figure 3, **13**).[17]

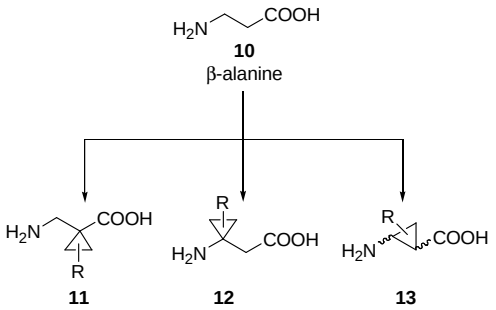
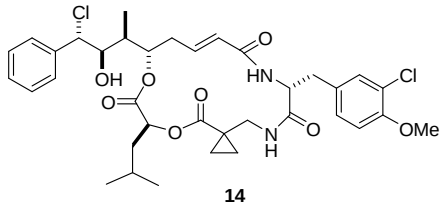


Figure 3. Cyclopropyl β-alanines.

The simplest representative of the first group, α-cyclopropyl-β-alanine (figure 3, **11** R=H), has been used for the synthesis of mimetics of natural depsipeptides belonging to the family of cryptophycins, which exhibit high activity against a broad spectrum of tumours (figure 4).[18] The augmented steric hindrance, due to the cyclopropane ring, proved to be suitable for protecting the ester bond of these depsipeptides from hydrolysis, increasing their stability and antitumor activity against leukaemia.[19]



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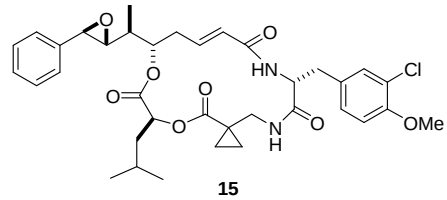

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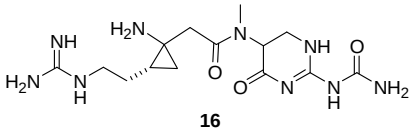
Figure 4. Cyclopropyl analogues of cryptophycins.

In another study, homooligomers of α-cyclopropyl-β-alanine (**11**, R=H) have been crystalized and studied by X-ray diffraction, revealing the formation of boatlike eight-membered H-bonded rings. This peculiar geometry, confirmed to be present even in solution, made hypothesize a "pleated ribbon" or a "flight of stairs" structure as a possible motif for a β-peptide consisting of α-cyclopropyl-β-alanine; such arrangement has no correspondents in the realm of α-peptides and proteins.[20]

The second group, composed by the β-cyclopropyl-β-amino acids, has been probably even more expanded, and several analogues, incorporating the side chains of natural amino acids, have been synthesized. For instance, a common synthetic route to serine, cysteine, arginine and lysine mimics have been developed; the latter two have been used for the synthesis of analogues of the antibiotics TAN-1057 A (**16**) and B (**17**) (figure 5).[21]



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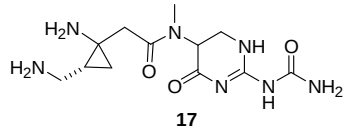

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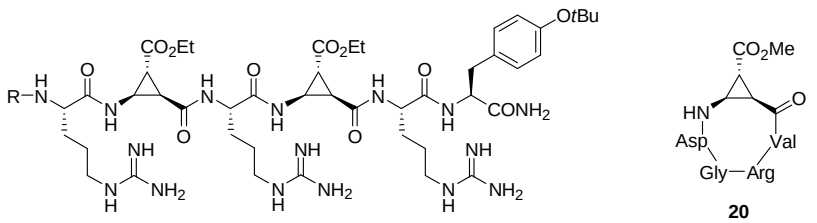
Figure 5. Cyclopropyl analogues of antibiotics TAN-1057 A and B.

Whereas these first two families of molecules are characterized by similar properties, those where the α and β-positions are tethered together are slightly different. The bridging of these two positions creates the most severely conformationally constrained β-alanine analogues possible, making these 2-aminocyclopropanecarboxylic acids (β-ACCs) extremely attractive as constituents of peptides. However, their utilization results pretty challenging, since β-ACCs belong to the class of vicinally donor-acceptor substituted cyclopropanes, which are extremely prone to undergo ring opening, resulting in their degradation. Nonetheless, adopting special synthetic strategies, this obstacle has been overcome and some β-ACCs-containing peptidomimetics have been obtained.[17] Among these products are the shortest known linear ligands with nanomolar affinity and good selectivity for the Neuropeptide Y1 receptor (figure 6, **18** and **19**), not to mention the potent nanomolar cyclopeptide ligand *cyclo*-(-Arg-Gly-Asp-(–)-β-Acc-Val-) (**20**) selective for integrin $\alpha_v\beta_3$ (see chapter 3 for a more detailed description of integrin ligands).[22,23]

β-ACCs derivatives demonstrated to produce strongly defined and stable conformations even in very short linear peptidomimetics; studies performed on oligomers of various sizes alternating units of β-ACCs and alanine indicated the formation of defined structures starting from the pentapeptides, while the extension of the sequence to seven residues resulted

enough to develop helical structures.[24] Therefore, β-ACCs can be acknowledged as building blocks for foldamers, behaving complementarily to the turn-inducer proline derivatives.



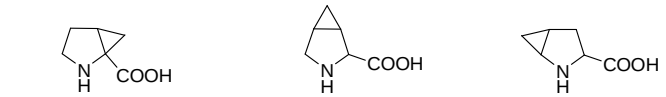
18 R = Ac-Arg-His-Tyr-Ile-Asp-Leu-
19 R = Ac-Asp-Leu-

20

Figure 6. β-ACCs-containing biologically active peptidomimetics.

2.2 Cyclopropane Proline Derivatives

Cyclopropane Prolines, commonly referred to as methanoprolines, represent another class of interesting constrained amino acids. By building the cyclopropane moiety on a proline heterocycle is possible to obtain three different regioisomers, namely 2,3-methanoproline, 3,4-methanoproline and 4,5-methanoproline (figure 7).



21 2,3-methanoproline

22 3,4-methanoproline

23 4,5-methanoproline

Figure 7. Methanoprolines.

The second regioisomer (**22)** is the only naturally occurring of the series; its *cis* form was discovered in 1969 in the fresh seeds of the american horse chestnut, *Aesculus parviflora*,[25] and successively found also in other plants as secondary metabolite.[13] Therefore, 3,4-methanoproline has also been the most investigated and the first synthesized;[26] subsequently, several different strategies for the synthesis of either its racemic mixture and its enantiopure forms have been proposed.[27-30] Early studies showed how this molecule was effective as male plant gametocide,[31] a useful tool to accomplish heterosis in crop plants, as well as inhibitor of the proline metabolism.[32] Moreover, 3,4-methanoproline has been utilized as model structure to design a wide variety of analogues with different functionalities bound to the methylene bridge. Derivatives bearing carboxyl groups on this position have been evaluated as constrained analogues of glutamic acid; one of them showed interesting activity towards NMDA kainate and mGLUr receptors (figure 8, **24**).[33,34]

On the other hand, the amino derivative **25** can be a new interesting rigid scaffold for peptide chemistry, able to work as an α- or γ- amino acid, exploiting the orthogonality of the protecting groups.[35] Finally, 3,4-methanoproline analogues of leucine (**26**), lysine (**27**), ornithine (**28**) and arginine (**29**) have been synthesised and efficiently employed for the construction of poly-L-proline type-II (PPII) secondary structure mimics (**30**), perfectly matching the conformational requirements.[36-42] This particular spatial arrangement,

characteristic of polyproline, is frequently exhibited also by recognition portions of proteins and by peptidic ligands, and is fundamental for binding with associated receptors.[43] Since, usually, nonprolyl amino acids, especially basic ones, present in PPII regions are critical for recognition mechanisms, these functionalized analogues can be very useful tools for the synthesis of active PPII mimics.[44]

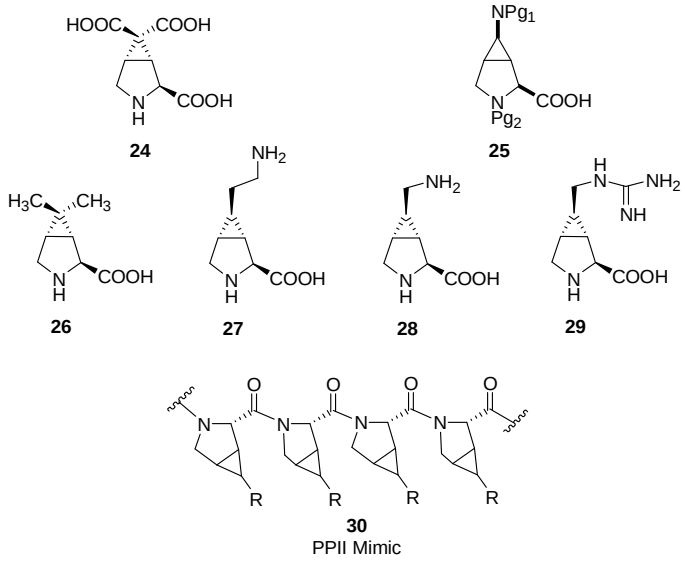


Figure 8. 3,4-Methanoproline derivatives.

The first artificial regioisomer, 2,3-methanoproline, was synthesised in 1989 and subsequently incorporated in a model dipeptide to investigate its conformational behaviour, showing a greater preference for the *cis* peptide bond at the ring nitrogen atom in comparison to the proline-containing correspondent (figure 9) (see chapter 7 for a similar comparative study we conducted on a cyclopropane pipecolic acid derivative).[11,45,46]

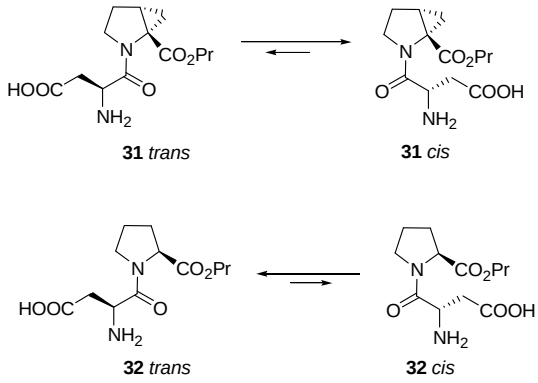
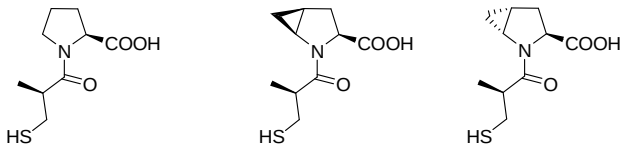


Figure 9. *cis*/*trans* isomerism in a dipeptide containing 2,3-methanoproline or proline.

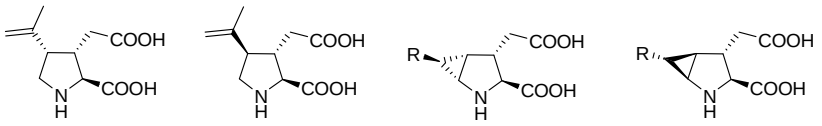
Finally, also the last regioisomer, 4,5-methanoproline, was efficiently synthesised in enantiopure forms for both the possible diasteroisomers.[47,48] These compounds, conformationally characterized by a prominent flattening of the 5-membered pyrrolidine ring, were employed for the synthesis of constrained analogues of captopril, a proline-derived angiotensin-converting-enzyme (ACE) inhibitor, resulting more active than captopril itself (figure 10).[49] Moreover, they showed to possess a catalytic activity for some of the reactions catalysed also by proline, exhibiting in some cases an outstanding enantioselectivity, exceeding the one obtained with proline.[50-52]



33 Captopril **34** **35**

Figure 10. Angiotensin-converting-enzyme (ACE) inhibitors.

Further differently substituted derivatives were also prepared to mimic the structure of kainic acids, natural ligands of glutamate receptors (figure 11); their biological activity, however, resulted low probably lacking the structural requirements needed.[53]



36 α-kainic acid **37** allo-kainic acid **38** R = H **39** R = vinyl **40** R = H **41** R = vinyl

Figure 11. Cyclopropyl analogues of kainic acids.

2.3 Cyclopropane Pipecolic Acid Derivatives

L-Pipecolic acid (figure 12, **42**) is a cyclic, naturally occurring non-proteinogenic α-amino acid, diffusely isolated in plants, fungi, microorganisms and human physiological fluids.[54] It is the major product of the degradation of lysine in human brain and it accumulates in the body fluids causing pipecolic acidemia in subjects suffering from Zellweger syndrome,[55] neonatal adrenoleukodystrophy,[56] and infantile Refsum disease.[57] Pipecolic acid and hydroxypipecolic acids (see chapter 8 for the synthesis of enantiopure *trans* 3-hydroxypipecolic acid) are also components of a wide range of pharmacologically active compounds, such as the immunosuppressive agents rapamycin (figure 12, **43**)[58] and FK506 (**44**),[59] the antitumor antibiotics sandramycin (**45**)[60], quinaldopeptine[61] and tetrazomine (**46**),[62] the cyclodepsipeptide antibiotic virginiamycin S1 (**47**)[63] and the serotonin receptor antagonist damipipecoline (**48**),[64] just to report some representative examples.

As already mentioned in chapter 1, pipecolic acid and pipecolic acid derivatives have been also extensively exploited for the construction of peptidomimetics. However, among the possible constrained derivatives, cyclopropane pipecolic acids have not received much attention so far and only few syntheses have been reported.

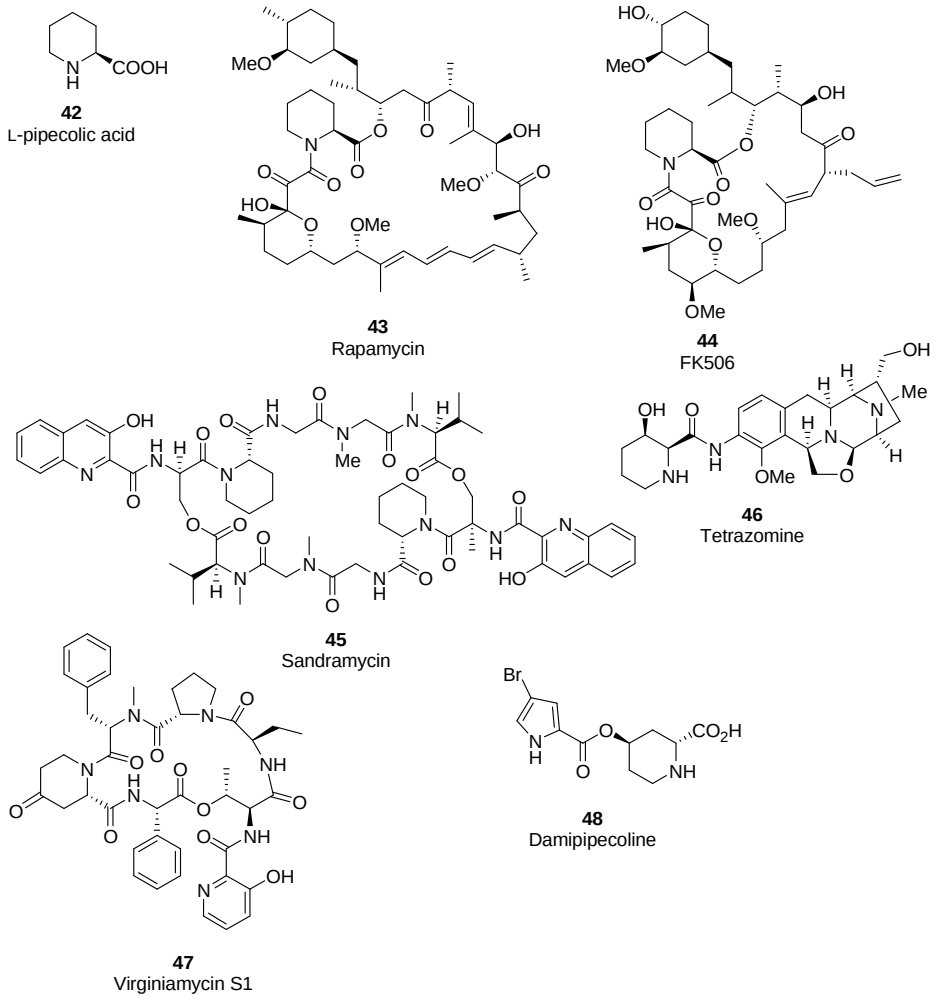
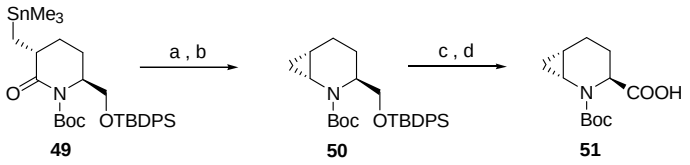


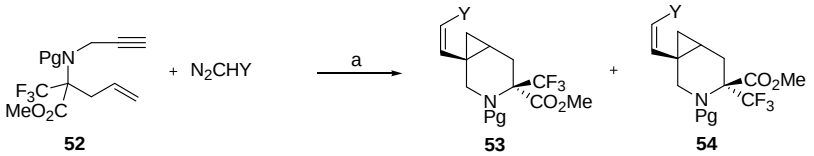
Figure 12. Bioactive compounds embedding pipecolic acids.

Hanessian's group described in 1997 the synthesis of both *cis* and *trans* 5,6-methanopipecolic acids in enantiopure forms, employing the same method used for the already cited 4,5-methanoprolines (see section 2.2).[48] This procedure exploits, as key step for the cyclopropane ring introduction, an intramolecular diasteroselective nucleophilic attack on the iminium ion, obtained by acid catalysis from a suitable hemiaminal intermediate formed *in situ* (scheme 1).
As already accounted for 4,5-methanoprolines, these products were incorporated in captopril rigid analogues too, which resulted, likewise the 4,5-mathanoproline derivatives, more active than captopril itself as angiotensin-converting-enzyme (ACE) inhibitors (see section 2.2).[49]



Scheme 1. Cyclopropane introduction in Hanessian's synthesis of 4,5-methanopipecolic acid. Reagents and conditions: a) $LiEt_3BH$, THF; b) pTsOH, $MeOH/CH_2Cl_2$; c) Bu_4NF, THF; d) $RuCl_3$, $NaIO_4$, $CCl_4/CH_3CN/H_2O$.

More recently, some fluorinated 4,5-methanopipecolic methyl ester derivatives have been synthesised in a racemic form by Dixneuf's group, exploring the scope of a peculiar ruthenium-catalysed tandem carbene addition/bicyclization of enynes (scheme 2).[65,66]



Scheme 2. Synthesis of fluorinated 4,5-methanopipecolic methyl ester derivatives. Reagents and conditions: a) [RuCl(cod)(Cp*)], Et_2O or dioxane or MeOH.

De Kimpe's group proposed the synthesis of a tricyclic cyclopropane derivative of Tic (see section 1.2), resulting in a doubly constrained analogue of phenylalanine (figure 13, **56**).[67] In this case, the three-membered ring was introduced by a cyclopropanation via dimethylsulfoxonium methylide of a suitable 1,2-dihydroisoquinoline derivative. Subsequently, the same group reported the synthesis of a regioisomer of the first product, which can be considered a doubly constrained analogue of ACC (see section 2.1) (figure 13, **57**).[68]

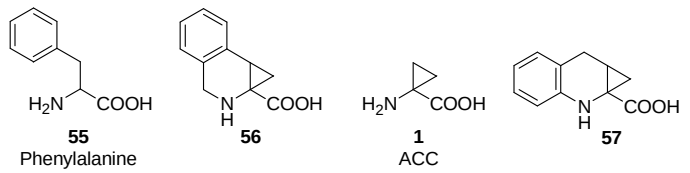
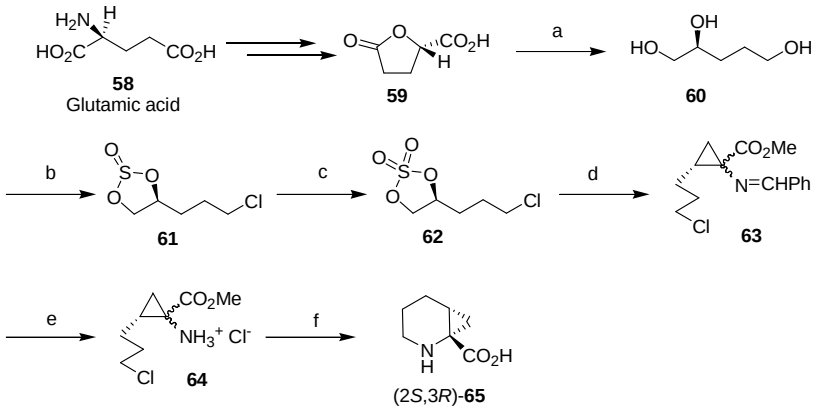


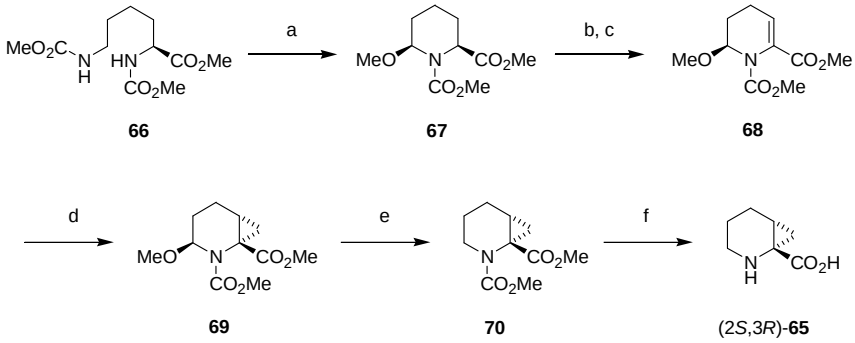
Figure 13. Tricyclic cyclopropane pipecolic acid derivatives.

Regarding 2,3-methanopipecolic acid, the model of which we chose to design the derivatives presented in this work, the first synthesis of the optically active form was presented in 1996 by Hercouet (scheme 3).[69]



Scheme 3. First synthesis of optically active 2,3-methanopipecolic acid. Reagents and Conditions: a) $BH_3{\cdot}Me_2S$, $CHCl_3$, 78%; b) $SOCl_2$, CCl_4, 72%; c) $NaIO_4$, $RuCl_3$ cat., 96%; d) $PhCH{=}NCH_2CO_2Me$, NaH, DME, 99%; e) 1N HCl/Et_2O, 86%; f) 1N NaOH, then 6N HCl, 59%.

The cyclopropane moiety was introduced by a diasteroselective alkylation of methylbenzylideneglycinate with chiral sulfate **62**, obtained starting from L-glutamic acid. Hydrolysis of imine **63** and subsequent treatment of the hydrochloride amino ester **64** with stoichiometric NaOH led to cyclization and obtainment of the desired product.
A second approach for the synthesis of optically active 2,3-methanopipecolic acid was proposed by Matsumura in 2000, starting from a diprotected L-lysine (scheme 4).[70]



Scheme 4. Matsumura's synthesis of enantiopure 2,3-methanopipecolic acid. Reagents and conditions: a) electrochemical oxidation, MeOH, then H_2SO_4, 47%; b) KHMDS, PhSSPh, 90%; c) *m*-CPBA, 92%; d) Me_3SOI, NaH, DMSO, 73%; e) $NaBH_4$, HCO_2H, 75%; f) Me_3SiI, $CHCl_3$, 50%.

The starting material was cyclized into the piperidine moiety by exploiting an electrochemical oxidation, followed by a diasteroselective acid-catalysed cyclization. Intermediate **67** was converted to 2,3-didehydropipecolate **68** employing a phenylthiolation of position 2, followed by oxidation. Then, the double bond was cyclopropanated by treatment with dimethylsulfoxonium methylide with a remarkable diasteroselection, explained in term of the steric hindrance exerted by the quasi-axial oriented 6-methoxy group. Finally, this chiral

auxiliary methoxy group was removed by reduction with $NaBH_4$ in formic acid, affording the protected desired product.
Considering that the syntheses listed above constitute an almost exhaustive survey of the efforts devoted to cyclopropane pipecolic acids so far, it is clear how, compared to proline analogues, this chemical space needs to be further explored, especially for what concerns substituted and diasteromerically and enantiomerically pure products. Moreover, to our knowledge, there were no reports about cyclopropane pipecolic acids embedded in peptides.
To enlarge the scope of these unnatural amino acids in the design and synthesis of highly selective and potent peptidomimetics, recently our group addressed the synthesis of both *cis* and *trans* enantiopure 4-hydroxy-2,3-methanopipecolic acids (or 5-hydroxy cyclopropane pipecolic acids, according to IUPAC numbering: see note at the end of the section) as new conformationally constrained homoserine analogues (figure 14, **71** and **72**).[71] Subsequently, also other polyhydroxylated derivatives have been prepared (figure 14, **73** and **74**), employing the same key reactions, which will be discussed later in this thesis (see chapters 4 and 5).[72]

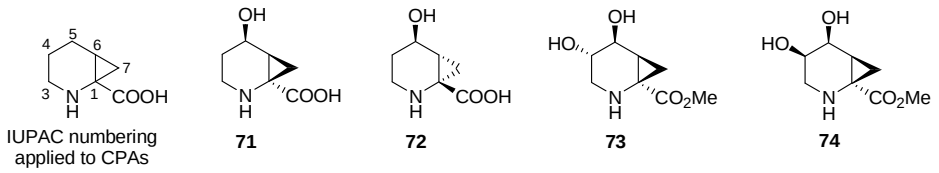


Figure 14. Hydroxylated 2,3-methanopipecolic acid derivatives synthesized by our group.

In this thesis we aimed to further increase the number of 2,3-methanopipecolic acids (or cyclopropane pipecolic acids: see note at the end of the section), introducing new hydroxy- and amino-substituted derivatives. Moreover, we tackled the synthesis of the first cyclopropane pipecolic acid-containing peptidomimetics; specifically, we designed new peptide ligands to target some representatives of the integrin family, a group of protein which will be discussed in the next chapter (see chapter 3).

Note: In this work, we will refer to our 2,3-methanopipecolic acids as CPAs, cyclopropane pipecolic acids, using the standard IUPAC numeration (figure 14), so that the positions tethered by the methylene bridge will be, actually, positions 1 and 6 (e.g. 4-hydroxy-2,3-methanopipecolic acid **71** will be referred to as 5-hydroxy cyclopropane pipecolic acid or, following entirely the IUPAC nomenclature, 5-hydroxy-2-aza-bicyclo[4.1.0]heptane-1-carboxylic acid).

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Chapter 3: Integrins

3.1 Integrins as Therapeutic Targets

The name integrin was attributed in 1986 to the first characterised representative of this large protein family to denote its role of integrating the extracellular and intracellular environments by the transmembrane association between the extracellular matrix (ECM) and the cytoskeleton (figure 1).[1,2] Actually, integrins are the major cell adhesion transmembrane receptors for ECM proteins (e.g. vitronectin and fibronectin) in animals, but they are also involved in cell-cell adhesion processes, as well as in the activation of intracellular signalling pathways mediated by growth factors, immunoglobulins, cytokines and matrix-degrading proteases. Integrin-mediated events are fundamental to modulate many aspects of cell behaviour, including proliferation, survival/apoptosis, shape, polarity, motility, gene expression and differentiation. Hence, every representative of the integrin family plays a key role in organism development (formation and remodelling of tissues and organs), immune response and haemostasis.[3-5]

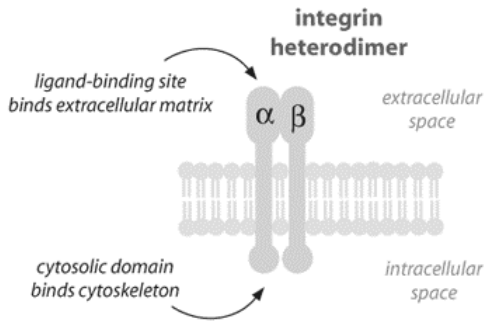


Figure 1. Integrin heterodimer.
(Adapted from "The Science Creative Quarterly", www.scq.ubc.ca, Jen PhilPott)

This group of receptors is composed by heterodimers of non-covalently associated α and β subunits. Each subunit is composed by an extracellular domain, a transmembrane domain and a short cytoplasmatic tail (figure 1). There are 24 known heterodimers, obtained by variously combining 18 α and 8 β different subunits, and characterised by different binding properties and different tissue distribution (figure 2). [6]

Integrins can be divided in 4 main subgroups, depending on the nature of their ligands, which usually contain short peptidic recognition sequences in their active sites. One subgroup comprehends those integrins which recognise ligands containing the short peptidic sequences LDV (lysine-aspartic acid-valine) or LDV-related (limited variability around a definable consensus sequence L/I-D/E-S/T/V was observed). Such proteins are typically expressed in leukocytes and regulate many aspects of immune response mechanisms (figure 2, circles).[2,7] Then, there are two smaller families, one of which is composed by integrins binding highly selectively to laminin (figure 2, hexagons), while the other one is constituted by collagen/laminin dual receptors, usually recognising the GFOGER peptidic sequence (figure 2, pentagons). Finally, the largest subgroup comprehends those integrins which recognise proteins containing an RGD (arginine-glycine-aspartic acid) tripeptide active site which, contrary to LDV, is invariant in all the related ligands (figure 2, triangles). The five α_V integrins

(including $\alpha_V\beta_3$ and $\alpha_V\beta_5$), $\alpha_5\beta_1$, $\alpha_8\beta_1$ and $\alpha_{IIb}\beta_3$ are all part of this subgroup.[6] They bind to a large number of ECM proteins and soluble vascular ligands and they are usually widely expressed in many different cells, such as platelets ($\alpha_{IIb}\beta_3$), osteoclasts ($\alpha_V\beta_3$), activated endothelial and epithelial cells, being involved in several physiological processes, such as platelet aggregation, bone resorption and angiogenesis.

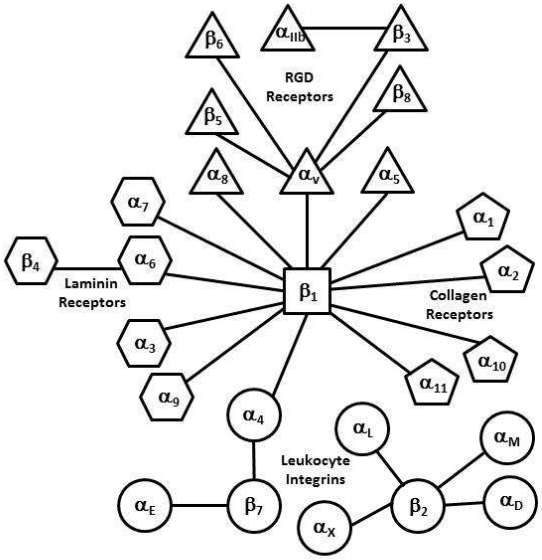


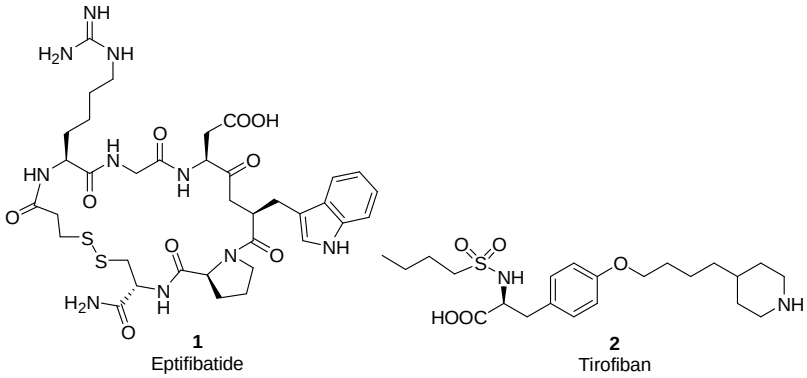
Figure 2. Known integrin subunit 24 combinations. Triangles: RGD receptors; pentagons: collagen/laminin dual receptors; hexagons: laminin receptors; circles: leukocyte integrins. β_1 is present in many subgroups, depending on the associated counterpart.
(Adapted from ref. [3])

Being involved in numerous physiological processes, integrins can also play a key role in many diseases, due to deficient or excessive expression and activity. Hence, integrin inhibition can be considered a valid therapeutic strategy to cope with several pathological conditions. This goal can be achieved developing suitable recombinant monoclonal antibodies, but also designing valuable peptide-derived or non-peptide peptidomimetic antagonists, containing or mimicking the appropriate short amino acid sequence.[7]

Integrin $\alpha_{IIb}\beta_3$ is responsible for platelets aggregation, by binding to soluble divalent fibrinogen; its importance in normal haemostasis is demonstrated by the genetic bleeding disorder, Glanzmann's thrombasthenia, characterized by uncontrolled bleeding and caused by a lack of, or mutations in, this integrin.[8] On the other hand, uncontrolled or aberrant platelet aggregation is a primary cause of arterial thrombosis, so that $\alpha_{IIb}\beta_3$ blocking can be a valid strategy to achieve a broad spectrum antithrombotic therapy to reduce the risk of acute cardiac ischemic events. Various drugs have been developed with this purpose, exhibiting an adequate efficacy; three of them have reached the market and are prescribed in case of acute coronary syndrome or during percutaneous coronary intervention: the recombinant antibody

abciximab (ReoPro),[9] the cyclic heptapeptide eptifibatide (Integrilin) (figure 3, **1**)[10] and the non-peptide mimetic tirofiban (Aggrastat) (figure 3, **2**).[11]

Leukocyte integrins, especially $\alpha_4\beta_1$, $\alpha_4\beta_7$ and $\alpha_L\beta_2$, are involved in the "extravasation" process of leukocytes from the blood stream to the sites of injury or infection, playing a fundamental role in immune response. As a matter of fact, mutations that block expression of the β_2 integrins are known to lead to severe immunodeficiency.[12] However, uncontrolled or excessive leukocyte migration can lead to a number of inflammatory disorder, such as rheumatoid arthritis, asthma, multiple sclerosis and other autoimmune diseases. Hence, inhibition of α_4 integrins has been proposed as therapeutic strategy for these pathologies, leading to the development of natalizumab (Tysabri, former Antegren), a monoclonal antibody inhibitor, marketed as drug for the treatment of multiple sclerosis and Crohn's disease.[13]



1 Eptifibatide **2** Tirofiban

Figure 3. Anti-$\alpha_{IIb}\beta_3$ marketed drugs.

Finally, $\alpha_5\beta_1$, various α_V integrins and, again, $\alpha_{IIb}\beta_3$, all part of the RGD-binding subgroup, are receiving an increasing attention, due to their strategic role in progression and dissemination of several types of cancer. No related drug has reached the market yet, but huge efforts have been dedicated to the comprehension of their mechanisms of action and to the development of suitable drug candidates for cancer treatment.[14] Since some of these receptors, specifically $\alpha_5\beta_1$ and $\alpha_V\beta_3$, are also the biological targets we chose to aim for with our CPA-containing peptidomimetics (CPA: cyclopropane pipecolic acid), they will be addressed separately in the next section.

3.2 Inhibition of $\alpha_V\beta_3$ and $\alpha_5\beta_1$ for Cancer Treatment

Together with $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ are, by far, the most studied proteins of the RGD-binding subgroup and a remarkable number of artificial antagonists has been designed for their inhibition.[15]

Integrin $\alpha_V\beta_3$ is mostly expressed in osteoclasts, where it participates to bone resorption process, and on active endothelial cells, where it mediates vascular angiogenesis. Normal angiogenesis is an essential process of foetal development, wound healing, ovulation, growth and development. On the other hand, $\alpha_V\beta_3$ is also widely overexpressed in several types of tumour cells and in the vascular tissue of the tumour microenvironment, promoting angiogenesis, which is a fundamental process also for solid tumours growth. Expanding solid

tumours reach a density point where the increased interstitial pressure inhibits the diffusion of metabolites, nutrients and oxygen, slowing down drastically this progression. Subsequently, many factors, such as metabolic and mechanical stress, inflammatory response and genetic mutations, can trigger the, so called, "angiogenic switch". This event, starting with a signalling cascade, culminates with the formation of new stable blood vessels, which permit tumour to leave its "dormancy state" and start expanding rapidly. Among all integrins, $\alpha_V\beta_3$ is the most strongly involved in this mechanism, playing a key role in every step of the process: the degradation of old endothelial cell basement membrane, proliferation and migration of new endothelial cells out of the original blood vessel, and stabilization of new blood vessels branching towards the tumour.[16,17]
Furthermore, $\alpha_V\beta_3$, together with $\alpha_{IIb}\beta_3$, promotes metastasis diffusion through lymphatic and haematogenous dissemination, and allows the metastatic deposit to become established through adhesion to ECM proteins. Again, $\alpha_V\beta_3$ is involved in the entire process, promoting extravasation from the primary tumour, cell adhesion, intravasation and tumour growth at the metastatic site.[18,19]
Accordingly, inhibition of $\alpha_V\beta_3$ has been considered a valid strategy for developing new anticancer therapies and many efforts have been dedicated to this goal.[20] Such result can be obtained blocking the RGD-binding site of the protein with an adequate ligand, which can be a monoclonal antibody, an RGD-based peptide or peptidomimetic, or a non-RGD-based antagonist mimicking the RGD sequence.[14] Countless examples of such products have been published in the last decades and we will focus on some of them later (see section 3.3); several exhibited a remarkable affinity towards $\alpha_V\beta_3$ and some reached clinical trials as antitumor chemotherapy.[21]

Tumours often overexpress, along with $\alpha_V\beta_3$, also $\alpha_5\beta_1$, the so called fibronectin receptor due to its high affinity towards this ECM protein, which has a similar proangiogenic function to $\alpha_V\beta_3$.[22] These two integrins seem to act synergistically, since $\alpha_V\beta_3$ has been shown to regulate the function of $\alpha_5\beta_1$ and vice versa via trans-dominant inhibition; so that blocking the activity of one of the two often demonstrated to trigger the overexpression and the overactivity of the other. This mechanism could be related with the ambiguous results obtained in clinical trials with many selective antagonists.[15] Usually, lack of selectivity towards a specific biological target is considered a safety issue, possibly leading to toxic effects. However, both selective and multi-integrin antagonists demonstrated to be generally safe, and the latter appeared more efficient.[23,24] These agents are probably non-toxic because the targeted integrins are only expressed or activated in remodelling tissues such as tumours.[25]
Therefore, a dual affinity towards both $\alpha_V\beta_3$ and $\alpha_5\beta_1$, as well as a multi-affinity towards a wider range of integrins, can be a desirable feature to obtain in an artificial ligand. Since the two integrins recognise the same RGD sequence and their binding sites are very similar (see section 3.3), this should not be a goal too difficult to achieve.

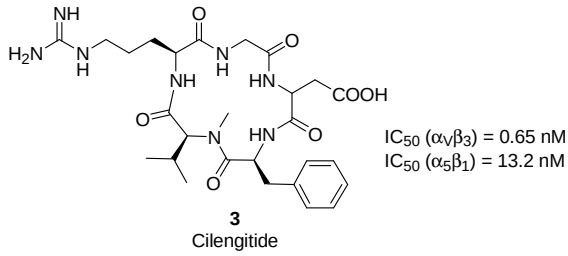
Besides antagonism, integrin artificial ligands can found also other applications relatively to cancer treatment. Their high affinity towards receptors overexpressed in cancer cells can be a valuable tool to build drug delivery systems. Artificial ligands can be linked to suitable cytotoxic molecules, directing the action of non-specific chemotherapy on cancer cells increasing their efficacy and reducing drastically their toxicity.[14]
The same principle can be applied in diagnostic field, delivering to the tumour radionuclides or dyes embedded in integrin targeting systems.

Therefore, highly decorated scaffolds are particularly useful for the construction of RGD-based ligands, since the functionalities not involved in the peptidic bonds can be exploited to covalently bind drugs or imaging devices, as well as to modulate the physical and chemical characteristics of the final products.

3.3 $\alpha_V\beta_3$ Ligands for Antagonism, Targeted Drug Delivery and Diagnostic

Since the first discovery in the early 1970s of the RGD sequence as a cell attachment site in fibronectin and its subsequent identification as the minimal integrin-binding sequence in many other ECM ligands, the structural basis of this recognition mechanism have been deeply investigated.[26,27]

It was observed that although many integrins recognize the RGD motif, they are also able, to a certain extent, to discriminate between different ligands. This behaviour was correlated with the ability of recognising only certain conformations of the RGD moiety, imposed by the surrounding secondary and tertiary structures of the different proteins. Lacking further indications, at first, artificial antagonists were designed in a "ligand-oriented" fashion, rationally exploring the conformational space (with the additional aid of NMR and molecular dynamics) and screening the activity of libraries of RGD-based peptides ("spatial screening"). In such a way Kessler's group developed Cilengitide (figure 4), the first small-molecule high affinity ligand for angiogenic integrins, starting from simple RGD-containing cyclic pentapeptides and proceeding with small modifications (D-amino acids introduction and N-methylations).[28-32]



3
Cilengitide

Figure 4. Cilengitide: first RGD-based subnanomolar ligand for angiogenic integrins.

In fact, only antibodies and disintegrins (a family of RGD-containing cysteine-rich peptides derived from viper venoms) had shown such biological activity before; anti-$\alpha_V\beta_3$ monoclonal antibodies hLM609 (etaracizumab, Vitaxin-2, Abegrin) and CNTO-95 (intetumumab, Centocor) were the first integrin related products to reach clinical trials as anticancer treatment.[25,33]

Cilengitide exhibited a remarkable nanomolar affinity towards $\alpha_V\beta_5$ and $\alpha_5\beta_1$, and an outstanding subnanomolar affinity towards $\alpha_V\beta_3$.[15] It was the only integrin-related antitumor drug candidate to have reached phase III clinical trials, specifically as treatment against glioblastoma.[34] Surprisingly, these trials did not furnish positive results and were suspended, demonstrating how much is still to be undisclosed about the role of integrins in cancer. On the other hand, phase II trials concerning the use of Cilengitide in combination with other chemotherapy are still ongoing.

Finally, in 2001, the crystal structure of the extracellular portion of $\alpha_V\beta_3$ was obtained; one year later also the crystal structure of $\alpha_V\beta_3$ complexed with Cilengitide was determined.[35,36]

These results help to elucidate the structural characteristics of $\alpha_V\beta_3$ binding pocket and a rational structure-based design of new ligands could arise. RGD binds at an interface between the α and β subunits, with the basic guanidinium residue fitting into a cleft in the α subunit and the acidic carboxylate residue pointing in the opposite direction and coordinating a cation bound in the β subunit (figure 5).

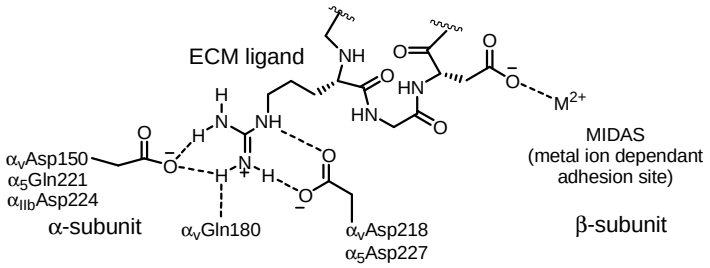


Figure 5. Key features of the RGD binding site.
(Adapted from ref. [15])

Arg and Asp act like an "electrostatic clamp" attaching to charged regions of the two subunits of the protein. Specifically, Arg forms salt-bridges with Asp150 and Asp218 of the α subunit, while Asp coordinates a variable divalent cation contained in a MIDAS (metal ion dependent adhesion site) in the β subunit. Moreover, in the case of Cilengitide a further hydrophobic interaction between D-Phe and β-Tyr122 was observed. Afterward, docking simulations, performed on complexed $\alpha_V\beta_3$ X-ray structure, allowed to establish some set parameters for affinity, such as the optimal distance between the pharmacophoric groups, helping in the design of both RGD-based and non-RGD-based ligands.[37] Previously, potent $\alpha_V\beta_3$ non-RGD-based ligands, such as SC-68448 (figure 6, **4**),[38] SCH221153 (**5**),[39] S-137 (**6**) and S-247 (**7**),[40] were identified only through the screening of large libraries of peptidomimetics, rationally refined accordingly to the structures of known ligands.

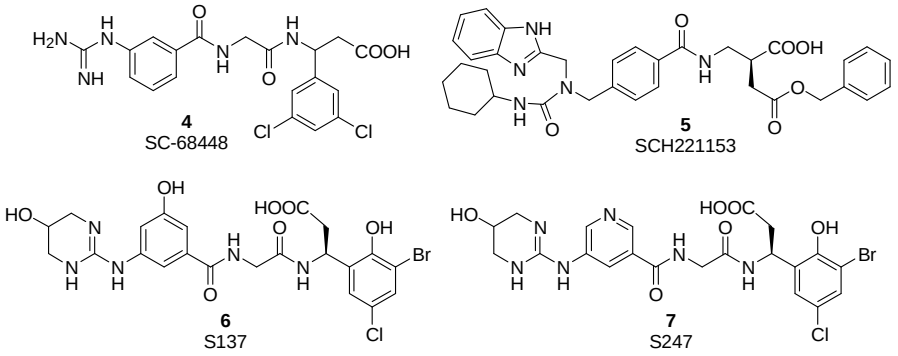


Figure 6. Potent non-RGD-based ligands for angiogenic integrins.

The other RGD-binding integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ displayed the same mechanism of interaction of $\alpha_v\beta_3$, differing only in binding site size and the identity of the arginine-binding residues

(figure 5).[41,42] Therefore, it is possible to obtain both selective and multi-integrin antagonists, depending on the specific structural features of the candidates.

Regarding conformational behaviour and selectivity, an interesting study was conducted cyclizing an RGD sequence together with the two enantiomers of cyclopropane amino acid β-Acc (see chapter 2) (figure 7), obtaining two diasteroisomers, one of which selective for $\alpha_v\beta_3$ (**8**), and the other exhibiting comparable affinity towards $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (**9**).[43]

Following Kessler's Cilengitide example, many ligands have been designed as conformationally constrained cyclopeptidomimetics to ensure a drastic reduction of the conformational space and guarantee a higher affinity (see chapter 1). Usually, these structures contain the native RGD sequence bound to a rigid central core of various nature, mainly a conformationally constrained artificial amino acid.

In this work we adopted the same concept to design our own RGD-based integrin ligands (see Scope of the work), following the path traced by many other research groups, which in the last years published a plethora of new ligands of this typology, often exhibiting nanomolar and subnanomolar activities.

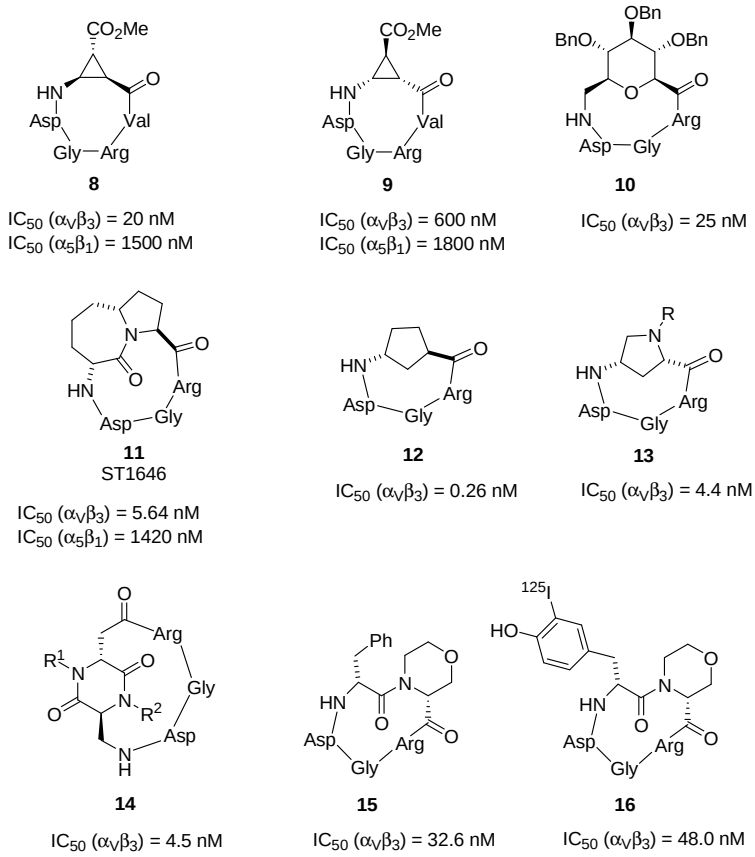


Figure 7. Potent RGD-based ligands for angiogenic integrins.

In 2000, Kessler's group itself developed new RGD-based ligands constructed on a sugar-derived core (figure 7, **10**), exhibiting high affinity towards $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$.[44] Moreover, in the same work, several variously decorated carbohydrates were embedded in cilengitide-like compounds in place of the valine side chain, with the aim of modulating the activity of the final products.

In 2001, Scolastico's group designed new ligands incorporating fused bicyclic lactams, one of which (ST1646) (figure 7, **11**) resulted active in the low nanomolar range towards $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and showed remarkable antiangiogenic properties.[45-47]

Afterward, Casiraghi and Zanardi developed two libraries of products based on γ-aminocyclopentane carboxylic acids (Acpca) and 4-aminoproline as central scaffolds; the two families contained respectively subnanomolar and low nanomolar ligands for $\alpha_v\beta_3$ (figure 7, **12** and **13**).[48-49] In the case of aminoproline derivatives the ring nitrogen, which at first was bound to activity-modulating groups, was also successfully employed for targeted delivery of imaging molecules (fluoresceine and DOTA) and cytotoxic drugs (paclitaxel).[50-51]

Another paclitaxel-containing drug delivery system was developed by Gennari's group employing a family of RGD-based ligands embedding bifunctional diketopiperazines (DKP) (figure 7, **14**); these paclitaxel conjugates were tested *in vitro* and *in vivo*, showing greater antitumor activity and lower toxicity than free paclitaxel.[52-54]

Finally, Guarna and co-workers established new morpholine-based RGD ligands (figure 7, **15**) as radiolabelled molecular imaging probe for angiogenesis, introducing a ^{125}I-containing tyrosine derivative in the peptidic sequence (figure 7, **16**).[55,56]

Besides the directly conjugated delivery systems designed by Zanardi and Gennari, another fascinating alternative is the use of nanocarriers as connection between integrin ligands and drugs or imaging molecules (figure 8).

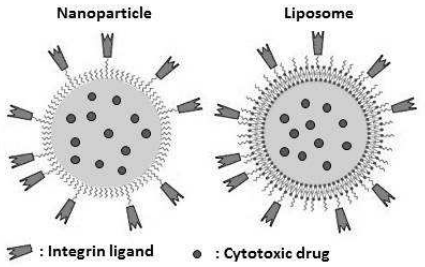


Figure 8. Nanocarriers for drug delivery.
(Adapted from R. Solaro, F. Chiellini, A. Battisti, *Materials*, **2010**, 3, 1928)

Basically, a nanocarrier (liposome, nanoparticle, micelle, etc.) can be loaded with a certain quantity of the selected drug and grafted on its surface with many copies of an integrin ligand. This way in a single event a larger amount of the drug can be delivered to its target. Moreover, nanocarriers offer other advantages; their size (20-400 nm) leads to a further "passive targeting" of tumours via the so called enhanced permeability and retention effect (EPR); in addition, because of the size of these systems, renal filtration is avoided, leading to prolonged blood circulation times and longer accessibility to target receptors within the tissue.[57,58] Lately, many efforts have been dedicated to the construction of such devices and a large number of clinical trials are ongoing.[14]

Our research was framed in this scenario and we hope that our contribution could be of use to progress in the field. As already mentioned, we tackled the synthesis of new RGD-based integrin ligands containing our cyclopropane pipecolic acids (CPAs) as central scaffolds and, because of the obvious structural similarities between aminoproline and CPAs, we chose Zanardi's work[50] as model for the design of the synthetic strategy for the construction of these CPA-containing peptidomimetics (see chapter 6).

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Scope of the Work

In the previous chapters we described how conformationally constrained amino acids are emerging privileged structures involved in many important applications, in particular as constituents of biologically active compounds, as well as conformational probes.
Although many efforts have been dedicated to the synthesis of constrained proline derivatives, the introduction of additional conformational restrictions in pipecolic acids by merging the piperidine with a cyclopropane ring has concerned very few examples. Extending this approach to other differently decorated cyclopropane pipecolic acids (CPAs) would certainly enlarge the scope of these unnatural amino acids in the design and synthesis of highly selective and potent peptide analogues in peptide-receptor recognition. To this aim, as already mentioned (see section 2.3), our group has recently published the synthesis of all four stereoisomers of 5-hydroxy-CPA as new conformationally constrained homoserine analogues to be employed as conformational probes and in the discovery of new drugs.
In this thesis we report our efforts to enlarge the series of CPAs by introducing hydroxy or amino groups at different positions of the piperidine ring (figure 1).

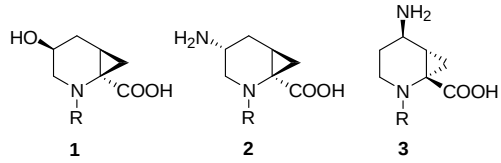


Figure 1. New CPAs synthesized within this thesis.

In addition, since to our knowledge there were no reports on cyclopropane pipecolic acids embodied in peptides, we wish to demonstrate that these new amino acid analogues are actually suitable templates for the preparation of both acyclic and cyclic peptidomimetics. In particular, we show that cyclopeptides can be built by exploiting the carboxylic group and a *cis*-amino functionality on the ring (figure 2, **A**), whereas acyclic peptides could be built on the α-amino acid moiety as in unconstrained pipecolic acids (figure 2, **B**).

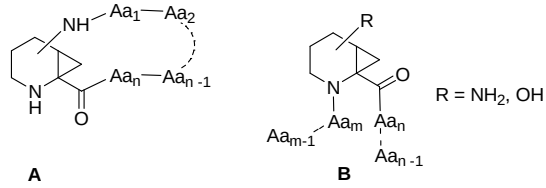


Figure 2. General structures of CPA-containing cyclic and acyclic peptides.

In view of possible applications of these templates in medicinal chemistry, the presence of the additional hydroxy (or amino) group can either provide further interactions with the active site of the target protein or be functionalized to attain higher potency and selectivity, and improve pharmacokinetics.

To prove the suitability of these compounds for preparing cyclic peptidomimetics, we chose to synthesize novel artificial ligands for RGD-binding angiogenic integrins (see chapter 3) by introducing suitable 4-amino- and 5-amino-CPA into an Arg-Gly-Asp (RGD) sequence (figure 3, **4** and **5**).
In this specific case, besides the above mentioned features, the additional amino group could also provide an anchoring position to build drug delivery systems (see section 3.3).

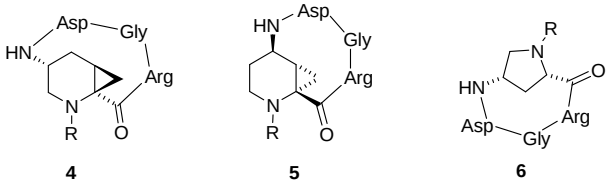


Figure 3. RGD-based integrin ligands.

Whereas the *cis* relative configuration of the carboxylic group and the amino functionality on the ring in the candidate scaffolds **2** and **3** was necessary to build a cyclopeptide, the particular absolute configurations (1*R*,4*R*,6*S* for **2** and 1*S*,5*R*,6*S* for **3**) were chosen on the basis of preliminary molecular modeling studies which showed that the RGD sequence in **4** and **5** best overlapped with that of potent 4-aminoproline derivative **6** (see section 3.3).
In addition, starting from **1** we designed a model linear tripeptide (figure 4, **7**) to investigate the conformational features offered by CPAs. Specifically, we evaluated the *cis*/*trans* isomerism about the pipecolic acid peptide bond and we compared this conformational behavior with that of published similar model tripeptides containing proline or pipecolic acid (figure 4, **8** and **9**).

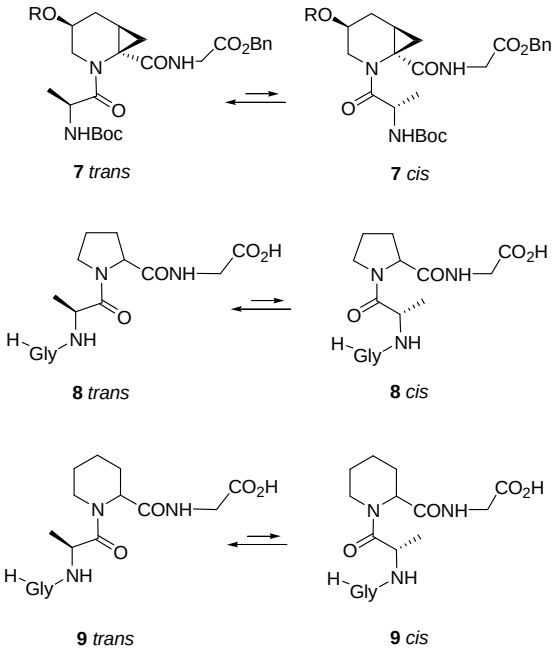
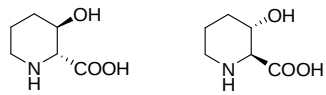


Figure 4. *Cis*/*trans* isomerism in CPA-containing and model tripeptides

Finally, we also designed a new synthetic strategy to obtain both enantiomers of *trans* 3-hydroxypipecolic acid (figure 5, **10**), which constitutes a non-natural variant of a structural motif often encountered in a variety of biologically active natural compounds. Additionally, being an artificial mono-substituted conformationally restricted amino acid (a constrained serine analogues), **10** could be employed for the construction of further RGD-based ligands as well as other biologically active peptidomimetics.



(–)-**10** (+)-**10**

Figure 5. *Trans* 3-hydroxypipecolic acid.

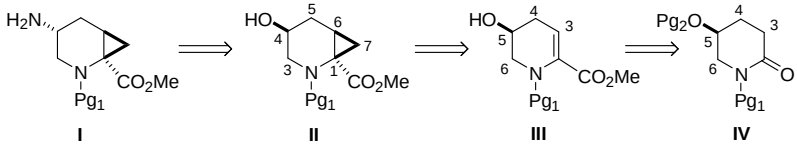
Results and Discussion

Chapter 4: Synthesis of 4-Substituted Cyclopropane Pipecolic Acids (4-R-CPAs)

4.1 General Synthetic Strategy

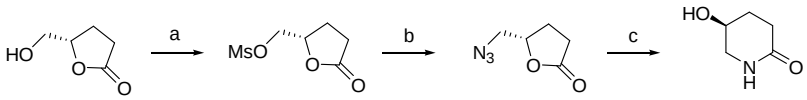
Our primary synthetic strategy towards 4-substituted-CPAs (scheme 1) entails the conversion of a suitably functionalized enantiopure lactam **IV** into the corresponding enecarbamate ester **III** by means of Pd-catalyzed methoxycarbonylation of the lactam-derived vinyl phosphate,[1] followed by the stereoselective OH-directed cyclopropanation of the double bond to give the cyclopropane pipecolic acid derivative **II**. Finally, functional groups manipulation can lead from the hydroxy derivative **II** to the amino-CPA **I** with a contemporary inversion of position 4 configuration.

As it will be illustrated later, slight modifications of this general procedure were employed for the synthesis of every CPA.



Scheme 1. Retrosynthetic Analysis for 4-R-CPAs.

Enantiopure 5-hydroxy-δ-valerolactam can be prepared from a lactone of which both enantiomers are commercially available, following the procedure reported by Herdeis in 1986 (scheme 2).[2] Accordingly, both enantiomers of the final products can be prepared in principle. On the other hand, as already mentioned (see Scope of the Work), at first we were interested in the synthesis of a selected specific enantiomer which is obtainable starting from (*S*)-(+)-γ-hydroxymethyl-γ-butyrolactone (**1**), the cheaper enantiomer derived from natural L-glutamic acid.[2]



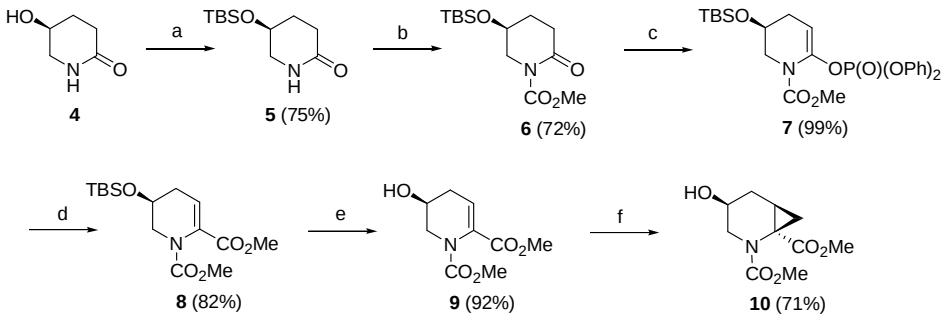
Scheme 2. Synthesis of enantiopure 5-hydroxy-lactam. Reagents and conditions: a) MsCl, Et_3N, CH_2Cl_2; b) NaN_3, [15]crown-5, CH_3CN, reflux; c) H_2, Pd/C, MeOH.

Hence, hydroxy-lactam **4** was obtained after reduction of azide **3** and subsequent spontaneous rearrangement of the resulting amine intermediate. With lactam **4** in hand, different protection schemes have been implemented for the hydroxy and the amide groups to enlarge the versatility of the final products.

4.2 Synthesis of N-CO_2Me Protected 4-R-CPAs

Initially, following a procedure already reported by our group,[3] enecarbamate ester **8** was obtained in four steps starting from lactam **4** (scheme 3). At first, the hydroxy group was protected as *tert*-butyldimethylsilyl ether and the ring nitrogen as methyl carbamate, resulting in diprotected intermediate **6**, which was quantitatively converted into the corresponding vinyl phosphate **7** by treatment with potassium bis(trimethylsilyl) amide (KHMDS) at –78 °C in tetrahydrofuran (THF), followed by the addition of diphenylchlorophosphate. Pd-catalysed methoxycarbonylation reaction of the phosphate in anhydrous DMF and at atmospheric pressure was carried out at 65 °C in the presence of an excess of MeOH to give key ester intermediate **8** in 44% yield after four steps.

Afterward, deprotection of the 5-OH group was accomplished to set the stage for the OH-directed stereoselective cyclopropanation of **9**.[4] Hydroxy group deprotection was performed experimenting two possible procedures. At first, it was carried out by using tetrabutylammonium fluoride (TBAF), but a partial elimination of *tert*-butyldimethylsilanol lowered the yield to 70%. As here reported, better results were obtained by using 3N HCl in acetonitrile, leading to a remarkable 92% yield.[5]

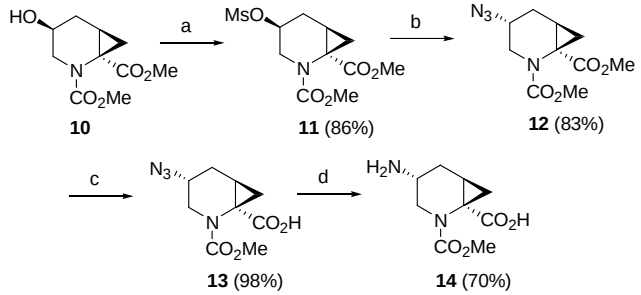


Scheme 3. Synthesis of 4-hydroxy-CPA. Reagents and conditions: a) TBSCl, Imidazole, DMF, 40 °C; b) MeOCOCl, *n*-BuLi, THF, –78 °C; c) KHMDS, $(PhO)_2P(O)Cl$, THF, –78 °C; d) $Pd(OAc)_2$, Ph_3P, CO, MeOH, Et_3N, DMF, 65 °C; e) 3N HCl, CH_3CN, 45 min; f) Et_2Zn, CH_2I_2, CH_2Cl_2, reflux.

The OH-directed cyclopropanation of homoallylic alcohol **9**, as expected, required a longer reaction time than what is usually required for allylic alcohols, such as the one previously used by our group for the synthesis of 5-OH-CPA (see section 2.3, 5.1 and 5.2).[6] We first investigated the use of Charette's zinc carbenoid ($2,4,6\text{-}Cl_3C_6H_2OZnCH_2I$),[7] which had provided the best results for 5-OH-CPA, but the reaction was sluggish at room temperature and never reached completion. Despite the low conversion rate, we were glad to observe that the hydroxy group still exerted a complete stereocontrol, thus leading to the *cis*-isomer **10** only. To attain complete conversion, we increased the temperature by carrying out the reaction in CH_2Cl_2 under reflux conditions and with two equivalents of the Wittig–Furukawa zinc carbenoid ($Zn(CH_2I)_2$).[8] Under these conditions, the reaction ended in 18 h to provide **10** still with complete stereoselectivity and in 71% yield after chromatography.[4]

With the 4-hydroxy-2-azabicyclo-[4.1.0]heptane-1-carboxylic acid derivative **10** in hand, the stage was set for the synthesis of the corresponding 4-amino- derivative **14** (scheme 4). The synthesis of **14**, which, as a δ-amino acid, is a dipeptide mimic, entailed *O*-mesylation (86%) followed by nucleophilic substitution with sodium azide to provide azido derivative **12** (83%)

with the correct *cis* stereochemistry of the two groups on the ring as required for the construction of a cyclopeptide. After hydrolysis of the ester group (93%) carried out with 1N NaOH in MeOH, hydrogenation over Pd/C at room temperature and atmospheric pressure provided, after 16 h, pure amino acid **14** in 70% yield.[4]



Scheme 4. Synthesis of 4-amino-CPA. Reagents and conditions: a) MsCl, Et_3N, CH_2Cl_2, –30 °C to 25 °C, 1 h; b) NaN_3, [15]crown-5, CH_3CN, reflux, 6 h; c) 1N NaOH, MeOH, RT, 24 h; d) H_2 (1 atm), 10% Pd/C, MeOH, RT, 16 h.

This approach allowed us to obtain, in a single run, almost 200 mg of pure **14**, which we used for the synthesis of the first cyclopeptide integrin ligand, as we report later (see section 6.2).

4.3 Stereochemical and Conformational Investigation on CPAs

The relative stereochemistry and preferred conformation of compounds **10** and **14** are easily assigned by the analysis of the coupling constants in the ^{1}H NMR spectra and by nuclear Overhauser effect (NOE) studies with the support of a molecular modelling study (figure 1).[9]

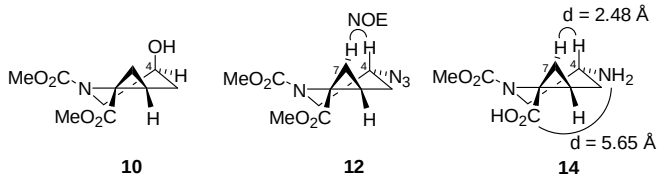


Figure 1. Stereochemical and conformational analysis of CPAs.

From the molecular modelling, it appears that the cyclopropane ring imposes a slightly distorted half-chair conformation on the piperidine ring with the 4-OH group axially oriented in compound **10**. In fact, 4-H resonates as a broad singlet at about δ=4.04 ppm owing to very small vicinal coupling constants, in accordance with its equatorial position. Moreover, the absence of any NOE between the same proton and the *endo* proton of the cyclopropane ring is in accordance to the *cis*-relative position of the OH and the cyclopropane ring. This is confirmed by the NOE correlation observed between 4-H and the *endo* proton of the cyclopropane ring in compound **12** obtained by S_N2 on **10** after its mesylation. In this compound, 4-H is axially oriented as suggested by the structured multiplet at about δ=3.45 ppm (two *trans*-diaxial ^3J couplings), and the observed NOE correlation with the cyclopropane proton that resonates at δ=0.8 ppm as a doublet of doublets is consistent with a *trans*-relative position between the cyclopropane and the azido group. The calculated distance between 4-H

and the cyclopropane *endo* proton, in its amino derivative **14**, is about 2.5 Å. Also for this compound, a molecular modelling analysis resulted in the half-chair conformation being the less energetic, and because of the equatorial position of the amino group, proton 4-H resonates at δ=3.1 ppm as a structured multiplet.

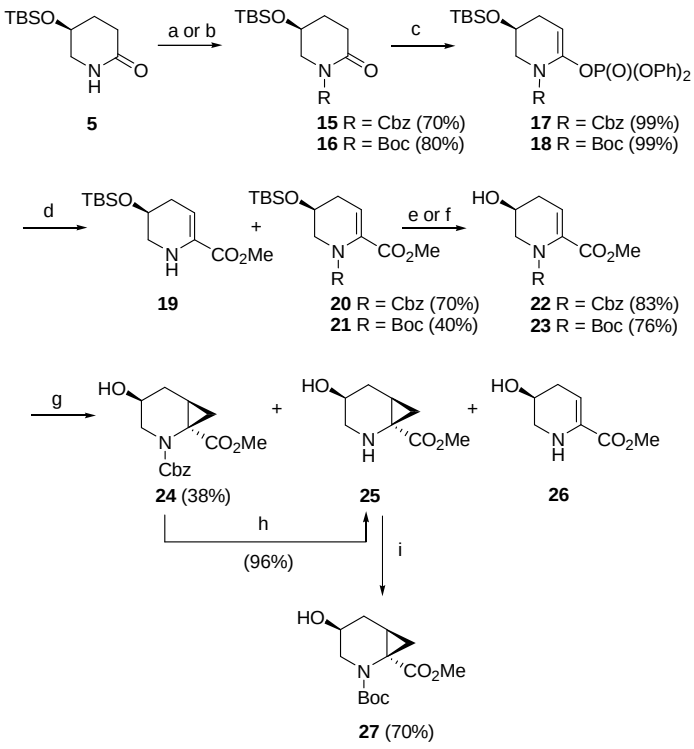
4.4 Synthesis of N-CO_2Bn- and N-CO_2*t*Bu-Protected 4-OH-CPAs

Since our CPAs were meant to be employed in peptide synthesis, we wanted to obtain final products the most versatile possible, where all the functional groups could be exploited for the further decoration of these scaffolds. Concerning this issue, it is fundamental to obtain CPA derivatives containing easily cleavable and possibly orthogonal protecting groups. Accordingly, protecting the ring nitrogen as methyl carbamate cannot be considered an optimal strategy, due to the harsh conditions necessary for its cleavage.

Therefore, we decided to tackle the synthesis of differently N-protected CPAs to guarantee the possibility of exploiting also the ring nitrogen either for peptide coupling or as anchoring point for other chemical entities (activity-modulating groups, physicochemical properties-modulating groups, drugs or imaging molecules).

At first, we tried to follow the same reaction sequence already successfully applied, introducing in place of the methyl carbamate alternatively a benzyl carbamate or a *tert*-butyl carbamate. Unfortunately, this approach resulted surprisingly quite troublesome because of two different and unexpected side reactions, leading to the premature partial or total loss of these protecting groups (scheme 5).

Initial conversion of lactam **5** into the N-Cbz- and N-Boc-protected vinyl phosphates **17** and **18**, which was carried out, as already reported, by treatment with potassium bis(trimethylsilyl)amide (KHMDS) at –78 °C and then trapping the enolates with diphenylchlorophosphate, was uneventful. However, whereas the Pd-catalyzed methoxycarbonylation of **17** carried out at 60 °C proceeded very well to give **20** in good yield (70%), after performing the same reaction on N-Boc-protected compound **18**, we observed the formation of carbonylated product **19**, which lacked the *tert*-butoxycarbonyl group, as the main product together with a minor amount of desired compound **21**. Under the best conditions, in the presence of a strong excess amount of MeOH (130 eq.) and at 75 °C, compound **21** was obtained in a 3:1 ratio with **19** and in 40% yield after chromatography on neutral alumina, whereas using the usual lower amount of MeOH (42 eq.) the yield was below 20% both at 55 °C and 75°C. Compound **19** was quite labile (it decomposed on standing at room temperature), and an attempt to protect its nitrogen atom to obtain again the corresponding *tert*-butyl carbamate **21** caused complete decomposition. It is worth to notice that our group described in the past a Pd-catalyzed process that leads to Weinreb amides in which the N-Boc protection was perfectly compatible with the carbonylative conditions, even if in that case the substrate was a more reactive triflate, the nucleophiles were amines and, above all, the reactions were conducted at room temperature.[10] On the other hand, Pd-catalyzed alkoxycarbonylations of lactam-derived vinyl phosphates were never been carried out with N-Boc-protected lactams but only with N-Ts-, N-Cbz-, N-acyl-, and N-CO_2Me-protected ones.[1,11]

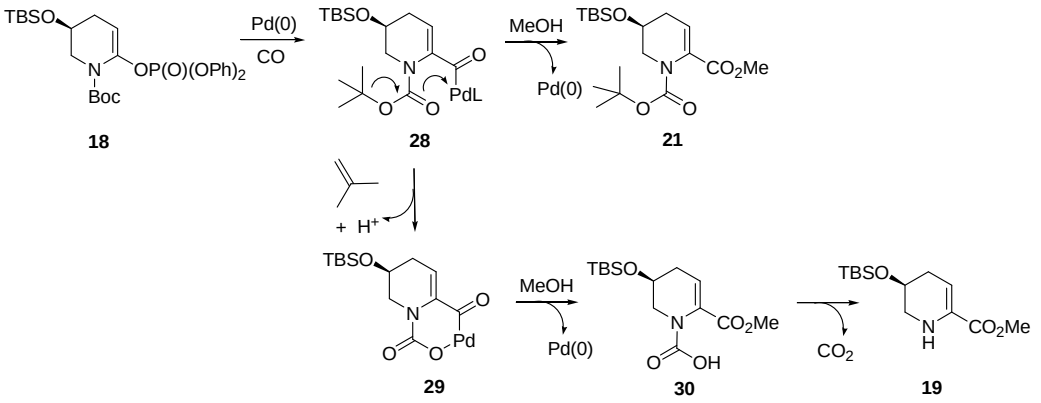


Scheme 5. Synthesis of N-CO_2Bn and N-CO_2tBu protected 4-OH-CPAs. Reagents and conditions: a) BnOCOCl, *n*-BuLi, THF, –78 °C; b) Boc_2O, Et_3N, 4-dimethylaminopyridine (DMAP), CH_2Cl_2, reflux, 5 h; c) KHMDS, $(PhO)_2P(O)Cl$, THF, –78 °C; d) $Pd(OAc)_2$, Ph_3P, CO, MeOH, Et_3N, DMF, 60 or 75 °C, 1.5 h; e) 3N HCl, CH_3CN, 1.5 h; f) TBAF, THF, 30 min; g) Et_2Zn, CH_2I_2, CH_2Cl_2, –15 °C to reflux, 16 h; h) H_2 (1 atm), 10% Pd/C, EtOAc, RT, 4 h; i) Boc_2O, Et_3N, MeOH, reflux, 4 h.

We proposed a possible mechanism for this undesired decarboxylation process (scheme 6). Following oxidative addition and subsequent CO insertion, the *tert*-butyl group should be lost concurrently to carbonyl attack to the Pd^{II} in the acyl–palladium complex **28** to produce intermediate **29**. Reductive elimination after addition of MeOH generated carbamic acid **30**, which underwent rapid decarboxylation to provide **19**. Increasing the relative amount of MeOH favoured coordination of MeOH to palladium in complex **28**, speeding up the rate of reductive elimination of **28** and thus the formation of desired compound **21**, although the yield was far from satisfactory.

Despite the low yield in compound **21**, we proceeded with the deprotection of the 5-OH group which, in this case, was necessarily carried out using TBAF, due to the presence of the acid labile N-Boc. As expected, we registered the formation of a certain amount of elimination product, but the yield in compound **23** was a more than satisfactory 76%. Unfortunately, when we tried its cyclopropanation by using the Wittig-Furukawa zinc carbenoid in refluxing CH_2Cl_2, another side reaction took place. Cyclopropanation of **23** occurred only in part accompanied by the complete loss of the Boc group to give an approximately 3:1 mixture of **25** and **26** after 16 h. As observed for **19**, also side product **26** resulted extremely unstable and decomposed

completely in an attempt to protect again its nitrogen atom. We conducted the cyclopropanation also at room temperature, but the outcome was even worse due to a slower rate of cyclopropanation and yet the contemporary complete loss of the Boc group.



Scheme 6. Possible mechanism for decarboxylation of N-Boc-protected derivative.

This was another unexpected result, since the N-Boc does seem perfectly tolerant to the Wittig–Furakawa cyclopropanation conditions,[12,13] but the longer reaction times and probably the relative position of the OH group with respect to the N protection, could account for our results. It is possible that coordination of both the 4-OH and Boc carbonyl groups to the Zn atom in a seven-membered cycle could facilitate loss of the *tert*-butyl cation from the N-protecting group. Actually, we found at least one case in which N-Boc loss was reported in the cyclopropanation of an N-Boc-protected tetrahydropyridine bearing a coordinating hydroxy group.[14]

We encountered the same complication, although to a lesser extent, in the cyclopropanation of N-Cbz-protected compound **22**, carried out according to the same procedure, which provided cyclopropane derivative **24** but only in 38% yield, when increasing the relative amount of zinc carbenoid to four equivalents.[4] Again, since cyclopropanation of the corresponding N-Cbz-protected allylic alcohol and 4,5-dihydroxy derivatives proceeded uneventfully at room temperature,[6,4] the same aforementioned reasons could account for this degradation process.

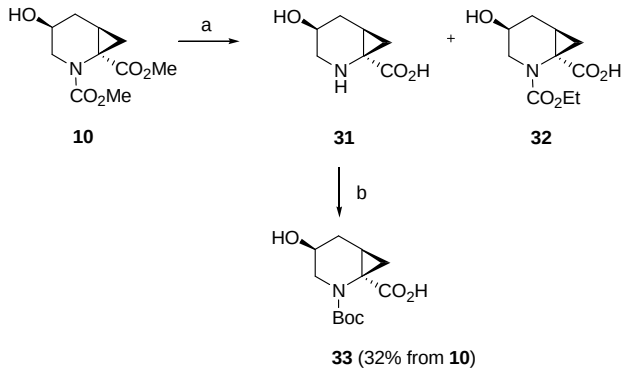
In any case, N-Boc-protected compound **27** could be prepared from **24** by removal of the Cbz group by hydrogenation (96% yield), followed by N protection with di-*tert*-butyl dicarbonate in **25**, which provided N-Boc-protected cyclopropane pipecolic acid derivative **27** in 70% yield.[4]

4.5 Further Attempts for the Synthesis of Differently N-Protected 4-OH-CPAs

Being obviously not completely satisfied with the above mentioned procedures, we decided to investigate alternative routes for the synthesis of differently N-protected CPAs.

At first, having already in hand an optimized strategy for the synthesis of N-CO_2Me-protected CPA **10**, we decided to start again from it and try the deprotection of this methyl carbamate. Previous attempts of acidic hydrolysis had led to complete decomposition of **10**, due to the reported lability to strong acid conditions of cyclopropyl amino acids.[15] Therefore, we tried a basic hydrolysis (KOH 25 eq. in refluxing EtOH) (scheme 7), knowing that the methyl ester

would be hydrolysed as well, but confident to be able to selectively and orthogonally protect again all the functional groups at a later stage.



Scheme 7. Basic hydrolysis of N-CO_2Me-protected CPA. Reagents and conditions: a) NaOH 25 eq., EtOH, reflux, 8h or 48h; b) Boc_2O, Et_3N, MeOH, reflux.

Refluxing the reaction for 8 h led to complete disappearance of **10**, but the expected product **31** was obtained in an inseparable approximately 1:1 mixture with the side product **32**, originated by the nucleophilic attack of the solvent on the carbamate. Retaining **32** an intermediate in desired product formation, we tried to apply longer reaction times and we observed that 48 h of reflux were necessary for the total disappearance of **32**. On the other hand, NMR of the crude showed that a certain amount of degradation products has been accumulated, whereas NMR of the first reaction crude (8 h attempt) was extremely clean except for the presence of the main side product. Our concerns found confirmation when we performed the selective N-protection of **31** to the corresponding *tert*-butyl carbamate **33** and we were able to isolate this product with only a 32% yield. Since nothing suggested that the protection had been troublesome, it is very likely that the low yield was caused by degradation in the hydrolysis step.

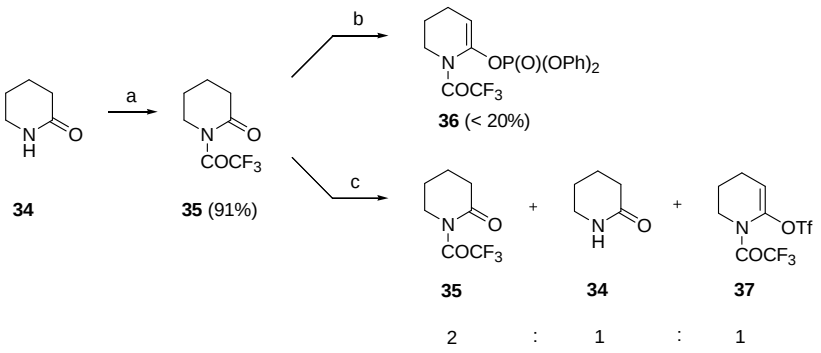
Along the way, we demonstrated that **31** can be selectively protected even if in a mixture with **32**, obtaining a new easily separable mixture of **33** and **32**. Therefore, 8 h hydrolysis, protection and separation could be employed in an iterative way to convert both **10** and **32** to **33** without degradation, but the procedure would be certainly time- and resource-consuming.

Subsequently, we tried a different approach, screening other possible more exotic protecting groups for the ring nitrogen to verify their compatibility with our synthetic strategy towards CPAs, using unsubstituted δ-valerolactam as model substrate.

Initially, we protected the lactam as trifluoroacetamide (-$NCOCF_3$) (scheme 8), since this group cannot undergo decarboxylation during the cyclopropanation step, and it is easily cleaved in aqueous basic conditions.[16]

Following a reported procedure,[17] the protection was performed with trifluoroacetic anhydride (TFAA) in toluene, giving pure protected known lactam **35** with excellent yield, although it resulted too unstable to be chromatographed. We tried to convert **35** in the correspondent vinyl phosphate **36** with the usual procedure, but degradation took place and the yield resulted very low (20% before purification). Probably, the basic conditions of the

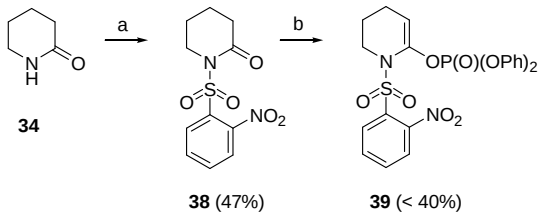
reaction and the work-up were harsh enough to cause protecting group loss and subsequent degradation.



Scheme 8. Protection of nitrogen ring as trifluoroacetamide. Reagents and conditions: a) TFAA, Toluene; b) KHMDS, $(PhO)_2P(O)Cl$, THF, –78 °C; c) Tf_2O, DTBMP, CH_2Cl_2.

Alternatively we attempted to convert **35** in the correspondent vinyl triflate **37** employing trifluoromethanesulfonic anhydride (Tf_2O) and di-*tert*-butyl-methylpyridine (DTBMP) in CH_2Cl_2. Unfortunately, in this case the conversion resulted very low and protecting group loss occurred at a certain extent anyway.

Therefore, being trifluoroacetamide not suitable for our synthetic strategy, we proceeded with the screening of a completely different protecting group, namely an *ortho*-nitrobenzenesulfonyl amide (scheme 9); this group should guarantee orthogonality, since it is removed under mild basic conditions only in the presence of thiols, being otherwise stable in both acidic and basic media.[18,19]

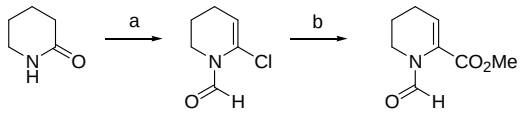


Scheme 9. Protection of nitrogen ring as *ortho*-nitrobenzenesulfonyl amide. Reagents and conditions: a) *n*-BuLi, *o*NBSCl, THF, –78 °C; b) KHMDS, $(PhO)_2P(O)Cl$, THF, –78°C.

Known protected lactam **38**[20] was obtained applying the standard procedure with *n*-BuLi and *ortho*-nitrobenzenesulfonyl chloride (*o*NBSCl) in THF at –78 °C but, surprisingly, even in this case the formation of vinyl phosphate **39** resulted troublesome. The reaction was conducted according to the usual procedure, but formation of degradation products was readily indicated by the almost black colour of the solution and then confirmed by the low yield (40% before purification).

Hence, we discarded also *ortho*-nitrobenzenesulfonyl amide as possible alternative N-protecting group and we proceeded with a further last attempt protecting the ring nitrogen as

formamide (scheme 10). This protecting group, similarly to N-CO_2Me, can be cleaved only in harsh acid or basic conditions but, analogously to dimethylformamide,[21] in principle it should be convertible in various functional groups, including amides.[22] Accordingly, linkers or peptide bond could be introduced without even deprotecting the amine.



34 **40** (70%) **41** (25%)

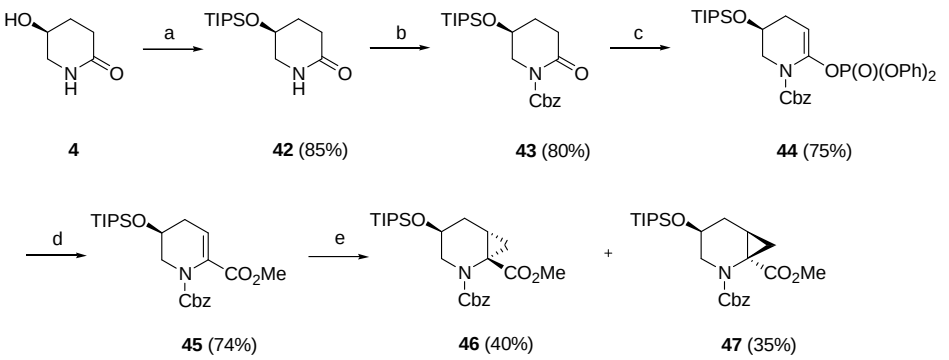
Scheme 10. Protection of nitrogen ring as formamide. Reagents and conditions: a) $POCl_3$, DMF, CH_2Cl_2, reflux; b) MeOH, CO, $Pd(OAc)_2$, Ph_3P, Et_3N, DMF, 75°C.

Following a reported procedure based on the Vilsmeier-Haack reaction,[23] upon treatment with $POCl_3$ and DMF in refluxing CH_2Cl_2 lactam **34** was protected and converted into the correspondent known vinyl chloride **40** in only one step.[24] Unfortunately, despite the sparing of a reaction step, this procedure resulted to hide a drawback, since vinyl chloride **40**, notwithstanding its low stability, demonstrated to be extremely less reactive than phosphate for the following methoxycarbonylation reaction. Complete conversion was obtained only augmenting temperature, reaction time and MeOH quantity (5 h at 75 °C with 70 eq. of MeOH), but in this conditions the yield resulted low anyway (25%), probably due to starting material degradation.
Therefore, even if this procedure resulted very useful in synthetic strategies including different Pd-catalyzed couplings, such as Suzuki or Sonogashira,[24] it is hardly applicable in the case of our Pd-catalyzed alkoxycarbonylation.

4.6 Optimised Synthesis of Differently N-Protected 4-OH-CPAs

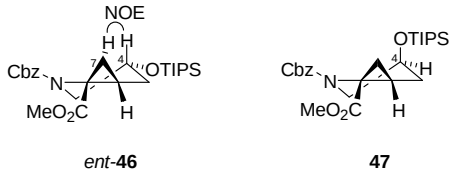
Having obtained poor results concerning the aforementioned protecting groups screening, we decided to go back to benzyl carbamate and change, instead, the conditions for the only troublesome cyclopropanation step (scheme 11).
Since the double bond in enecarbamate esters such **45** is conjugated to a carboxylic group, it is possible to perform the cyclopropanation even by exploiting Michael-type reactions of sulfur methylides;[25] such procedures are conducted in transition metals-free basic media, ensuring the compatibility of benzyl carbamate. Obviously, OH-directed *cis* stereoselectivity would not be possible anymore, lacking the coordinating Zn-carbenoid. On the other hand, a bulky homoallylic protecting group, such as tri-isopropyl silyl ether (-OTIPS), could direct the cyclopropanation onto the opposite face of the double bond providing a *trans* selectivity, as already reported for OTBS-protected allylic alcohols in the synthesis of *trans*-5-OH-CPA.[6]
To investigate this possibility, we synthesized known protected lactam **42**[26] and we transformed it into the correspondent enecarbamate ester **45** employing the usual procedures.
The cyclopropanation step was conducted in dry DMSO, preforming *in situ* the reactive dimethylsulfoxonium methylide upon treatment of trimethylsulfoxonium iodide (TMSOI) with NaH. In this case the synthesis worked fine (total yield: 75%),[27] even if almost no facial selectivity was observed, whereas in the synthesis of 5-OH-CPA a 7:1 *trans*/*cis* ratio had been obtained.[6] We hypothesized that this lack of selectivity had to be ascribed to the larger

distance from the double bond of the homoallylic bulky group, despite its axial disposition (confirmed by NMR).



Scheme 11. Optimised synthesis of N-CO_2Bn protected 4-OH-CPAs. Reagents and conditions: a) TIPSCl, Imidazole, DMF, 40 °C; b) *n*-BuLi, CbzCl, THF, –78 °C; c) KHMDS, $(PhO)_2P(O)Cl$, THF, –78 °C; d) MeOH, CO, $Pd(OAc)_2$, Ph_3P, Et_3N, DMF, 65 °C; e) NaH, TMSOI, DMSO.

Nonetheless, *trans* product **46** and *cis* product **47** resulted surprisingly easy to separate by column chromatography, so that this procedure can be considered an efficient method for the simultaneous synthesis of *trans*-4-OH-CPA **46** and *cis*-4-OH-CPA **47** which can be both subsequently employed as conformationally constrained amino acids for peptide synthesis, as we report later for compound **47** (see chapter 7).
The relative *cis* stereochemistry of **47** was readily assigned because of its identity to the compound obtained by protecting as tri-isopropyl silyl ether the 4-OH of compound **24**; consequently, the opposite *trans* stereochemistry was assigned to **46**. The preferred conformation of both **46** and **47** were assigned by nuclear Overhauser effect (NOE) studies (figure 2).

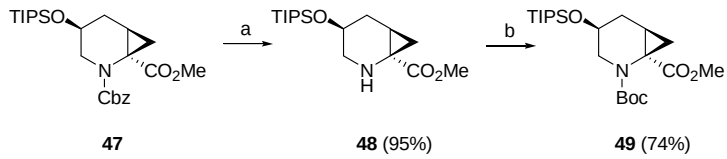


ent-**46** **47**

Figure 2. NOE studies on CPAs

In compound **46** the NOE correlation observed between 4-H and the *endo* proton of the cyclopropane ring is in accordance with an axial orientation of 4-H and a *trans*-relative position between the OTIPS and the cyclopropane ring. On the contrary, no NOE was observed between 4-H and the *endo* proton of the cyclopropane ring in compound **47**, demonstrating an equatorial orientation of 4-H and a *cis*-relative position of the OTIPS and the cyclopropane ring. In both the compounds, as usual, the methyl ester is forced in an axial orientation to remove the $A^{(1,3)}$ strain with the N-protecting group.

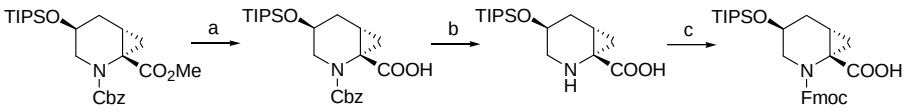
Additionally, to enlarge the scope of this synthetic strategy, we demonstrated even in this case the possibility to easily obtain also the N-CO_2tBu-protected derivative **49** in two steps starting from **47** (scheme 12) by applying the same procedure already described for **27**.[28]



47 **48** (95%) **49** (74%)

Scheme 12. Synthesis of N-CO_2tBu protected 4-OH-CPA. Reagents and conditions: a) H_2 (1 atm), 10% Pd/C, EtOAc, RT, 4 h; b) Boc_2O, Et_3N, MeOH, reflux.

Finally, starting from *trans* diasteroisomer **46** we tackled the protection of the amino group as N-9-fluorenylmethyloxycarbonyl (N-Fmoc) (scheme 13), which is, besides N-Boc, the other protecting group of choice for solid-phase peptide synthesis (SPPS).
In this case we wanted to obtain a product readily utilizable for peptide synthesis, exhibiting contemporarily an N-Fmoc-protected amino group and a free carboxylic acid accessible for peptide coupling.
Accordingly, methyl ester of **46** was selectively hydrolysed in a mixture of 1 N NaOH and MeOH, which had to be heated to 50 °C for 24 h, since differently than in other cases (see sections 4.2, 5.3 and 7.1), the reaction did not proceed at room temperature. This was probably due to the low solubility of the substrate in the reaction mixture or to the remarkable steric hindrance experienced by the methyl ester, possessing a *cis* stereochemistry relative to the bulky –OTIPS group. Nonetheless, product **50** was obtained with a high yield (88%) and converted to free amino acid **51** by means of the usual hydrogenation. Finally, amino group was protected as N-Fmoc by treating **51** with N-(9-fluorenylmethoxycarbonyloxy) succinimide (FmocOSu) in a mixture of aqueous Na_2CO_3 and THF, affording final product **52** in 81% yield.[29]



46 **50** (88%) **51** (94%) **52** (81%)

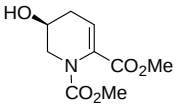
Scheme 13. Synthesis of N-Fmoc protected 4-OH-CPA. Reagents and conditions: a) 1 N NaOH, MeOH, 50 °C, 24 h; b) H_2 (1 atm), 10% Pd/C, EtOAc, RT; c) FmocOSu, Na_2CO_3 aq. THF.

The strategies depicted in scheme 11, 12 and 13 represent the most efficient way, designed so far, to obtain versatile 4-substituted-CPAs. Rigid amino acid scaffolds **46** and **47**, bearing three all orthogonally protected functional groups, were obtained straightforwardly in five steps starting from enantiopure hydroxy-lactam **4** and affording almost half a gram of each diasteroisomer in a single run. Moreover, as demonstrated above, **46** and **47** can be further modified according to the current synthetic needs.

4.7 Experimental

General

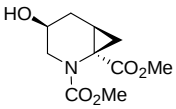
Chromatographic separations were performed under pressure on silica gel 60 (Merck, 70–230 mesh), unless otherwise stated, using flash column techniques; R_f values refer to thin-layer chromatography (TLC) carried out on 0.25 mm silica gel plates with the same eluent indicated for column chromatography. ^{1}H (400 and 200 MHz) and ^{13}C (50.33 and 100.4 MHz) NMR spectra were recorded using Varian Inova and Mercury spectrometers in $CDCl_3$ at 25 °C unless otherwise stated. Mass spectra were carried out by direct inlet using an LCQ Fleet Ion Trap LC/MS system (Thermo Fisher Scientific) with an ESI interface in the positive mode. Microanalyses were carried out with a Perkin-Elmer 2400/2 elemental analyser. Optical rotations were determined with a JASCO DIP-370 instrument. Anhydrous solvents were either commercial or prepared according to standard techniques.



Dimethyl (5*S*)-5-hydroxy-5,6-dihydropyridine-1,2(4*H*)-dicarboxylate [(–)-9]

Compound **8**[3] (1.04 g, 3.16 mmol) was dissolved in CH_3CN (130 mL) and, after cooling to 0 °C, a 3 N solution of HCl (130 mL) was added dropwise. The cooling bath was removed and the mixture was left under stirring for 45 min. Afterward, a satd. solution of $NaHCO_3$ (420 mL) was slowly added until pH 7 was reached, the mixture was extracted with EtOAc (5 x 280 mL) and the combined extractions were dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane/EtOAc, 1:3; R_f 0.26), to afford pure **9** (681 mg, 92%) as a white solid.

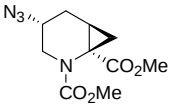
M.p. 101.5-104.0 °C. $[\alpha]_D^{25}$ –25.0 (*c* 0.40, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 6.08 (t, *J* = 3.9 Hz, 1 H, 3-H), 4.18–4.14 (m, 1 H, 5-H), 3.81–3.76 (m, 4 H, 6-H_{eq} + OCH_3), 3.73 (s, 3 H, OCH_3), 3.53 (dd, *J* = 13.1, 2.1 Hz, 1 H, 6-H_{ax}), 2.54 (ddd, *J* = 19.8, 5.6, 3.9 Hz, 1 H, 4-H), 2.22 (dt, *J* = 19.8, 3.9 Hz, 1 H, 4-H'), 2.01 (bs, 1 H, OH). ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 164.7 (CO), 155.6 (NCO), 132.0 (C-2), 121.4 (C-3), 64.1 (C-5), 53.5 (OCH_3), 52.4 (OCH_3), 49.5 (C-6), 32.2 (C-4). MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 238 (5) $[M + Na]^+$, 194 (100). Elemental analysis calcd. (%) for $C_9H_{13}NO_5$ (215.20): C, 50.23; H, 6.09; N, 6.51. Found: C, 50.15; H, 6.21; N, 6.62.



Dimethyl (1*R*,4*S*,6*R*)-4-hydroxy-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-10]

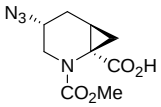
Et_2Zn (6.32 mL of a 1M solution in hexane, 6.32 mmol) and CH_2I_2 (1.01 mL, 12.6 mmol) were added dropwise under an N_2 atmosphere to a solution of **9** (681 mg, 3.16 mmol) in anhydrous CH_2Cl_2 (30 mL) cooled to –10 °C. After 1 h the cooling bath was removed and the reaction mixture was left under stirring for 1 h at room temperature, then heated to reflux for 18 h. The suspension was then cooled in an ice bath and a satd. solution of NH_4Cl (30 mL) was added dropwise under vigorous stirring. The cooling bath was removed and when the solution became clear, the layers were separated, the aqueous layer was extracted with CH_2Cl_2 (3 × 30

mL) and the combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by flash chromatography (*n*-hexane/EtOAc, 1:3; R_f 0.24), to afford pure **10** (514 mg, 71%) as a colourless oil.
$[\alpha]_D^{21}$ +27.8 (*c* 1.36, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (2.5:1 mixture of rotamers) δ (ppm): 4.04–3.96 (m, 2 H, 4-H both rotamers + 3-H_{eq} major rotamer), 3.81 (dd, *J* = 13.6, 4.1 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.72 (s, 3 H, OCH_3, minor rotamer), 3.70 (s, 3 H, OCH_3, minor rotamer), 3.69 (s, 3 H, OCH_3, major rotamer), 3.67 (s, 3 H, OCH_3, major rotamer), 3.04 (dd, *J* = 13.6, 2.3 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.87 (dd, *J* = 13.3, 1.4 Hz, 1 H, 3-H_{ax}, major rotamer), 2.40 (bs, 1 H, OH, major rotamer), 2.32 (bs, 1 H, OH, minor rotamer), 2.14–2.00 (m, 1 H, 5-H, both rotamers), 1.93–1.88 (m, 1 H, 5-H', both rotamers), 1.82 (dd, *J* = 10.1, 4.6 Hz, 1 H, 7-H, both rotamers), 1.69–1.59 (m, 1 H, 6-H, both rotamers), 1.22 (dd, *J* = 8.0, 4.6 Hz, 1 H, 7-H', major rotamer), 1.19–1.17 (m, 1 H, 7-H', minor rotamer). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 173.0 (CO), 158.5 (NCO), 64.8 (C-4), 53.1 (OCH_3), 52.3 (OCH_3), 46.7 (C-3), 38.3 (C-1), 27.8, 23.9, 22.5. MS (ESI) m/z (%): 481 (76) $[2M + Na]^+$, 252 (100) $[M + Na]^+$. Elemental analysis calcd. (%) for $C_{10}H_{15}NO_5$ (229.23): C, 52.40; H, 6.60; N, 6.11. Found: C, 52.33; H, 6.74; N, 5.99.



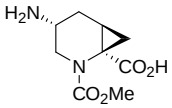
Dimethyl (1*R*,4*R*,6*S*)-4-azido-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(–)-12]
MsCl (195 µL, 2.47 mmol) was added dropwise to a solution of **10** (436 mg, 1.90 mmol) and triethylamine (343 µL, 2.47 mmol) in anhydrous CH_2Cl_2 (10 mL) cooled to –30 °C. After 5 min the cooling bath was removed and the reaction mixture was left under stirring for 1 h at room temperature. Then a 0.5 N solution of HCl (1 mL) was added dropwise and after 10 min the mixture was extracted with CH_2Cl_2 (3 x 5 mL). The combined organic layers were washed with water (3 x 5 mL) and dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane/EtOAc 1:2, R_f 0.24) to afford compound **11** (502 mg, 86%) as a colourless oil.
^{1}H NMR (400 MHz, $CDCl_3$) (2:1 mixture of rotamers) δ (ppm): 5.00-4.97 (m, 1 H, 4-H, major rotamer), 4.95-4.90 (m, 1 H, 4-H, minor rotamer), 4.32 (dd, *J* = 14.3, 3.7 Hz, 1 H, 3-H_{eq}, major rotamer), 4.18 (dd, *J* = 14.3, 4.0 Hz, 3-H_{eq}, minor rotamer), 3.75 (s, 3 H, OCH_3, minor rotamer), 3.74 (s, 3 H, OCH_3, minor rotamer), 3.73 (s, 3 H, OCH_3, major rotamer), 3.71 (s, 3 H, OCH_3, major rotamer), 3.09 (dd, *J* = 14.3, 2.0 Hz, 1 H, 3-H_{ax}, minor rotamer), 3.02 (s, 3 H, SCH_3, major rotamer), 2.98 (s, 3 H, SCH_3, minor rotamer), 2.94 (dd, *J* = 14.3, 1.6 Hz, 1 H, 3-H_{ax}, major rotamer), 2.34–2.15 (m, 2 H, 5-H + 5-H', both rotamers), 1.97 (dd, *J* = 9.9, 5.2 Hz, 1 H, 7-H, minor rotamer), 1.91 (dd, *J* = 10.0, 5.1 Hz, 1 H, 7-H, major rotamer), 1.76–1.66 (m, 1 H, 6-H, both rotamers), 1.16-1.11 (m, 1 H, 7-H', both rotamers).
Mesylate **11** was dissolved in anhydrous acetonitrile (10 mL) and [15]crown-5 (65 µL, 0.33 mmol) and NaN_3 (159 mg, 2.45 mmol) were added to the resulting solution under an N_2 atmosphere. The mixture was heated to reflux for 6 h and then stirred at room temperature for 12 h. Afterward, the mixture was filtered through a silica/celite 1:1 pad and the filtrate concentrated under *vacuum*. The residue was purified by flash chromatography (*n*-hexane/EtOAc, 3:1; R_f 0.21) affording pure **12** (344 mg, 83%) as a colourless oil.

$[\alpha]_D^{22}$ –18.4 (*c* 0.68, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) (1.8:1 mixture of rotamers) δ (ppm): 4.08 (dd, *J* = 13.1, 4.7 Hz, 1 H, 3-H_{eq}, major rotamer), 3.92 (dd, *J* = 13.1, 4.6 Hz, 3-H_{eq}, minor rotamer), 3.73 (s, 3 H, OCH_3, minor rotamer), 3.70 (s, 3 H, OCH_3, major rotamer), 3.69 (s, 3 H, OCH_3, major rotamer), 3.68 (s, 3 H, OCH_3, minor rotamer), 3.51-3.42 (m, 1 H, 4-H, both rotamers), 2.83 (dd, *J* = 13.1, 8.6 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.76 (dd, *J* = 13.1, 8.2 Hz, 1 H, 3-H_{ax}, major rotamer), 2.02-1.94 (m, 3 H, 5-H + 5-H' both rotamers + 7-H minor rotamer), 1.89 (dd, *J* = 9.8, 4.9 Hz, 1 H, 7-H, major rotamer), 1.85-1.80 (m, 1 H, 6-H, both rotamers), 0.86-0.80 (m, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.2 (CO), 156.9 (NCO), 55.1 (C-4), 53.1 (OCH_3), 52.5 (OCH_3), 44.9 (C-3), 37.7 (C-1), 27.2, 23.6, 22.6. MS (ESI) *m/z* (%): 277 (100) $[M + Na]^+$. Elemental analysis calcd. (%) for $C_{10}H_{14}N_4O_4$ (254.24): C, 47.24; H, 5.55; N, 22.04. Found: C, 47.01; H, 5.31; N, 22.43.



(1*R*,4*R*,6*S*)-4-Azido-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(–)-13]

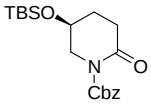
A 1 N solution of NaOH (2 mL) was added to a solution of **12** (344 mg, 1.35 mmol) in methanol (3 mL) and the resulting mixture was vigorously stirred for 24 h at room temperature. Afterward, the methanol was evaporated, the remaining aqueous layer was acidified to pH 3 by adding a 1 N solution of HCl, and the product was extracted with $CHCl_3$ (5 x 5 mL). Then the aqueous layer was further acidified to pH 1 and the product was extracted again with $CHCl_3$ (5 x 5 mL). The combined organic layers were dried over Na_2SO_4 and after filtration and evaporation of the solvent, compound **13** (302 mg, 93%) was obtained as a thick colourless oil. $[\alpha]_D^{27}$ –17.9 (*c* 0.75, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) (1.6:1 mixture of rotamers) δ (ppm): 8.70 (bs, 1 H, COOH), 4.10-4.06 (m, 1 H, 3-H_{eq}, major rotamer), 3.93-3.90 (m, 1 H, 3-H_{eq}, minor rotamer), 3.74 (s, 3 H, OCH_3, both rotamers), 3.52-3.47 (m, 1 H, 4-H, both rotamers), 2.88-2.76 (m, 1 H, 3-H_{ax}, both rotamers), 2.02-1.87 (m, 4 H, 5-H + 5-H' + 6-H + 7-H, both rotamers), 0.89-0.86 (m, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 177.7 (COOH), 157.0 (NCO), 55.3 (C-4), 53.4 (OCH_3), 45.3 (C-3), 37.6 (C-1), 27.5, 24.5, 24.1. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 263 (23) $[M + Na]^+$, 220 (46), 206 (100), 187 (57). Elemental analysis calcd. (%) for $C_9H_{12}N_4O_4$ (240.22): C, 45.00; H, 5.04; N, 23.32. Found: C, 45.35; H, 4.88; N, 23.63.



(1*R*,4*R*,6*S*)-4-Amino-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid (14)

Pd/C (10%, 40 mg) was added to a solution of **13** (302 mg, 1.26 mmol) in anhydrous methanol (25 mL) under an N_2 atmosphere. The resulting suspension was stirred vigorously under a H_2 atmosphere (balloon) at room temperature for 16 h. After filtration through a celite layer and evaporation of the solvent, pure **14** (189 mg, 70%) was obtained as a white solid directly used in the next step without purification.

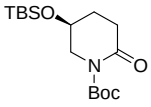
^{1}H NMR (400 MHz, CD_3OD) (2.1:1 mixture of rotamers) δ (ppm): 4.16 (dd, *J* = 12.5, 3.1 Hz, 1 H, 3-H_{eq}, major rotamer), 4.08 (dd, *J* = 13.0, 3.8 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.73 (s, 3 H, OCH_3, major rotamer), 3.71 (s, 3 H, OCH_3, minor rotamer), 3.10-3.03 (m, 1 H, 4-H, both rotamers), 2.92-2.84 (m, 1 H, 3-H_{ax}, minor rotamer), 2.79 (dd, *J* = 12.5, 10.5 Hz, 1 H, 3-H_{ax}, major rotamer), 2.27 (dd, *J* = 13.6, 5.4 Hz, 1 H, 5-H, major rotamer), 2.23-2.20 (m, 1 H, 5-H, minor rotamer), 1.98-1.89 (m, 1 H, 5-H', both rotamers), 1.87-1.70 (m, 2 H, 6-H + 7-H, both rotamers), 0.75 (dd, *J* = 7.03, 4.3 Hz, 1 H, 7-H', major rotamer), 0.70-0.67 (m, 1 H, 7-H', minor rotamer). MS/MS (ESI of $[M]^+$) *m/z* (%): 214 (11) $[M]^+$, 182 (100), 154 (16).



(5*S*)-Benzyl 5-(*tert*-butyldimethylsilanyloxy)-2-oxopiperidine-1-carboxylate [(+)-15]

n-BuLi (306 µL of a 1.6 M solution in hexane, 0.49 mmol) was added dropwise, under an N_2 atmosphere, to a solution of lactam **5**[2] (112 mg, 0.49 mmol) in anhydrous THF (5 mL) cooled to –78 °C, keeping the temperature below –70 °C during the addition. The mixture was stirred for 15 min and then benzyl chloroformate (77 µL, 0.54 mmol) was added dropwise. After 10 min the cooling bath was removed and the temperature was allowed to reach 0 °C. Keeping the reaction flask in an ice bath, a satd. solution of $NaHCO_3$ (2 mL) was added and the mixture was stirred for 10 min. Afterward, the layers were separated, the aqueous layer was extracted with CH_2Cl_2 (3 x 2 mL) and the combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by flash chromatography (*n*-hexane/EtOAc, 4:1; R_f 0.21), to afford pure **15** (125 mg, 70%) as a white solid.

M.p. 35.6-36.8 °C. $[\alpha]_D^{22}$ +12.2 (*c* 0.68, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.44-7.41 (m, 2 H, Ph), 7.38-7.29 (m, 3 H, Ph), 5.29 (s, 2 H, C*H*$_2$Ph), 4.19-4.15 (m, 1 H, 5-H), 3.75 (ddd, *J* = 13.3, 4.7, 1.6 Hz, 1 H, 6-H), 3.68 (dd, *J* = 13.3, 3.7 Hz, 1 H, 6-H'), 2.76 (ddd, *J* = 17.2, 9.4, 6.6 Hz, 1 H, 3-H), 2.46 (ddd, *J* = 17.2, 6.2, 5.7 Hz, 1 H, 3-H'), 2.00-1.92 (m, 1 H, 4-H), 1.90-1.82 (m, 1 H, 4-H'), 0.86 [s, 9 H, $SiC(CH_3)_3$], 0.07 [s, 3 H, $Si(CH_3)_2$], 0.06 [s, 3 H, $Si(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 170.7 (CO), 154.1 (N*C*O_2Bn), 135.5 (C_{arom}), 128.5 (2 C, C_{arom}), 128.2 (C_{arom}), 128.0 (2 C, C_{arom}), 68.5 (*C*H_2Ph), 64.2 (C-5), 52.9 (C-6), 30.9 (C-3), 28.9 (C-4), 25.6 [3 C, $SiC(CH_3)_3$], 18.0 [$SiC(CH_3)_3$], –4.9 [2 C, $Si(CH_3)_2$]. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 386 (37) $[M + Na]^+$, 342 (100). Elemental analysis calcd. (%) for $C_{19}H_{29}NO_4Si$ (363.52): C, 62.78; H, 8.04; N, 3.85. Found: C, 62.40; H, 7.67; N, 3.98.

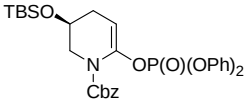


(5*S*)-*tert*-Butyl 5-(*tert*-butyldimethylsilanyloxy)-2-oxopiperidine-1-carboxylate [(+)-16]

Triethylamine (1.14 mL, 8.15 mmol), Boc_2O (1.64 g, 7.41 mmol) and DMAP (91 mg, 0.741 mmol) were added to a solution of lactam **5**[2] (1.70 g, 7.41 mmol) in anhydrous CH_2Cl_2 (45 mL), under an N_2 atmosphere. The resulting reaction mixture was heated to reflux for 5 h, adding further Boc_2O (2.46 g, 11.1 mmol) portionwise over the first 3 h. Afterward, water (55 mL) was added, the layers were separated and the aqueous one was extracted with CH_2Cl_2 (3 x 25 mL). The combined organic layers were washed with a 5% solution of $KHSO_4$ (55 mL), a satd.

solution of $NaHCO_3$ (55 mL), water (55 mL) and brine (55 mL) and then dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by flash chromatography (*n*-hexane/EtOAc, 5:1; R_f 0.23), affording pure **16** (1.95 g, 80%) as a white solid.

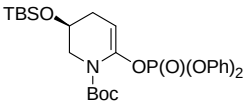
M.p. 34.0-36.0 °C. $[\alpha]_D^{24}$ +7.8 (*c* 0.99, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 4.17-4.12 (m, 1 H, 5-H), 3.68-3.59 (m, 2 H, 6-H + 6-H'), 2.71 (ddd, *J* = 17.2, 9.0, 6.7 Hz, 1 H, 3-H), 2.42 (dt, *J* = 17.2, 6.2 Hz, 1 H, 3-H'), 1.99-1.91 (m, 1 H, 4-H), 1.86-1.78 (m, 1 H, 4-H'), 1.52 [s, 9 H, $OC(CH_3)_3$], 0.88 [s, 9 H, $SiC(CH_3)_3$], 0.08 [s, 6 H, $Si(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 170.9 (CO), 152.5 (NCO_2tBu), 82.9 [$OC(CH_3)_3$], 64.4 (C-5), 52.4 (C-6), 31.1 (C-3), 29.0 (C-4), 28.0 [3 C, $OC(CH_3)_3$], 25.7 [3 C, $SiC(CH_3)_3$], 18.0 [$SiC(CH_3)_3$], –4.9 [2 C, $Si(CH_3)_2$]. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 352 (15) $[M + Na]^+$, 252 (100). Elemental analysis calcd. (%) for $C_{16}H_{31}NO_4Si$ (329.51): C, 58.32; H, 9.48; N, 4.25. Found: C, 57.89; H, 9.95; N, 4.75.



(3*S*)-Benzyl 6-[(diphenoxyphosphoryl)oxy]-3-(*tert*-butyldimethylsilanyloxy)-3,4-dihydropyridine-1(2*H*)-carboxylate (17)

A solution of **15** (125 mg, 0.34 mmol) in anhydrous THF (840 µL) was added dropwise to a solution of KHMDS (850 µL of a 0.5 M solution in toluene, 0.425 mmol) in anhydrous THF (2.2 mL) cooled to –78 °C, under an N_2 atmosphere, and the resulting mixture was stirred for 1.5 h at –78 °C. Afterward, a solution of diphenyl chlorophosphate (88 µL, 0.425 mmol) in anhydrous THF (650 µL) was added dropwise and the reaction was left under stirring for 1 h at –78 °C, before allowing the temperature to reach 0 °C. Then a 5% NaOH aqueous solution (6.5 mL) was slowly added and the product was extracted with Et_2O (3 × 4 mL). The combined organic layers were washed with 5% NaOH (5 mL) and dried over anhydrous K_2CO_3 for 30 min. After filtration and evaporation of the solvent (without heating and leaving a small volume of solvent), the crude phosphate was chromatographed (*n*-hexane/EtOAc, 4:1 with 1% Et_3N; R_f 0.22), affording **17** (201 mg, 99%) as a pale yellow oil, which was immediately used in the next step.

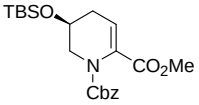
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.36-7.24 (m, 10 H, Ph), 7.20-7.15 (m, 5 H, Ph), 5.10-5.02 (m, 3 H, AB system CH_2Ph + 5-H), 4.04-3.99 (m, 1 H, 3-H), 3.76 (dd, *J* = 12.7, 6.1 Hz, 1 H, 2-H), 3.44 (dd, *J* = 12.7, 2.1 Hz, 1 H, 2-H'), 2.38 (dq, *J* = 18.2, 4.3 Hz, 1 H, 4-H), 2.07 (dq, *J* = 18.2, 3.9 Hz, 1 H, 4-H'), 0.85 [s, 9 H, $SiC(CH_3)_3$], 0.05 [s, 3 H, $Si(CH_3)_2$], 0.04 [s, 3 H, $Si(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 154.6 (NCO_2Bn), 150.3 (2 C, C_{arom}), 139.5 (C-6), 135.8 (C_{arom}), 129.7 (4 C, C_{arom}), 128.4 (2 C, C_{arom}), 128.0 (2 C, C_{arom}), 127.9 (C_{arom}), 125.2 (2 C, C_{arom}), 120.1 (4 C, C_{arom}), 98.5 (C-5), 67.9 (CH_2Ph), 64.7 (C-3), 51.5 (C-2), 32.0 (C-4), 25.7 [3 C, $SiC(CH_3)_3$], 18.0 [$SiC(CH_3)_3$], –5.0 [2 C, $Si(CH_3)_2$]. MS (ESI) *m/z* (%): 1213 (100) $[2M + Na]^+$, 596 (20) $[M + 1]^+$.



(3*S*)-*tert*-Butyl 6-[(diphenoxyphosphoryl)oxy]-3-(*tert*-butyldimethylsilanyloxy)-3,4-dihydropyridine-1(2*H*)-carboxylate (18)

Prepared as reported for **17**, starting from **16** (1.95 g, 5.92 mmol). After flash chromatography (*n*-hexane/EtOAc, 4:1 with 1% Et_3N; R_f 0.46), compound **18** (3.29 g, 99%) was obtained as a pale yellow oil, which was immediately used in the next step.

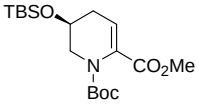
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.36-7.17 (m, 10 H, Ph), 5.04-5.01 (m, 1 H, 5-H), 4.01-3.96 (m, 1 H, 3-H), 3.59-3.49 (m, 2 H, 2-H + 2-H'), 2.43-2.35 (m, 1 H, 4-H), 2.06 (dq, *J* = 18.1, 4.2 Hz, 1 H, 4-H'), 1.42 [s, 9 H, $OC(CH_3)_3$], 0.89 [s, 9 H, $SiC(CH_3)_3$], 0.08 [s, 6 H, $Si(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 153.4 (NCO_2tBu), 150.5 (2 C, C_{arom}), 140.1 (C-6), 129.9 (4 C, C_{arom}), 125.6 (2 C, C_{arom}), 120.3 (4 C, C_{arom}), 98.0 (C-5), 81.8 [$OC(CH_3)_3$], 65.3 (C-3), 51.2 (C-2), 32.3 (C-4), 28.3 [3 C, $OC(CH_3)_3$], 26.0 [3 C, $SiC(CH_3)_3$], 18.4 [$SiC(CH_3)_3$], –4.6 [2 C, $Si(CH_3)_2$].



(5*S*)-1-Benzyl 2-methyl 5-(*tert*-butyldimethylsilanyloxy)-5,6-dihydropyridine-1,2(4*H*)-dicarboxylate [(+)-20]

$Pd(OAc)_2$ (7.6 mg, 0.034 mmol) and Ph_3P (19.7 mg, 0.075 mmol) were added to a solution of phosphate **17** (201 mg, 0.34 mmol) in anhydrous DMF (860 µL), under an N_2 atmosphere. The flask was flushed and saturated with carbon monoxide and the mixture was vigorously stirred for 10 min at 25 °C under carbon monoxide atmosphere. Afterward, Et_3N (92 µL, 0.68 mmol) and anhydrous methanol (578 µL, 14.3 mmol) were added and the mixture was stirred at 60 °C (external bath) for 1.5 h under carbon monoxide atmosphere (balloon). After cooling, water (8 mL) was added, the product was extracted with Et_2O (4 × 8 mL) and the combined organic extracts were dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane/EtOAc, 6:1; R_f 0.17), to afford pure **20** (97 mg, 70%) as a thick colourless oil.

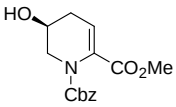
$[\alpha]_D^{19}$ +10.8 (*c* 0.99, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.37-7.29 (m, 5 H, Ph), 6.04 (t, *J* = 3.9 Hz, 1 H, 3-H), 5.12 (AB system, 2 H, CH_2Ph), 4.05-4.03 (m, 1 H, 5-H), 3.60-3.55 (m, 5 H, 6-H + 6-H' + OCH_3), 2.45 (ddd, *J* = 19.3, 5.5, 3.9 Hz, 1 H, 4-H), 2.14 (dt, *J* = 19.3, 3.9 Hz, 1 H, 4-H'), 0.86 [s, 9 H, $SiC(CH_3)_3$], 0.06 [s, 6 H, $Si(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 164.7 (CO), 154.6 (NCO_2Bn), 135.8 (C_{arom}), 132.0 (C-2), 128.4 (2 C, C_{arom}), 128.3 (2 C, C_{arom}), 128.2 (C_{arom}), 121.5 (C-3), 68.0 (CH_2Ph), 64.6 (C-5), 51.9 (C-6), 49.8 (OCH_3), 33.3 (C-4), 25.7 [3 C, $SiC(CH_3)_3$], 18.0 [($SiC(CH_3)_3$], –4.9 [2 C, $Si(CH_3)_2$]. MS (ESI) *m/z* (%): 833 (100) $[2M + Na]^+$, 406 (20) $[M + 1]^+$. Elemental analysis calcd. (%) for $C_{21}H_{31}NO_5Si$ (405.56): C, 62.19; H, 7.70; N, 3.45. Found: C, 62.32; H, 7.44; N, 3.38.



(5*S*)-1-*tert*-Butyl 2-methyl 5-(*tert*-butyldimethylsilanyloxy)-5,6-dihydropyridine-1,2(4*H*)-dicarboxylate [(+)-21]

Prepared as reported for **20**, starting from **18** (3.29 g, 5.86 mmol), using a triple amount of anhydrous methanol (29.9 mL, 738 mmol) and carrying out the reaction at 75 °C. The crude was purified by flash chromatography on neutral aluminium oxide of activity II (prepared from Fluka, 100-125 mesh, Brockmann Activity I) (*n*-hexane/EtOAc, 20:1; R_f 0.31), affording pure **21** (871 mg, 40%) as a colourless oil.

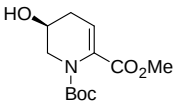
$[\alpha]_D^{23}$ +22.8 (*c* 0.76, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 5.93 (t, *J* = 3.9 Hz, 1 H, 3-H), 4.03-3.95 (m, 1 H, 5-H), 3.77 (s, 3 H, OCH_3), 3.64-3.38 (m, 2 H, 6-H + 6-H'), 2.44 (ddd, *J* = 19.3, 5.7, 3.9 Hz, 1 H, 4-H), 2.12 (ddd, *J* = 19.3, 5.2, 3.9 Hz, 1 H, 4-H'), 1.42 [s, 9 H, $OC(CH_3)_3$], 0.87 [s, 9 H, $SiC(CH_3)_3$], 0.08 [s, 6 H, $Si(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 165.2 (CO), 153.3 (NCO_2tBu), 132.4 (C-2), 120.1 (C-3), 81.3 [$OC(CH_3)_3$], 64.8 (C-5), 51.9 (C-6), 49.1 (OCH_3), 33.3 (C-4), 28.0 [3 C, $OC(CH_3)_3$], 25.8 [3 C, $SiC(CH_3)_3$], 18.1 [($SiC(CH_3)_3$], – 4.9 [2 C, $Si(CH_3)2$]. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 394 (12) $[M + Na]^+$, 338 (100), 294 (57). Elemental analysis calcd. (%) for $C_{18}H_{33}NO_5Si$ (371.54): C, 58.19; H, 8.95; N, 3.77. Found: C, 58.50; H, 9.21; N, 3.82.



1-Benzyl 2-methyl (5*S*)-5-hydroxy-5,6-dihydropyridine-1,2(4*H*)-dicarboxylate [(–)-22]

Prepared as reported for **9**, starting from **20** (97 mg, 0.24 mmol) and carrying out the reaction for 1.5 h. After flash chromatography (*n*-hexane/EtOAc, 1:1; R_f 0.19), pure **22** (58 mg, 83%) was obtained as a white solid.

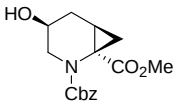
M.p. 87.8-89.2 °C. $[\alpha]_D^{25}$ –14.6 (*c* 0.65, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.38-7.29 (m, 5 H, Ph), 6.06 (t, *J* = 3.9 Hz, 1 H, 3-H), 5.19 (part A of an AB system, *J* = 12.1 Hz, 1 H, CH_2Ph), 5.12 (part B of an AB system, *J* = 12.1 Hz, 1 H, CH_2Ph), 4.17-4.16 (m, 1 H, 5-H), 3.82 (dd, *J* = 12.9, 6.1 Hz, 1 H, 6-H), 3.58-3.54 (m, 4 H, 6-H' + OCH_3), 2.53 (ddd, *J* = 19.7, 5.0, 3.9 Hz, 1 H, 4-H), 2.22 (dt, *J* = 19.7, 3.9 Hz, 1 H, 4-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) δ (ppm): 164.5 (CO), 154.9 (NCO_2Bn), 135.7 (C_{arom}), 132.1 (C-2), 128.5 (2 C, C_{arom}), 128.3 (2 C, C_{arom}), 128.2 (C_{arom}), 121.0 (C-3), 68.2 (CH_2Ph), 64.1 (C-5), 52.0 (OCH_3), 49.4 (C-6), 32.1 (C-4). MS (ESI) *m/z* (%): 292 (100) $[M + 1]^+$. Elemental analysis calcd. (%) for $C_{15}H_{17}NO_5$ (291.30): C, 61.85; H, 5.88; N, 4.81. Found C, 62.11; H, 5.62; N, 4.62.



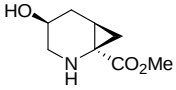
1-*tert*-Butyl 2-methyl (5*S*)-5-hydroxy-5,6-dihydropyridine-1,2(4*H*)-dicarboxylate [(–)-23]

TBAF (2.67 mL of a 1 M solution in THF, 2.67 mmol) was slowly added to a solution of **21** (871 mg, 2.34 mmol) in anhydrous THF (100 mL), under an N_2 atmosphere. After 30 min the solvent was evaporated under *vacuum* and the residue was taken up in Et_2O (100 mL). The solvent was

evaporated again and the residue was purified by flash chromatography (*n*-hexane/EtOAc, 1:1; R_f 0.24), affording pure **23** (458 mg, 76%) as a white solid.
M.p. 116.0-118.0 °C. $[\alpha]_D^{22}$ –21.0 (*c* 0.68, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 5.97 (t, *J* = 3.9 Hz, 1 H, 3-H), 4.14–4.10 (m, 1 H, 5-H), 3.77 (s, 3 H, OCH_3), 3.66 (dd, *J* = 12.9, 6.3 Hz, 1 H, 6-H_{eq}), 3.54 (dd, *J* = 12.9, 2.5 Hz, 1 H, 6-H_{ax}), 2.51 (ddd, *J* = 19.7, 5.7, 3.9 Hz, 1 H, 4-H), 2.40 (bs, 1 H, OH), 2.19 (dt, *J* = 19.7, 3.9 Hz, 1 H, 4-H'), 1.42 [s, 9 H, $OC(CH_3)_3$]. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 165.0 (CO), 153.8 (NCO_2tBu), 132.3 (C-2), 120.1 (C-3), 81.7 [$OC(CH_3)_3$], 64.1 (C-5), 51.9 (OCH_3), 48.9 (C-6), 32.0 (C-4), 27.9 [3 C, $OC(CH_3)_3$]. MS (ESI) *m/z* (%): 537 (100) $[2M + Na]^+$, 258 (43) $[M + 1]^+$. Elemental analysis calcd. (%) for $C_{12}H_{19}NO_5$ (257.28): C, 56.02; H, 7.44; N, 5.44. Found: C, 56.37; H, 7.68; N, 5.45.

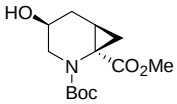


2-Benzyl 1-methyl (1*R*,4*S*,6*R*)-4-hydroxy-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-24]
Prepared as reported for **10**, starting from **22** (58 mg, 0.20 mmol) and using a double amount of Et_2Zn (800 µL of a 1 M solution in hexane, 0.80 mmol) and CH_2I_2 (128 µL, 1.60 mmol). After purification by flash chromatography (*n*-hexane/EtOAc, 1:1; R_f 0.21) pure **24** (23 mg, 38%) was obtained as a colourless oil.
$[\alpha]_D^{25}$ +22.7 (*c* 0.92, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (2.8:1 mixture of rotamers) δ (ppm): 7.36-7.25 (m, 5 H, Ph), 5.26 (part A of an AB system, *J* = 12.5 Hz, 1 H, CH_2Ph, major rotamer), 5.18 (AB system, 2 H, CH_2Ph, minor rotamer), 5.08 (part B of an AB system, *J* = 12.5 Hz, 1 H, CH_2Ph, major rotamer), 4.06-3.99 (m, 2 H, 4-H both rotamers + 3-H_{eq} major rotamer), 3.86 (dd, *J* = 13.6, 4.3 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.69 (s, 3 H, OCH_3, minor rotamer), 3.55 (s, 3 H, OCH_3, major rotamer), 3.07 (dd, *J* = 13.6, 2.5 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.92-2.88 (m, 1 H, 3-H_{ax}, major rotamer), 2.16-1.91 (m, 3 H, 5-H both rotamers + 5-H' both rotamers + OH), 1.85 (dd, *J* = 10.2, 4.7 Hz, 1 H, 7-H, both rotamers), 1.72-1.60 (m, 1 H, 6-H, both rotamers), 1.26 (dd, *J* = 8.0, 4.7 Hz, 1 H, 7-H', major rotamer), 1.24-1.19 (m, 1 H, 7-H', minor rotamer). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.9 (CO), 157.7 (NCO), 136.7 (C_{arom}), 128.4 (2 C, C_{arom}), 127.8 (C_{arom}), 127.4 (2 C, C_{arom}), 67.3 (CH_2Ph), 64.9 (C-4), 52.2 (OCH_3), 46.8 (C-3), 38.4 (C-1), 27.9, 24.1, 22.5. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 306 (3) $[M + 1]^+$, 262 (100). Elemental analysis calcd. (%) for $C_{16}H_{19}NO_5$ (305.33): C, 62.94; H, 6.27; N, 4.59. Found: C, 62.88; H, 6.39; N, 4.44.



Methyl (1*R*,4*S*,6*R*)-4-hydroxy-2-azabicyclo[4.1.0]heptane 1-carboxylate [(+)-25]
Pd/C (5 mg, 10%) was added to a solution of **24** (23 mg, 0.075 mmol) in ethyl acetate (1.5 mL), under an N_2 atmosphere. The resulting suspension was stirred under a H_2 atmosphere (balloon) at room temperature for 4 h. After filtration over a celite layer and evaporation of the solvent, pure **25** (12 mg, 96%) was obtained as a colourless oil.
$[\alpha]_D^{24}$ +91.0 (*c* 0.91, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.88-3.85 (m, 1 H, 4-H), 3.71 (s, 3 H, OCH_3), 2.83 (ddd, *J* = 12.7, 3.9, 1.4 Hz, 1 H, 3-H), 2.69-2.65 (m, 3 H, 3-H' + OH + NH), 2.10-

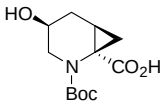
1.98 (m, 2 H, 5-H + 5-H'), 1.64-1.54 (m, 2 H, 6-H + 7-H), 1.15 (dd, *J* = 7.03, 3.5 Hz, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) δ (ppm): 175.4 (CO), 64.0 (C-4), 52.4 (OCH_3), 47.5 (C-3), 39.5 (C-1), 28.7, 26.1, 19.6. MS (ESI) *m/z* (%): 172 (100) $[M + 1]^+$. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 172 (9) $[M + 1]^+$, 112 (100). Elemental analysis calcd. (%) for $C_8H_{13}NO_3$ (171.19): C, 56.13; H, 7.65; N, 8.18. Found: C, 56.37; H, 7.36; N, 8.01.



2-*tert*-Butyl 1-methyl (1*R*,4*S*,6*R*)-4-hydroxy-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-27]

Triethylamine (20 µL, 0.14 mmol) and Boc_2O (31 mg, 0.14 mmol) were added to a solution of **25** (12 mg, 0.070 mmol) in anhydrous methanol (2 mL), under an N_2 atmosphere, and the resulting reaction mixture was heated to reflux for 4 h. Afterward, water (3 mL) was added and the product was extracted with CH_2Cl_2 (5 x 2 mL). The combined organic extracts were washed with a 5% solution of $KHSO_4$ (3 mL), a satd. solution of $NaHCO_3$ (3 mL), water (3 mL) and brine (3 mL) and then dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by flash chromatography (*n*-hexane/EtOAc, 1:2; R_f 0.34), affording pure **27** (13 mg, 70%) as a white gummy solid.

$[\alpha]_D^{24}$ +22.5 (*c* 0.60, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (3.6:1 mixture of rotamers) δ (ppm): 4.08-4.04 (m, 1 H, 4-H, major rotamer), 4.00-3.96 (m, 2 H, 3-H_{eq} major rotamer, 4-H minor rotamer), 3.73-3.72 (m, 1 H, 3-H_{eq}, minor rotamer), 3.70 (s, 3 H, OCH_3, major rotamer), 3.69 (s, 3 H, OCH_3, minor rotamer), 3.06 (dd, *J* = 13.6, 2.6 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.84 (dd, *J* = 13.7, 1.8 Hz, 1 H, 3-H_{ax}, major rotamer), 2.06-1.99 (m, 1 H, 5-H, both rotamers), 1.96-1.92 (m, 1 H, 5-H', both rotamers), 1.82 (dd, *J* = 10.0, 4.6 Hz, 1 H, 7-H, both rotamers), 1.73-1.58 (m, 2 H, 6-H both rotamers + OH), 1.48 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.43 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.18 (dd, *J* = 8.0, 4.6 Hz, 1 H, 7-H', major rotamer), 1.16-1.14 (m, 1 H, 7-H', minor rotamer). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 173.4 (CO), 157.0 (NCO), 80.3 [$OC(CH_3)_3$], 65.5 (C-4), 52.3 (OCH_3), 46.2 (C-3), 38.9 (C-1), 28.5 [3 C, $OC(CH_3)_3$], 28.1, 24.2, 22.8. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 294 (6) $[M + Na]^+$, 238 (21), 194 (100). Elemental analysis calcd. (%) for $C_{13}H_{21}NO_5$ (271.31): C, 57.55; H, 7.80; N, 5.16. Found: C, 57.81; H, 7.62; N, 4.93.



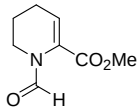
(1*R*,4*S*,6*R*)-4-Hydroxy-2-(*tert*-buthoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(+)-33]

A solution of **10** (78 mg, 0.34 mmol) and KOH (477 mg, 8.5 mmol) in ethanol (4.6 mL) was heated to reflux for 48 h. Afterward, the mixture was allowed to reach room temperature and neutralized with a 1.25 M solution of HCl in MeOH. After filtration over a celite layer and evaporation of the solvent, the deprotected amino acid **31** was obtained as an orange solid directly used in the next step without purification.

^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 4.09-4.07 (m, 1 H, 4-H), 3.11 (dd, *J* = 12.7, 3.7 Hz, 1 H, 3-H), 2.87 (dd, *J* = 13.7, 1.2 Hz, 1 H, 3-H'), 2.07 (t, *J* = 4.0 Hz, 2 H, 5-H), 1.75-1.71 (m, 1 H, 6-H), 1.56-1.48 (m, 1 H, 7-H), 1.30-1.22 (m, 1 H, 7-H'). MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 112 (100).

Amino acid **31** was dissolved in anhydrous methanol (4 mL) and triethylamine (190 µl, 3.16 mmol) and Boc_2O (300 mg, 3.16 mmol) were added to the resulting solution, under an N_2 atmosphere. The reaction mixture was heated to reflux for 24 h, then the solvent was evaporated under vacuum and the residue was dissolved in H_2O (5 mL). $NaH_2PO_4{\cdot}2H_2O$ (50 mg) was added to the resulting solution, which was then cooled to 0 °C, acidified to pH 2, adding a 1 N solution of HCl, and stirred for 30 min at 0 °C. Afterward, the product was extracted with EtOAc (6 x 5 mL) and the combined organic extracts were dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (CH_2Cl_2/methanol 10:1, R_f 0.25), to afford compound **33** (28 mg, 32% from **10**) as a pale yellow oil.

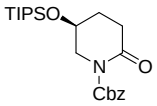
$[\alpha]_D^{20}$ +7.3 (*c* 0.75, CH_3OH). ^{1}H NMR (400 MHz, CD_3OD) (3:1 mixture of rotamers) δ (ppm): 3.95-3.91 (m, 1 H, 4-H, major rotamer), 3.90-3.88 (m, 1 H, 4-H, minor rotamer), 3.69 (dd, *J* = 13.0, 4.9 Hz, 1 H, 3-H_{eq} major rotamer), 3.64 (dd, *J* = 12.9, 5.1 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.10 (dd, *J* = 12.9, 3.3 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.97 (dd, *J* = 13.0, 3.0 Hz, 1 H, 3-H_{ax}, major rotamer), 2.17-2.10 (m, 1 H, 5-H, both rotamers), 1.80 (dd, *J* = 9.8, 4.7 Hz, 7-H, minor rotamer), 1.75 (dd, *J* = 9.5, 4.3 Hz, 7-H, major rotamer), 1.70-1.59 (m, 2 H, 5-H' + 6-H, both rotamers), 1.48 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.45 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.08 (dd, *J* = 7.4, 4.3 Hz, 1 H, 7-H', major rotamer), 1.06-1.04 (m, 1 H, 7-H', minor rotamer). ^{13}C NMR (100.4 MHz, CD_3OD) (major rotamer) δ (ppm): 177.2 (CO), 158.5 (NCO), 81.2 [$OC(CH_3)_3$], 66.1 (C-4), 47.5 (C-3), 39.6 (C-1), 30.0, 28.7 [3 C, $OC(CH_3)_3$], 24.4, 23.8. MS (ESI) *m/z* (%): 537 (100) $[2M + Na]^+$, 280 (65) $[M + Na]^+$.



Methyl 1-formyl-1,4,5,6-tetrahydropyridine-2-carboxylate (41)

Prepared as reported for **20**, starting from **40**[24] (204 mg, 1.4 mmol), using a double amount of anhydrous methanol (4 mL, 99 mmol) and carrying out the reaction at 75 °C for 5 h. After purification by flash chromatography (*n*-hexane/EtOAc, 2:1; R_f 0.22) pure **41** (60 mg, 25%) was obtained as a pale yellow oil.

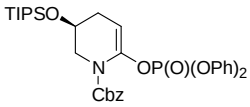
^{1}H NMR ($CDCl_3$, 200 MHz) (5.5:1 mixture of rotamers) δ (ppm): 8.55 (s, 1 H, CHO, major rotamer), 8.01 (s, 1 H, CHO, minor rotamer), 6.42 (t, *J* = 4.0 Hz, 1 H, 3-H, major rotamer), 6.16 (t, *J* = 3.8 Hz, 1 H, 3-H, minor rotamer), 3.81 (s, 3 H, OCH_3, major rotamer), 3.78 (s, 3 H, OCH_3, minor rotamer), 3.71-3.66 (m, 2 H, 6-H + 6-H', major rotamer), 3.57-3.51 (m, 2 H, 6-H + 6-H', minor rotamer), 2.34-2.26 (m, 2 H, 4-H + 4-H', both rotamers), 1.92-1.74 (m, 2 H, 5-H + 5-H', both rotamers). ^{13}C NMR ($CDCl_3$, 50 MHz) (major rotamer) δ (ppm): 163.5 (CO), 161.9 (CHO), 130.1 (C-2), 125.5 (C-3), 52.4 (OCH_3), 38.8 (C-6), 23.4 (C-4), 21.8 (C-5). MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 170 (3) $[M + 1]^+$, 142 (84), 138 (100).



(5*S*)-Benzyl 5-(triisopropylsilanyloxy)-2-oxopiperidine-1-carboxylate [(+)-43]

Prepared as reported for **15**, starting from **42**[25] (1.46 g, 5.38 mmol). After purification by flash chromatography (*n*-hexane/EtOAc, 4:1; R_f 0.28) pure **43** (1.75 g, 80%) was obtained as a colourless oil.

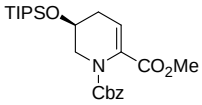
$[\alpha]_D^{20}$ +10.7 (*c* 1.00, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.42-7.40 (m, 2 H, Ph), 7.36-7.25 (m, 3 H, Ph), 5.27 (s, 2 H, C*H*$_2$Ph), 4.29-4.25 (m, 1 H, 5-H), 3.84 (ddd, *J* = 13.1, 4.7, 1.5 Hz, 1 H, 6-H), 3.69 (dd, *J* = 13.1, 3.3 Hz, 1 H, 6-H'), 2.77 (ddd, *J* = 17.2, 9.9, 7.1 Hz, 1 H, 3-H), 2.46 (dt, *J* = 17.2, 5.9 Hz, 1 H, 3-H'), 2.02-1.86 (m, 2 H, 4-H + 4-H'), 1.02 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 170.8 (CO), 154.0 (N*C*O_2Bn), 135.4 (C_{arom}), 128.5 (2 C, C_{arom}), 128.2 (C_{arom}), 128.0 (2 C, C_{arom}), 68.4 (*C*H_2Ph), 64.2 (C-5), 53.0 (C-6), 30.9 (C-3), 29.0 (C-4), 17.9 {6 C, Si[CH(*C*$H_3)_2]_3$}, 12.1 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 833 (100) $[2M + Na]^+$, 406 (26) $[M + 1]^+$. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 362 (72), 272 (100), 228 (29).



(3*S*)-Benzyl 6-[(diphenoxyphosphoryl)oxy]-3-(triisopropylsilanyloxy)-3,4-dihydropyridine-1(2*H*)-carboxylate [(–)-44]

Prepared as reported for **17**, starting from **43** (1.75 g, 4.31 mmol). After flash chromatography (*n*-hexane/EtOAc, 8:1 with 1% Et_3N; R_f 0.23), pure **44** (2.06 g, 75%) was obtained as a colourless oil, which was immediately used in the next step.

$[\alpha]_D^{23}$ –2.6 (*c* 0.70, $CDCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.35-7.24 (m, 10 H, Ph), 7.20-7.15 (m, 5 H, Ph), 5.10-5.02 (m, 3 H, C*H*$_2$Ph + 5-H), 4.13-4.11 (m, 1 H, 3-H), 3.75 (dd, *J* = 12.5, 6.4 Hz, 1 H, 2-H), 3.57 (dd, *J* = 12.5, 2.4 Hz, 1 H, 2-H'), 2.43 (dq, *J* = 18.1, 4.7 Hz, 1 H, 4-H), 2.14 (dq, *J* = 18.1, 4.0 Hz, 1 H, 4-H'), 1.03 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 154.4 (N*C*O_2Bn), 150.4 (2 C, C_{arom}), 139.5 (C-6), 135.7 (C_{arom}), 129.7 (4 C, C_{arom}), 128.4 (2 C, C_{arom}), 128.1 (2 C, C_{arom}), 128.0 (C_{arom}), 125.5 (2 C, C_{arom}), 120.1 (4 C, C_{arom}), 98.6 (C-5), 67.9 (*C*H_2Ph), 64.9 (C-3), 51.6 (C-2), 32.3 (C-4) 17.9 {6 C, Si[CH(*C*$H_3)_2]_3$}, 12.1 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 1298 (100) $[2M + Na]^+$, 638 (8) $[M + 1]^+$.

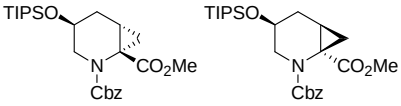


(5*S*)-1-Benzyl 2-methyl 5-(triisopropylsilanyloxy)-5,6-dihydropyridine-1,2(4*H*)-dicarboxylate [(+)-45]

Prepared as reported for **20**, starting from **44** (1.95 g, 3.06 mmol). After flash chromatography (*n*-hexane/EtOAc, 9:1; R_f 0.17), pure **45** (1.02 g, 74%) was obtained as a thick colourless oil.

$[\alpha]_D^{22}$ +16.8 (*c* 0.96, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.36-7.29 (m, 5 H, Ph), 6.04 (t, *J* = 3.7 Hz, 1 H, 3-H), 5.20-5.07 (m, 2 H, C*H*$_2$Ph), 4.13 (bs, 1 H, 5-H), 3.78-3.71 (m, 1 H, 6-H), 3.55 (bs, 4 H, 6-H' + OCH_3), 2.51 (ddd, *J* = 19.5, 5.5, 3.9 Hz, 1 H, 4-H), 2.19 (dt, *J* = 19.5, 4.5 Hz, 1 H, 4-H'), 1.03 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}. ^{13}C NMR ($CDCl_3$, 100.4 MHz) δ (ppm): 164.7

(CO), 154.4 (N*C*O_2Bn), 135.7 (C_{arom}), 131.9 (C-2), 128.4 (2 C, C_{arom}), 128.3 (2 C, C_{arom}), 128.2 (C_{arom}), 121.5 (C-3), 68.0 (*C*H_2Ph), 64.7 (C-5), 52.0 (C-6), 49.9 (OCH_3), 33.6 (C-4), 17.9 {6 C, Si[CH(*C*H_3)$_2$]$_3$}, 12.1 {3 C, Si[*C*H(CH$_3$)$_2$]$_3$}. MS (ESI) *m/z* (%): 917 (100) [2M + Na]$^+$, 448 (67) [M + 1]$^+$. MS/MS (ESI of [M + 1]$^+$) *m/z* (%): 404 (100). Elemental analysis calcd. (%) for $C_{24}H_{37}NO_5Si$ (447.64): C, 64.39; H, 8.33; N, 3.13. Found: C, 63.67; H, 8.95; N, 3.57.



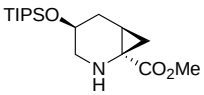
2-Benzyl 1-Methyl (1*S*,4*S*,6*S*)-4-(triisopropylsilanyloxy)-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-46] and 2-Benzyl 1-Methyl (1*R*,4*S*,6*R*)-4-(triisopropylsilanyloxy)-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-47]

NaH (60% in weight in mineral oil, 97 mg, 2.42 mmol), previously washed with dry *n*-hexane (2 × 5 mL), was suspended in dry DMSO (10 mL), under an N_2 atmosphere. Trimethylsulfoxonium iodide (709 mg, 3.22 mmol) was added to the resulting suspension in three portions and the mixture was stirred at room temperature for 30 min, until complete disappearance of the precipitate. Then, a cooling bath at 15 °C was applied and a solution of **45** (722 mg, 1.61 mmol) in dry DMSO (5 mL) was added dropwise to the reaction mixture. The cooling bath was removed and the reaction mixture was stirred at room temperature for 45 min. Afterward, a cooling bath at 15 °C was applied again and water (100 mL) was added to the mixture, which was then extracted with Et_2O (5 × 75 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated, affording a colourless oil containing a 1.4:1 mixture of *trans* (**46**) and *cis* (**47**) isomers. The two isomers were separated and purified by flash chromatography (*n*-hexane/EtOAc, 9:1, R_f 0.27 and R_f 0.15), to afford pure **46** (301 mg, 40%) and pure **47** (264 mg, 35%) both as thick colourless oils.

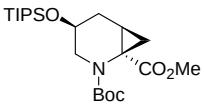
46: $[\alpha]_D^{21}$ +11.4 (*c* 0.95, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) (2.3:1 mixture of rotamers) δ (ppm): 7.36-7.27 (m, 5 H, Ph, both rotamers), 5.28 (part A of an AB system, *J* = 12.6 Hz, 1 H, C*H*$_2$Ph, major rotamer), 5.16 (AB system, *J* = 12.3 Hz, 2 H, C*H*$_2$Ph, minor rotamer), 5.06 (part B of an AB system, *J* = 12.6 Hz, 1 H, C*H*$_2$Ph, major rotamer), 3.95-3.84 (m, 2 H, 4-H major rotamer + 3-H_{eq} both rotamers), 3.79-3.72 (m, 1 H, 4-H minor rotamer), 3.69 (s, 3 H, OCH_3, minor rotamer), 3.52 (s, 3 H, OCH_3, major rotamer), 2.93-2.84 (m, 1 H, 3-H_{ax}, both rotamers), 2.10-2.03 (m, 1 H, 5-H, major rotamer), 2.01-1.79 (m, 4 H, 5-H minor rotamer + 5-H' minor rotamer + 6-H both rotamers + 7-H both rotamers), 1.73-1.65 (m, 1 H, 5-H', major rotamer), 1.03 {s, 21 H, Si[C*H*(CH$_3$)$_2$]$_3$ + Si[CH(C*H*$_3$)$_2$]$_3$, major rotamer}, 0.99 {s, 21 H, Si[C*H*(CH$_3$)$_2$]$_3$ + Si[CH(C*H*$_3$)$_2$]$_3$, minor rotamer}, 0.89-0.84 (m, 1 H, 7-H', minor rotamer), 0.81 (dd, *J* = 7.4, 4.7 Hz, 1 H, 7-H', major rotamer). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.9 (CO), 156.5 (NCO), 136.7 (C_{arom}), 128.4 (2 C, C_{arom}), 127.8 (C_{arom}), 127.6 (2 C, C_{arom}), 67.1 (*C*H_2Ph), 65.8 (C-4), 52.1 (OCH_3), 49.2 (C-3), 37.4 (C-1), 31.2, 24.1, 23.3, 17.9 {6 C, Si[CH(*C*H$_3$)$_2$]$_3$}, 12.1 {3 C, Si[*C*H(CH$_3$)$_2$]$_3$}. MS (ESI) *m/z* (%): 945 (14) [2M + Na]$^+$, 462 (100) [M + 1]$^+$. MS/MS (ESI of [M + 1]$^+$) *m/z* (%): 429 (81), 418 (58), 295 (100), 287 (63).

47: $[\alpha]_D^{22}$ +19.5 (*c* 1.74, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (2.5:1 mixture of rotamers) δ (ppm): 7.37-7.23 (m, 5 H, Ph), 5.27-5.22 (m, 1 H, C*H*$_2$Ph, both rotamers), 5.05-5.00 (m, 1 H, C*H*$_2$Ph, both rotamers), 4.11-4.03 (m, 2 H, 4-H both rotamers + 3-H_{eq} major rotamer), 3.95 (dd, *J* = 13.2, 3.9 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.69 (s, 3 H, OCH_3, minor rotamer), 3.52 (s, 3 H, OCH_3, major rotamer), 2.92 (dd, *J* = 13.2, 1.6 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.88-2.78 (m, 1 H, 3-H_{ax}, major rotamer), 2.04-1.96 (m, 2 H, 5-H both rotamers + 5-H' major rotamer), 1.92-1.88 (m, 2

H, 5-H' minor rotamer + 7-H minor rotamer), 1.83 (dd, *J* = 9.9, 4.1 Hz, 1 H, 7-H, major rotamer), 1.69-1.59 (m, 1 H, 6-H, both rotamers), 1.36 (dd, *J* = 8.0, 4.1 Hz, 1 H, 7-H', both rotamers), 1.02 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$, major rotamer}, 0.96 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$, minor rotamer}. ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 173.4 (CO), 157.1 (NCO), 137.1 (C_{arom}), 128.2 (2 C, C_{arom}), 127.7 (C_{arom}), 127.6 (2 C, C_{arom}), 67.0 (*C*H_2Ph), 65.8 (C-4), 52.2 (OCH_3), 47.2 (C-3), 38.3 (C-1), 29.3, 23.7, 22.8, 18.0 {6 C, Si[CH(*C*$H_3)_2]_3$}, 12.1 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 945 (100) $[2M + Na]^+$, 462 (12) $[M + 1]^+$. Elemental analysis calcd. (%) for $C_{25}H_{39}NO_5Si$ (461.67): C, 65.04; H, 8.51; N, 3.03. Found: C, 65.24; H, 8.27; N, 3.37.

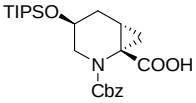


Methyl (1*R*,4*S*,6*R*)-4-triisopropylsilanyloxy-2-azabicyclo[4.1.0]heptane 1-carboxylate [(+)-48]
Prepared as reported for **25**, starting from **47** (53 mg, 0.11 mmol). After filtration and evaporation of the solvent, compound **48** (36 mg, 95%) was obtained as a colourless oil.
$[\alpha]_D^{21}$ +42.8 (*c* 1.12, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.82 (d, *J* = 3.5 Hz, 1 H, 4-H), 3.69 (s, 3 H, OCH_3), 2.75 (ddd, *J* = 13.6, 3.5, 1.1 Hz, 1 H, 3-H_{eq}), 2.54 (d, *J* = 13.6 Hz, 1 H, 3-H_{ax}), 2.08-1.98 (m, 2 H, 5-H + 5-H'), 1.92 (bs, 1 H, NH), 1.60-1.51 (m, 2 H, 6-H + 7-H), 1.34-1.28 (m, 1 H, 7-H'), 1.01 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}. ^{13}C NMR (100.4 MHz, $CDCl_3$) δ (ppm): 176.0 (CO), 64.4 (C-4), 52.3 (OCH_3), 48.3 (C-3), 38.2 (C-1), 29.0, 26.6, 20.4, 18.0 {6 C, Si[CH(*C*$H_3)_2]_3$}, 12.0 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 677 (61) $[2M + Na]^+$, 328 (100) $[M + 1]^+$. Elemental analysis calcd. (%) for $C_{17}H_{33}NO_3Si$ (327.53): C, 62.34; H, 10.16; N, 4.28. Found: C, 62.30; H, 9.86; N, 4.07.



2-*tert*-Butyl 1-methyl (1*R*,4*S*,6*R*)-4-triisopropylsilanyloxy-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-49]
Prepared as reported for **27**, starting from **48** (36 mg, 0.11 mmol). After flash chromatography (*n*-hexane/EtOAc, 8:1; R_f 0.23), pure **49** (35 mg, 74%) was obtained as a colourless oil.
$[\alpha]_D^{23}$ +17.5 (*c* 1.15, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (2.5:1 mixture of rotamers) δ (ppm): 4.10-4.06 (m, 1 H, 4-H, major rotamer), 4.04-3.98 (m, 2 H, 3-H_{eq} major rotamer, 4-H minor rotamer), 3.87-3.80 (m, 1 H, 3-H_{eq}, minor rotamer), 3.69 (s, 3 H, OCH_3, major rotamer), 3.68 (s, 3 H, OCH_3, minor rotamer), 2.89 (dd, *J* = 13.3, 1.8 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.74 (dd, *J* = 13.0, 1.5 Hz, 1 H, 3-H_{ax}, major rotamer), 2.01-1.90 (m, 2 H, 5-H + 5-H', both rotamers), 1.87 (dd, *J* = 9.9, 4.4 Hz, 1 H, 7-H, minor rotamer), 1.77 (dd, *J* = 10.0, 4.2 Hz, 1 H, 7-H, major rotamer), 1.63-1.55 (m, 1 H, 6-H, both rotamers), 1.46 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.41 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.29 (dd, *J* = 7.9, 4.2 Hz, 1 H, 7-H', both rotamers), 1.03 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}. ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 173.7 (CO), 156.0 (NCO), 79.5 [O*C*$(CH_3)_3$], 66.0 (C-4), 52.0 (OCH_3), 46.1 (C-3), 38.4 (C-1), 29.2, 28.3 [3 C, OC(*C*$H_3)_3$], 23.4, 22.9, 18.0 {6 C, Si[CH(*C*$H_3)_2]_3$}, 12.0 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%):

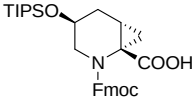
877 (74) $[2M + Na]^+$, 450 (36) $[M + Na]^+$, 328 (98). MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 395 (27), 351 (100).



(1*S*,4*S*,6*S*)-4-triisopropylsilanyloxy-2-(benzyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(+)-50]

NaOH (2 mL of a 1 N solution in water, 2 mmol) was added to a solution of **46** (418 mg, 0.91 mmol) in methanol (4 mL) and the resulting mixture was vigorously stirred for 24 h at 50 °C. Afterward, the methanol was evaporated, the remaining aqueous layer was acidified to pH 3 by adding a 1 N solution of HCl. The resulting suspension was extracted with CH_2Cl_2 (4 x 30 mL) and the combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent, compound **50** (355 mg, 88%) was obtained as a thick pale yellow oil.

$[\alpha]_D^{25}$ +10.9 (*c* 0.65, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) (1.7:1 mixture of rotamers) δ (ppm): 9.02 (bs, 1 H, COOH), 7.34-7.29 (m, 5 H, Ph, both rotamers), 5.22-5.03 (m, 2 H, C*H*$_2$Ph, both rotamers), 3.98-3.89 (m, 1 H, 3-H$_{eq}$, both rotamers), 3.87-3.82 (m, 1 H, 4-H, major rotamer), 3.69-3.60 (m, 1 H, 4-H minor rotamer), 2.83 (dd, *J* = 12.6, 6.8 Hz, 1 H, 3-H$_{ax}$, major rotamer), 2.79-2.72 (m, 1 H, 3-H$_{ax}$, minor rotamer), 2.07-1.97 (m, 1 H, 5-H, both rotamers), 1.94-1.87 (m, 2 H, 6-H + 7-H, both rotamers), 1.84-1.78 (m, 1 H, 5-H', both rotamers), 1.04 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$, major rotamer}, 0.98 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$, minor rotamer}, 0.85-0.78 (m, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 178.0 (COOH), 156.4 (NCO), 136.7 (C$_{arom}$), 128.4 (2 C, C$_{arom}$), 128.0 (2 C, C$_{arom}$), 127.4 (C$_{arom}$), 67.2 (*C*H$_2$Ph), 65.8 (C-4), 49.0 (C-3), 37.6 (C-1), 31.3, 24.9, 24.4, 17.9 {6 C, Si[CH(*C*H$_3)_2]_3$}, 12.1 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 933 (23) $[2M + K]^+$, 448 (100) $[M + 1]^+$.



(1*S*,4*S*,6*S*)-4-triisopropylsilanyloxy-2-(9-Fluorenylmethoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(+)-52]

Pd/C (87 mg, 10%) was added to a solution of **50** (355 mg, 0.79 mmol) in ethyl acetate (25 mL), under an N_2 atmosphere. The resulting suspension was stirred under a H_2 atmosphere (balloon) at room temperature for 18 h. Afterward, methanol (50 mL) was added and the mixture was filtered over a celite layer to remove the catalyst. Subsequent evaporation of the solvent led to **51** (233 mg, 94%) as a pale yellow solid, which was employed for the next step without further purification.

^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 4.02-3.96 (m, 1 H, 4-H), 2.96-2.92 (m, 1 H, 3-H), 2.91-2.86 (m, 1 H, 3-H'), 2.13-2.06 (m, 1 H, 5-H), 2.05-1.99 (m, 1 H, 5-H'), 1.84-1.74 (m, 1 H, 6-H), 1.56 (dd, *J* = 10.0, 5.8 Hz, 1 H, 7-H), 1.10 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}, 0.93 (dd, *J* = 7.5, 5.8 Hz, 1 H, 7-H'). MS (ESI) *m/z* (%): 649 (100) $[2M + Na]^+$, 336 (43) $[M + Na]^+$, 314 (17) $[M + 1]^+$. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 140 (100).

Amino acid **51** was suspended in THF (1.7 mL) and a 10% solution of Na_2CO_3 in water (2 mL) was added to this suspension. The resulting mixture was cooled to 0 °C and a solution of Fmoc-OSu (275 mg, 0.81 mmol) in THF (5 mL) was added. This reaction mixture was vigorously stirred at room temperature for 24 h. Afterward, the solvent was evaporated under *vacuum*, the resulting residue was taken up in EtOAc (12 mL) and a satd. solution of NH_4Cl (12 mL) was added. The mixture was extracted with EtOAc (3 x 12 mL) and the combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent the crude was purified by flash chromatography (*n*-hexane/EtOAc, 4:1 + 0.5% CH_3COOH; R_f 0.16) to afford compound **52** (321 mg, 81%) as a white solid.
M.p. 58.1-60.3 °C. $[\alpha]_D^{24}$ +1.2 (*c* 0.82, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) (2:1 mixture of rotamers) δ (ppm): 9.11 (bs, 1 H, COOH), 7.73-7.64 (m, 2 H, Fmoc), 7.58-7.50 (m, 2 H, Fmoc), 7.37-7.28 (m, 2 H, Fmoc), 7.27-7.20 (m, 2 H, Fmoc), 4.51-4.28 (m, 2 H, CH_2 Fmoc), 4.20-4.08 (m, 1 H, CH Fmoc), 3.90 (dd, *J* = 12.7, 4.2 Hz, 1 H, 3-H_{eq}, major rotamer), 3.87-3.81 (m, 1 H, 3-H_{eq}, minor rotamer), 3.71-3.60 (m, 1 H, 4-H, both rotamers), 2.78-2.62 (m, 1 H, 3-H_{ax}, both rotamers), 1.96-1.72 (m, 3 H, 5-H + 5-H' + 6-H, both rotamers), 1.67-1.56 (m, 1 H, 7-H, both rotamers), 1.01 {s, 21 H, Si[C*H*$(CH_3)_2]_3$ + Si[CH(C*H*$_3)_2]_3$}, 0.63-0.51 (m, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 177.7 (COOH), 156.4 (NCO), 143.8 (2 C, C_{arom}), 141.2 (2 C, C_{arom}), 127.5 (2 C, C_{arom}), 126.9 (2 C, C_{arom}), 125.0 (2 C, C_{arom}), 119.8 (2 C, C_{arom}), 67.4 (CH_2 Fmoc), 65.8 (C-4), 48.7 (C-3), 47.3 (CH Fmoc), 37.8 (C-1), 31.4, 29.7, 25.0, 17.9 {6 C, Si[CH(*C*$H_3)_2]_3$}, 12.1 {3 C, Si[*C*H$(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 1093 (100) $[2M + Na]^+$, 558 (9) $[M + Na]^+$.

4.8 References

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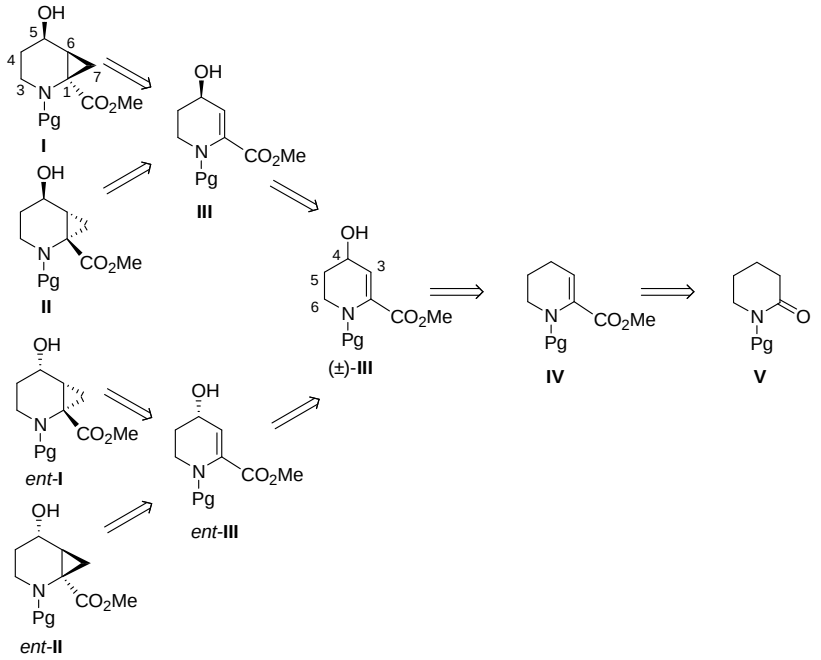
[27] Unpublished results
[28] Unpublished results
[29] Unpublished results

Chapter 5: Synthesis of 5-Substituted Cyclopropane Pipecolic Acids (5-R-CPAs)

5.1 General Synthetic Strategy towards 5-OH-CPAs

As previously mentioned, our group already published in 2011 the synthesis of both *cis* and *trans* 5-OH-CPAs as new conformationally constrained homoserine analogues (see section 2.3).[1] These compounds were obtained by applying a diastereodivergent procedure (scheme 1) starting from achiral commercial δ-valerolactam (**V**).



Scheme 1. Retrosynthetic analysis for 5-OH-CPAs.

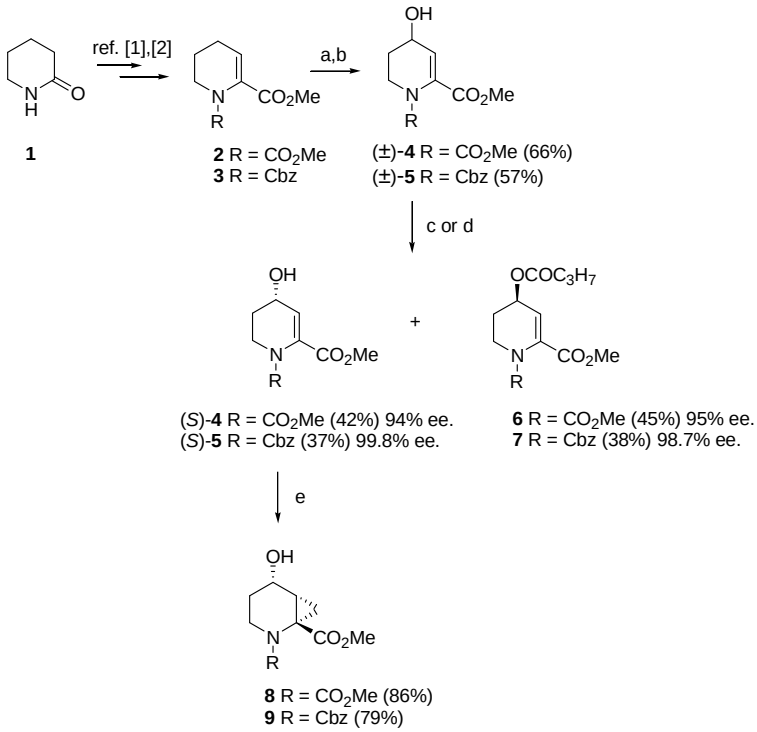
Enecarbamate esters **IV** were synthesised by means of the usual Pd-catalyzed methoxycarbonylation of the lactam-derived vinyl phosphates and then oxidized on the allylic position to get the racemic allylic alcohols **III**. Afterward, a highly enantioselective lipase-catalysed kinetic resolution provided the two enantioenriched forms of **III**,[2] which in turn were cyclopropanated to obtain the *cis* (**I**) or the *trans* (**II**) products, depending on the reaction conditions. Under the best conditions for OH-directed cyclopropanation, Charette's Zn-carbenoid (2,4,6-$Cl_3C_6H_2OZnCH_2I$)[3] provided *cis*-4-OH-CPAs (**I**) in the highest yield (73–86%) and with complete facial selectivity. The *trans* selectivity in Michael-type addition of dimethylsulfoxonium methylide[4] on suitably OH-protected allylic alcohols was 1:4 to 1:7 in DMSO and diastereopure *trans* isomers (**II**) were obtained by chromatography after OH-deprotection in 57–73% yield.

Hence, starting from the aforementioned published synthetic strategy, in this work we wanted to enlarge the series of 5-substituted cyclopropane pipecolic acids (5-R-CPAs) by introducing one N-CO_2tBu-protected 5-OH derivative as well as completely new 5-NH_2-CPAs.

5.2 Synthesis of N-CO_2*t*Bu-Protected 5-OH-CPA

The synthesis of both N-CO_2Me- (**8**) and N-CO_2Bn-protected (**9**) *cis* 5-OH-CPAs were already effectively accomplished in 2011 (scheme 2),[1] since in the case of allylic alcohols also benzyl carbamate protection demonstrated to be perfectly compatible with the use of Zn-carbenoids in cyclopropanation step.

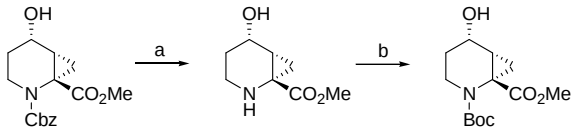
In these procedures, both racemic allylic alcohols **4** and **5** were obtained by allylic oxidation of the correspondent enecarbamate esters **2** and **3** carried out with N-bromosuccinimide (NBS) in the presence of a catalytic amount of azobisisobutyronitrile (AIBN), followed by hydrolysis with $ZnCl_2$ in wet acetone. Subsequently, enantioenriched (*S*)-**4** and (*S*)-**5** were isolated by exploiting kinetic resolutions realized through enantioselective enzyme-catalysed esterification of (*R*)-**4** and (*R*)-**5**, carried out in the presence of PS "AMANO" IM lipase as a catalyst and vinyl butyrate as an acylating agent. Finally, (*S*)-**4** and (*S*)-**5** were efficiently cyclopropanated using Charette's Zn-carbenoid (2,4,6-$Cl_3C_6H_2OZnCH_2I$), formed *in situ* from Et_2Zn and CH_2I_2 in the presence of 2,4,6-trichlorophenol (TCP), affording both **8** and **9** with remarkable yields and total diastereoselectivity.



Scheme 2. Synthesis of *cis*-5-OH-CPAs. Reagents and conditions: a) NBS, AIBN, $CCl_4/CHCl_3$ 9:1, reflux; b) $ZnCl_2$, aq. acetone; c) vinyl butyrate, PS-"AMANO"-IM lipase, THF, 30 °C, 6 h; d) vinyl butyrate, PS-"AMANO"-IM lipase, TBME, 30 °C (two sequential kinetic resolutions); e) Et_2Zn, CH_2I_2, TCP, CH_2Cl_2, –40 °C to 25 °C.

Starting from these two nicely synthesised cyclopropanated products, we wanted to investigate the possibility to generate further new CPAs.

Considering that we already demonstrated how N-CO_2*t*Bu-protected CPAs are easily accessible from N-CO_2Bn-protected correspondent (see chapter 4), we firstly tackled such synthesis, applying the same procedure already described for 4-OH-CPA (scheme 3).



Scheme 3. Synthesis of N-CO_2*t*Bu-Protected 5-OH-CPA. Reagents and conditions: a) H_2 (1 atm), 10% Pd/C, EtOAc, RT, 4 h; b) Boc_2O, Et_3N, MeOH, reflux, 4 h.

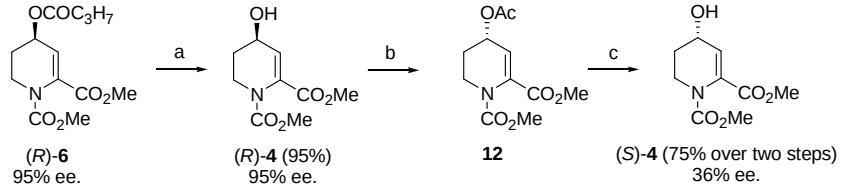
Accordingly, N-Boc protected amino acid **11** was prepared in 66% yield[5] after hydrogenation of known N-Cbz derivative **9**.[1]

In such a way, we established once again our amino acids as suitable building blocks for peptide synthesis, since their amino groups can be easily protected with those which are considered the protecting groups of choice in solid-phase peptide synthesis (SPPS).

5.3 Synthesis of 5-NH_2-CPAs

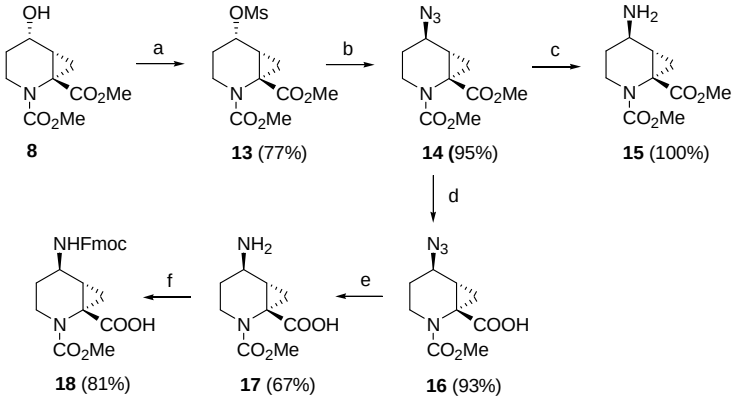
We planned the synthesis of 5-amino-CPAs with the same purpose we did for 4-amino-CPA, that was the construction of CPA-containing new integrin ligands. Consequently, even in this case we selected the best absolute configuration for our amino acids, according to preliminary molecular modelling studies (see Scope of the work). Specifically, we determined that we needed allylic alcohol (*S*)-**4** as starting material to obtain the stereochemistry of choice in the final products.

The synthesis of racemic alcohol **4** and its enzymatic kinetic resolution (EKR) were carried out as already described (scheme 2). Since the resolution provided just less than 50% of the requisite enantiopure stereoisomer (*S*)-**4** from the racemic alcohol, butyrate (*R*)-**6** was hydrolysed and subjected to Mitsunobu reaction to invert the C-4 configuration and thus increase the amount of the desired 4*S* enantiomer (scheme 4). This procedure was conducted treating (*R*)-**4** with Ph_3P and diisopropyl azodicarboxylate (DIAD) in the presence of acetic acid as an acylating agent and final product (*S*)-**4** was obtained in good yield (75% over two steps) after hydrolysis of the acetate.



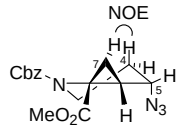
Scheme 4. Inversion of C-4 configuration in allylic alcohol **4** by means of a Mitsunobu reaction. Reagents and conditions: a) MeONa, MeOH, 0 °C; b) Ph_3P, DIAD, CH_3COOH, THF, 0°C; c) MeONa, MeOH, 0 °C.

However, as this reaction was carried out on an allylic alcohol,[6] prone to stable carbocation formation, racemization took place to a certain extent and it furnished alcohol (*S*)-**4** with only 36% *ee*.
Even though enzymatic kinetic resolution and Mitsunobu could be potentially employed in an iterative way to convert all the *R* enantiomer to the *S* correspondent, the procedure would be certainly time- and resource-consuming, not to mention the partial loss of material in the process, due to the non-quantitative yields.
Thus, for the synthesis of 5-amino-CPA and its peptidomimetic derivative we used just the (*S*)-**4** directly obtained from the EKR, that is with a 94% *ee* (97:3 *er*), relying on the fact that HPLC purification of the final peptidomimetic product would provide a diastereopure compound.
(*S*)-**4** was cyclopropanated as already reported (scheme 2) and, applying the same procedure already described for 5-R-CPAs (see section 4.2), 4-OH-CPA (**8**) was converted into azido derivative **14** (scheme 5),[5] which displays the correct *cis* stereochemistry of the two groups on the ring as required for the construction of a cyclopeptide.



Scheme 5. Synthesis of 5-amino-CPAs. Reagent and conditions: a) MsCl, Et_3N, CH_2Cl_2, –30 to 25 °C, 1 h; b) NaN_3, [15]crown-5, CH_3CN, reflux, 6 h; c) H_2 (1 atm), 10% Pd/C, MeOH, RT, 16 h. d) 1N NaOH, MeOH; e) H_2, 10% Pd/C, MeOH; f) FmocOSu, Na_2CO_3 aq., THF, 25 °C, 24h.

Relative stereochemistry of **14** was assigned as a consequence of the bimolecular nucleophilic displacement, which afforded the product from known compound **8**[1], whereas the preferred conformation was assigned, as usual, by the analysis of the coupling constants in the ^{1}H NMR spectra and by nuclear Overhauser effect (NOE) studies (figure 1). The 5-H proton resonates at about δ=4 ppm as a broad multiplet owing to very low coupling constant values in accordance with an equatorial position. Moreover, an NOE effect between *endo* 7-H and axial 4-H further confirms the stereochemical assignment.

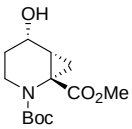


ent-**14**

Figure 1. NOE studies on key intermediate **14**

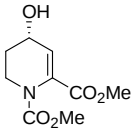
From **14** we synthesised two different amino derivatives (**15** and **18**), according to the subsequent coupling strategy we desired to adopt. Product **15**, readily accessible with quantitative yield by hydrogenation of **14**, exhibits a free amino group, while the carboxylic function is still protected as methyl ester.[5] Therefore, such compound can firstly form a peptide bond with its amino function. On the contrary, compound **18** is protected as N-Fmoc on the amino group and bears a free carboxylic function, which can be promptly coupled. To obtain this further derivative, suitable even for solid-phase peptide synthesis (SPPS), at first we carried out the hydrolysis of the methyl ester of **14** that, differently from what previously reported (see section 4.6), occurred at room temperature in 24 h leading to intermediate **16** with good yield (93%). Afterward, the azido group was reduced to amine by the usual procedure, even if in this case the resulting free amino acid **17** resulted insoluble in the reaction media (methanol), probably due to the formation of the zwitterion, and precipitated as soon as formed, mixing with the solid catalyst. Therefore, at the end of the reaction, before filtering off Pd/C, addition of water was necessary to dissolve **17**. In such a way we managed to isolate this product, even if the yield of the reaction (67%) was slightly lowered by probable partial material loss during the process. Finally, we carried out the N-Fmoc protection with N-(9-fluorenylmethoxycarbonyloxy)succinimide (FmocOSu) in a mixture of aqueous Na_2CO_3 and THF, affording final product **18** with 81% yield.[7]
In this way we could obtain an amount of suitable protected amino acids **15** and **18** sufficient to accomplish peptide synthesis; in particular, 200 mg of **18** were synthesised in a single run. Accordingly, as we report later (see chapter 6), both the compounds were used to tackle the construction of a new peptidomimetic integrin ligand.

5.4 Experimental



2-*tert*-Butyl 1-Methyl (1*S*,5*S*,6*R*)-5-Hydroxy-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(–)-11]
Triethylamine (25 µL, 0.18 mmol) and Boc_2O (40 mg, 0.18 mmol) were added to a solution of **10**[1] (15 mg, 0.088 mmol) in anhydrous methanol (2 mL), under an N_2 atmosphere, and the resulting reaction mixture was heated to reflux for 4 h. Afterward, water (3 mL) was added and the product was extracted with CH_2Cl_2 (5 x 2 mL). The combined organic extracts were washed with a 5% solution of $KHSO_4$ (3 mL), a satd. solution of $NaHCO_3$ (3 mL), water (3 mL) and brine (3 mL) and then dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by flash chromatography (*n*-hexane/EtOAc, 1:2; R_f 0.38), affording pure **11** (16 mg, 66%) as a white solid.
M. p. 124.5-125.7 °C. $[\alpha]_D^{23}$ –31.7 (*c* 0.70, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (3:1 mixture of rotamers) δ (ppm): 4.37-4.31 (m, 1 H, 5-H, both rotamers), 3.98 (dt, *J* = 13.5, 3.7 Hz, 1 H, 3-H_{eq}, major rotamer), 3.81 (dt, *J* = 10.9, 3.6 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.71 (s, 3 H, OCH_3 major rotamer), 3.70 (s, 3 H, OCH_3, minor rotamer), 2.77 (t, *J* = 10.9 Hz, 3-H_{ax}, minor rotamer), 2.66 (td, *J* = 13.5, 1.9 Hz, 3-H_{ax}, major rotamer), 2.00-1.91 (m, 2 H, 4-H + 6-H, both rotamers), 1.85

(dd, *J* = 9.9, 5.0 Hz, 1 H, 7-H, both rotamers), 1.64 (bs, 1 H, OH, both rotamers), 1.47 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.42 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.27-1.15 (m, 1 H, 4-H', both rotamers), 1.07 (dd, *J* = 6.4, 4.6 Hz, 1 H, 7-H', minor rotamer), 1.01 (dd, *J* = 7.6, 5.0 Hz, 1 H, 7-H', major rotamer). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.6 (CO), 155.3 (NCO), 80.4 [$OC(CH_3)_3$], 64.7 (C-5), 52.5 (OCH_3), 42.0, 40.3, 31.2, 29.3, 28.5 [3 C, $OC(CH_3)_3$], 19.7. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 272 (16) $[M + 1]^+$, 240 (55), 198 (100). Elemental analysis calcd. (%) for $C_{13}H_{21}NO_5$ (271.31): C, 57.55; H, 7.80; N, 5.16. Found: C, 57.18; H, 7.58; N, 4.90.

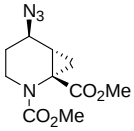


Conversion of Dimethyl (*R*)-4-Hydroxy-5,6-dihydropyridine-1,2(4H)-dicarboxylate [(+)-4] into (*S*)-4-Hydroxy-5,6-dihydropyridine-1,2(4H)-dicarboxylate [(–)-4] by Mitsunobu Reaction

A solution of (*R*)-**4**[2] (95% *ee*)(105 mg, 0.49 mmol) and Ph_3P (195 mg, 0.74 mmol) in dry THF (3.5 mL) was added to a solution of diisopropyl azodicarboxylate (DIAD) (146 μL, 0.74 mmol) and acetic acid (42 μL, 0.74 mmol) in dry THF (3.5 mL) cooled to 0 °C. The resulting mixture was stirred for 1.5 h at 0 °C, then the solvent was removed under *vacuum*. The residue was taken up in a *n*-hexane-Et_2O 1:1 mixture (5 mL), which was then filtered and evaporated under *vacuum*. The residue was chromatographed (CH_2Cl_2/Et_2O, 50:1; R_f 0.26) to afford **12** (in a 4:1 ratio with a residual impurity of reduced DIAD) as a colourless oil.

Acetate **12** was hydrolysed according to published procedure,[2] to afford pure (*S*)-**4** (79 mg, 75% over two steps) exhibiting a 36% enantiomeric excess, determined by comparison of the observed optical rotation with the specific rotation of the enantiopure correspondent.

Spectroscopic data as reported in the literature.[2]



Dimethyl (1*S*,5*R*,6*S*)-5-Azido-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-14]

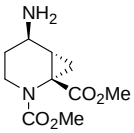
MsCl (31 μL, 0.39 mmol) was added dropwise to a solution of **8**[1] (70 mg, 0.31 mmol) and triethylamine (54 μL, 0.39 mmol) in anhydrous CH_2Cl_2 (3 mL) cooled to –30 °C. After 5 min the cooling bath was removed and the reaction mixture was left under stirring for 2 h at room temperature. Then a 0.5 N solution of HCl (150 μL) was added dropwise and after 10 min the mixture was extracted with CH_2Cl_2 (3 x 1 mL). The combined organic layers were washed with water (3 x 1 mL) and dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (pure Et_2O, R_f 0.34) to afford compound **13** (72 mg, 77%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) (2:1 mixture of rotamers) δ (ppm): 5.34-5.28 (m, 1 H, 5-H, both rotamers), 4.11 (dt, *J* = 13.6, 3.5 Hz, 1 H, 3-H_{eq}, major rotamer), 3.95 (dt, *J* = 13.3, 3.7 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.74 (s, 3 H, OCH_3, minor rotamer), 3.72 (s, 3 H, OCH_3, major rotamer), 3.71 (s, 3 H, OCH_3, both rotamers), 3.07 (s, 3 H, SCH_3, both rotamers), 2.86 (t, *J* = 13.3 Hz, 3-H_{ax},

minor rotamer), 2.77 (td, *J* = 13.6, 1.9 Hz, 3-H_{ax}, major rotamer), 2.17-2.11 (m, 2 H, 4-H + 6-H, both rotamers), 2.07 (dd, *J* = 9.8, 5.5 Hz, 1 H, 7-H, minor rotamer), 2.01 (dd, *J* = 9.8, 5.5 Hz, 1 H, 7-H, major rotamer), 1.54-1.44 (m, 1 H, 4-H', both rotamers), 1.26-1.18 (m, 1 H, 7-H', both rotamers).

Mesylate **13** was dissolved in anhydrous acetonitrile (2 mL) and [15]crown-5 (10 µL, 0.05 mmol) and NaN_3 (22 mg, 0.34 mmol) were added to the resulting solution under an N_2 atmosphere. The mixture was heated to reflux for 6 h and then stirred at room temperature for 12 h. Afterward, the mixture was filtered through a silica/celite 1:1 pad and the filtrate concentrated under *vacuum*. The residue was purified by flash chromatography (*n*-hexane/EtOAc, 3:1; R_f 0.21) affording pure **14** (57 mg, 95%) as a white solid.

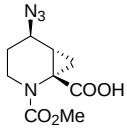
M. p. 72.1-75.4 °C. $[\alpha]_D^{19}$ +28.5 (*c* 1.02, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (2:1 mixture of rotamers) δ (ppm): 4.00 (bs, 1 H, 5-H, both rotamers), 3.87 (dt, *J* = 13.2, 3.9 Hz, 1 H, 3-H_{eq}, major rotamer), 3.75-3.65 (m, 7 H, 3-H_{eq} minor rotamer + 2 x OCH_3 both rotamers), 3.03 (td, *J* = 13.3, 1.9 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.94 (td, *J* = 13.2, 2.3 Hz, 1 H, 3-H_{ax}, major rotamer), 2.00 (dd, *J* = 10.2, 5.5 Hz, 1 H, 7-H_{exo}, minor rotamer), 1.93 (dd, *J* = 10.5, 5.5 Hz, 1 H, 7-H_{exo}, major rotamer), 1.83-1.72 (m, 2 H, 4-H_{eq} + 6-H, both rotamers), 1.58-1.49 (m, 1 H, 4-H_{ax}, both rotamers), 0.87-0.79 (m, 1 H, 7-H_{endo}, both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 171.9 (CO), 156.8 (NCO), 54.7 (C-5), 53.1 (OCH_3), 52.7 (OCH_3), 38.4, 36.7, 27.9, 27.8, 21.0. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 255 (5) $[M + 1]^+$, 223 (100), 212 (16). Elemental analysis calcd. (%) for $C_{10}H_{14}N_4O_4$ (254.24): C, 47.24; H, 5.55; N, 22.04. Found: C, 47.21; H, 5.31; N, 22.41.



Dimethyl (1*S*,5*R*,6*S*)-5-Amino-2-azabicyclo[4.1.0]heptane-1,2-dicarboxylate [(+)-15]

Pd/C (10%, 7 mg) was added to a solution of **14** (57 mg, 0.22 mmol) in anhydrous methanol (4 mL), under an N_2 atmosphere. The resulting suspension was vigorously stirred under a H_2 atmosphere (balloon) at room temperature for 24 h. After filtration over a celite layer and evaporation of the solvent, pure **15** (51 mg, 100%) was obtained as a colourless oil.

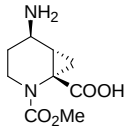
$[\alpha]_D^{23}$ +14.7 (*c* 0.88, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (1.5:1 mixture of rotamers) δ (ppm): 3.75-3.57 (m, 8 H, 5-H + 3-H_{eq} + 2 x OCH_3, both rotamers), 3.23-3.17 (m, 1 H, 3-H_{ax}, minor rotamer), 3.16-3.10 (m, 1 H, 3-H_{ax}, major rotamer), 1.91 (dd, *J* = 10.2, 5.5 Hz, 1 H, 7-H_{exo}, minor rotamer), 1.84 (dd, *J* = 10.0, 5.3 Hz, 1 H, 7-H_{exo}, major rotamer), 1.64-1.45 (m, 3 H, 4-H_{eq} + 4-H_{ax} + 6-H, both rotamers), 1.33 (bs, 2 H, NH_2), 0.82-0.76 (m, 1 H, 7-H_{endo}, both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 173.0 (CO), 157.2 (NCO), 53.0 (OCH_3), 52.5 (OCH_3), 45.0 (C-5), 38.2, 37.7, 32.8, 31.2, 21.7. MS/MS (ESI of $[M + 1]^+$) *m/z* (%): 229 (2) $[M + 1]^+$, 212 (44), 197 (50), 154 (100). Elemental analysis calcd. (%) for $C_{10}H_{16}N_2O_4$ (228.25): C, 52.62; H, 7.07; N, 12.27. Found: C, 52.75; H, 6.84; N, 12.25.



(1*S*,5*R*,6*S*)-5-Azido-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(+)-16]

A 1 N solution of NaOH (1.23 mL) was added to a solution of **14** (208 mg, 0.82 mmol) in MeOH (2 mL) and the resulting mixture was vigorously stirred for 24 h at room temperature. Afterward, the methanol was evaporated and the remaining aqueous layer was washed with Et_2O (5 mL). Then, the aqueous layer was acidified to pH 3, adding a 1 N solution of HCl, and the product was extracted with $CHCl_3$ (5 x 5 mL). Finally, the aqueous layer was further acidified to pH 1 and the product was extracted again with $CHCl_3$ (5 x 5 mL). The combined organic layers were dried over Na_2SO_4 and after filtration and evaporation of the solvent, compound **16** (183 mg, 93%) was obtained as a white solid.

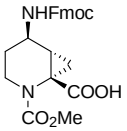
$[\alpha]_D^{21}$ +30.7 (*c* 0.98, $CHCl_3$) (*ee* 94%). ^{1}H NMR (400 MHz, $CDCl_3$) (1.5:1 mixture of rotamers) δ (ppm): 10.21 (bs, 1 H, COOH), 4.04-4.00 (m, 1 H, 5-H, both rotamers), 3.89 (dt, *J* = 11.3, 4.1 Hz, 1 H, 3-H_{eq}, major rotamer), 3.78-3.69 (m, 4 H, 3-H_{eq} minor rotamer + OCH_3 both rotamers), 3.07 (t, *J* = 11.9 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.97 (t, *J* = 11.3 Hz, 1 H, 3-H_{ax}, major rotamer), 2.06-1.87 (m, 2 H, 6-H + 7-H_{exo}, both rotamers), 1.82-1.75 (m, 1 H, 4-H, both rotamers), 1.60-1.52 (m, 1 H, 4-H', both rotamers), 0.95-0.88 (m, 1 H, 7-H_{endo}, both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 177.2 (CO), 156.9 (NCO), 54.7 (C-5), 53.2 (OCH_3), 38.0, 36.7, 28.6, 27.6, 21.5. MS (ESI) m/z (%): 239 (100) $[M - 1]^-$. Elemental analysis calcd. (%) for $C_9H_{12}N_4O_4$ (240.22): C, 45.00; H, 5.04; N, 23.32. Found: C, 45.21; H, 4.87; N, 23.01.



(1*S*,5*R*,6*S*)-5-Amino-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid (17)

10% Pd/C (24 mg) was added to a solution of **16** (183 mg, 0.76 mmol) in anhydrous MeOH (15 mL), under an N_2 atmosphere. The resulting suspension was stirred vigorously under a H_2 atmosphere (balloon) at room temperature. During the reaction, the resulting product precipitated, due to the formation of the zwitterion. After 24 h, H_2O (100 mL) was added and the resulting mixture was filtered over a celite layer. After evaporation of the solvent, pure **17** (123 mg, 67%) was obtained as a white solid, directly used in the next step without further purifications.

^{1}H NMR (400 MHz, D_2O) (2:1 mixture of rotamers) δ (ppm): 3.79-3.72 (m, 1 H, 3-H_{eq}, both rotamers), 3.71 (s, 3 H, OCH_3, minor rotamer), 3.70 (s, 3 H, OCH_3, major rotamer), 3.62-3.58 (m, 1 H, 5-H, major rotamer), 3.57-3.54 (m, 1 H, 5-H, minor rotamer), 3.20-3.07 (m, 1 H, 3-H_{ax}, both rotamers), 1.84-1.77 (m, 3 H, 4-H + 4-H' + 7-H, both rotamers), 1.69-1.61 (m, 1 H, 6-H, both rotamers), 0.94-0.87 (m, 1 H, 7-H', both rotamers). MS (ESI) m/z (%): 215 (67) $[M + 1]^+$.



(1*S*,5*R*,6*S*)-5-(9-Fluorenylmethoxycarbonylamino)-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(–)-18]

A 10% aqueous solution of Na_2CO_3 (1.6 mL) was added to a suspension of amino acid **17** (120 mg, 0.56 mmol) in THF (1.3 mL). A solution of Fmoc-*O*Su (189 mg, 0.56 mmol) in THF (3.8 mL) was added to the resulting mixture, cooled to 0 °C, which was then vigorously stirred at room temperature for 24 h. Afterward, the solvent was evaporated under *vacuum* and the residue was taken up in EtOAc (6 mL). Then, a satd. solution of NH_4Cl (6 mL) was added and the mixture was extracted with EtOAc (5 x 6 mL). The aqueous layer was acidified to pH 2, adding a 1 N solution of HCl, and extracted again with EtOAc (5 x 6 mL). The combined organic layers were dried over Na_2SO_4 and after filtration and evaporation of the solvent the crude was purified by flash chromatography ($MeOH/CH_2Cl_2$ 1:20, then $MeOH/CH_2Cl_2$ 1:10, R_f 0.38) to afford compound **18** (197 mg, 81%) as a white solid.

M.p. 171-173 °C (dec). $[\alpha]_D^{22}$ –16.1 (*c* 1.60, $CHCl_3$) (*ee* 94%). ^{1}H NMR (400 MHz, CD_3OD) (mixture of rotamers) δ (ppm): 7.76 (d, *J* = 7.5 Hz, 2 H, Fmoc), 7.63-7.61 (m, 2 H, Fmoc), 7.36 (t, *J* = 7.5 Hz, 2 H, Fmoc), 7.28 (t, *J* = 7.5 Hz, 2 H, Fmoc), 4.40-4.32 (m, 2 H, CH_2 Fmoc), 4.16 (bs, 1 H, CH Fmoc), 3.81 (bs, 1 H, 5-H), 3.75-3.60 (m, 4 H, 3-H_{eq} + OCH_3), 3.09-3.00 (m, 1 H, 3-H_{ax}), 1.79-1.76 (m, 1 H, 7-H), 1.66-1.56 (m, 3 H, 4-H + 4-H' + 6-H), 0.78-0.70 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, CD_3OD) (major rotamer) δ (ppm): 159.3 (C_q), 158.8 (C_q), 158.1 (C_q), 145.3 (2 C, $C_{q,arom}$), 142.6 (2 C, $C_{q,arom}$), 128.7 (2 C, C_{arom}), 128.1 (2 C, C_{arom}), 126.1 (2 C, C_{arom}), 121.0 (2 C, C_{arom}), 67.4 (CH_2 Fmoc), 53.3 (OCH_3), 48.5 (CH Fmoc), 46.1 (C-5), 40.5 (C-4), 39.0 (C-3), 29.2 (C-6), 29.0 (C-4), 21.0 (C-7). MS (ESI) m/z (%): 435 (100) $[M - 1]^-$. Elemental analysis calcd. (%) for $C_{24}H_{24}N_2O_6$ (436.46): C, 66.04; H, 5.54; N, 6.42. Found: C, 66.23; H, 5.29; N, 6.09.

5.5 References

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Chapter 6: Cyclic RGD-based New Integrin Ligands Containing CPAs as Central Scaffolds

6.1 Design of New Integrin Ligands

To assess the suitability of CPAs for building peptidomimetics, we decided to embed selected 4-amino-CPA (**1**) and 5-amino-CPA (**3**) (figure 1) into RGD-containing cyclic peptides (**2** and **4**), which were then evaluated as possible new ligands for angiogenic integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (see chapter 3).

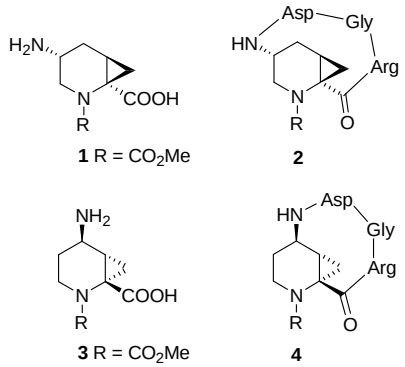


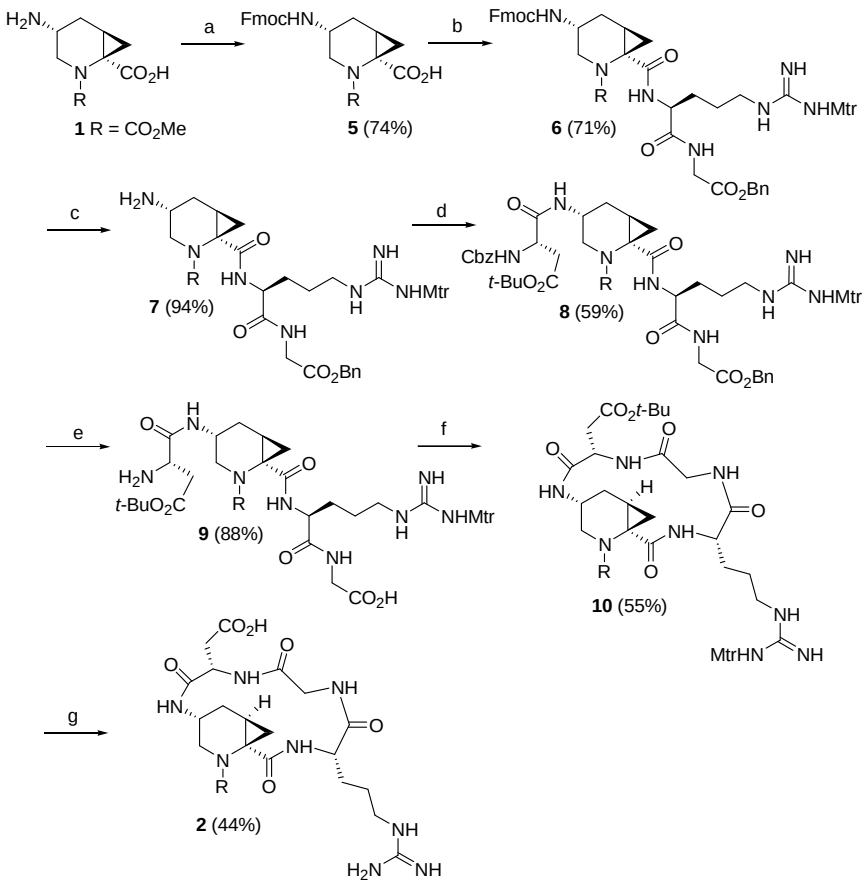
Figure 1. Selected CPAs as scaffolds for new integrin ligands

We were in fact interested in evaluating whether the different relative position (and spatial orientation) of the amino and carboxylic groups on which the RGD sequence is fixed would affect the potency of the peptidomimetic towards the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. In this way we could determine whether the two templates (**1** and **3**) would provide an optimal platform for the synthesis of integrin ligands as potential therapeutics or for targeted delivery of drugs or diagnostics (see section 3.3 and Scope of the work). Accordingly, after synthesis and biological evaluation, we also performed an accurate study (NMR experiments and molecular modelling) concerning the structures of both peptide candidates **2** and **4**, comparing their conformational behaviors and trying to rationalize their biological activities.

6.2 Synthesis of *Cyclo*[RGD-4-NHCPA]

The synthesis of **2** (scheme 1) was carried out in solution according to the procedure reported by Battistini et al. for the synthesis of aminoproline-containing integrin ligands (see Scope of the work and section 3.3).[1]

Thus, after protection of the amino group as N-Fmoc (74% yield) by the usual procedure (see section 5.3), carboxylic acid **5** was coupled to home-made dipeptide H-Arg(Mtr)-Gly-OBn[1] (Mtr: 4-methoxy-2,3,6,-trimethylbenzenesulphonyl), by using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) as the coupling reagent in THF at 35 °C, which provided, after four days, tripeptide **6** in 71% yield (chromatographic purification). In comparison to the corresponding step carried out for the synthesis of the aminoproline derivative,[1] which was complete in 12 h, in our case the reaction required much longer reaction times, probably due to the remarkable steric hindrance experienced by the carboxylic group of our molecule. This behaviour was generally always observed in every peptide coupling performed on CPAs.



Scheme 1. Synthesis of RGD-based integrin ligand containing 4-NH_2-CPA as central scaffold. Reagents and conditions: a) FmocOSu, Na_2CO_3 aq., THF, 18 h; b) DEPBT, DIPEA, H-Arg(Mtr)-Gly-OBn, THF, 35 °C, 4 d; c) CH_2Cl_2/Et_2NH 1:1, 3 h; d) DEPBT, DIPEA, Z-Asp(O*t*Bu)-OH, THF, 35 °C, 4 d; e) H_2 (1 atm), 10% Pd/C, EtOH, 24 h; f) DEPBT, DIPEA, THF, 35 °C, 4 d; g) TFA/TIS/H_2O 95:2.5:2.5, 18 h.

Deprotection of the 5-amino group in **6** was carried out in a CH_2Cl_2/diethylamine 1:1 mixture and provided uneventfully compound **7** (94% yield), which was coupled to Z-Asp(O*t*Bu)-OH as above to give tetrapeptide **8** in 59% yield after chromatography. Catalytic hydrogenation over 10% Pd/C allowed concurrent deprotection of both chain terminals to give tetrapeptide **9** in 88% yield. With the exposed amino and carboxylic functionalities in place, the construction of macrocycle **10** was first attempted by exposing a slightly high-diluted solution (3.5 mM) of **9** in DMF to the three-component system 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt)/collidine at room temperature as reported by Battistini et al.[1] However, after chromatographic separation on silica gel, we were unable to recover any fraction that contained a product with the right mass-to-charge ratio (checked by ESI-MS).

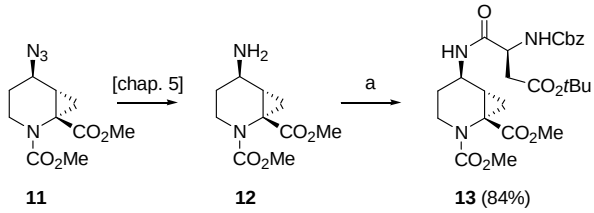
Therefore, we opted for an alternative setting of the cyclization procedure, resorting to the initial coupling conditions by working in THF and by making use of DEPBT as the coupling

reagent, since it had already proved effective on our substrates. Thus compound **9** was (incompletely) dissolved in THF (3.5 mM) and treated with DEPBT and N,N-diisopropylethylamine (DIPEA), which caused the complete solubilisation of the starting material. After 4 days at 35 °C, TLC monitoring revealed the complete disappearance of the starting material and the formation of a new spot. After chromatography of the crude reaction mixture, a major product was obtained (55% yield), the mass spectrum of which revealed a molecular ion at m/z 793 $[M+1]^+$ consistent with the cyclopeptide structure of **10**; its ^1H and ^{13}C NMR spectra resulted perfectly compatible as well. Eventually, exhaustive deprotection of all functionalities (except the N piperidine ring), which was carried out in a 95:2.5:2.5 trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O mixture, provided after semipreparative HPLC purification (see appendix 1 for analytical HPLC of pure **2**) cyclopeptide **2** (molecular ion at m/z 525 $[M+1]^+$), which was fully characterized (see appendix 1 for full characterisation) and tested as a new potential integrin binder.[2]

6.3 Synthesis of *Cyclo*[RGD-5-NHCPA]

Initially, we tried a different synthetic approach for the construction of peptidomimetic **4** with the aim of shorten the procedure applied for the first ligand. Specifically, we attempted to couple first the amino group of the CPA to the aspartic acid, so that to avoid the N-Fmoc protection/deprotection steps, exploiting the fact that the carboxylic group of CPA comes out from our synthetic strategy already protected as methyl ester.

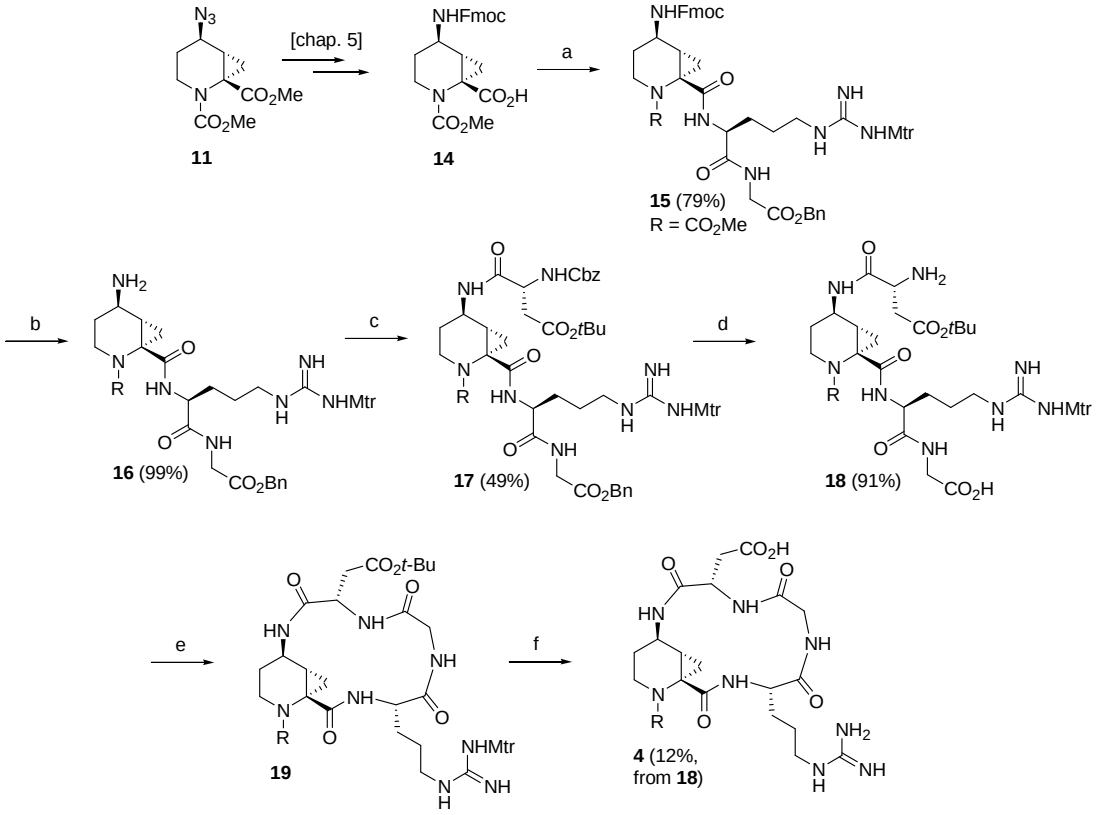
Therefore, as previously reported (see chapter 5), we converted azido derivative **11** into 5-amino CPA methyl ester **12** and tried to use directly this as starting material for assembling the target compound (scheme 2).



Scheme 2. Reagents and conditions: a) Z-Asp(O*t*Bu)OH, DEPBT, DIPEA, THF.

However, whilst the coupling of the 5-amino group with Z-Asp(O*t*Bu)OH was successful, the selective hydrolysis of the methyl ester (necessary to link the Arg-Gly dipeptide) was troublesome. Despite the two protections (methyl and *t*-butyl esters) are generally considered orthogonal, in this molecule this proved untrue and we never managed to obtain selectively the free carboxylic acid on CPA. This was probably due to the unusual excessive stability conferred to the methyl ester by the remarkable surrounding steric hindrance, as already observed on another occasion (see section 4.6). Usual procedure (1 N NaOH/MeOH at room temperature) surprisingly led to contemporary cleavage of both methyl and *t*-butyl ester, while attempts to use porcine liver esterase (PLE) in a selective enzymatic hydrolysis afforded only the unchanged starting compound **13**.

Thus, we resort to the first longer procedure, which had already proven effective for the construction of peptidomimetic **2**, and we applied it also for the synthesis of **4** (scheme 3).



Scheme 3. Synthesis of RGD-based integrin ligand containing 5-NH_2-CPA as central scaffold. Reagents and conditions: a) DEPBT, DIPEA, H-Arg(Mtr)-Gly-OBn, THF, 35 °C, 4 d; b) CH_2Cl_2/Et_2NH 1:1, 25 °C, 4.5 h; c) DEPBT, DIPEA, Z-Asp(O*t*Bu)-OH, THF, 35 °C, 4 d; d) H_2 (1 atm), 10% Pd/C, EtOH, 25 °C, 24 h; e) DEPBT, DIPEA,THF, 35 °C, 4 d; f) TFA/TIS/H_2O (95:2.5:2.5), 25 °C, 18 h.

Accordingly, the suitably N-Fmoc-protected amino acid **14** was prepared from **11** as described in chapter 5 and used as the starting substrate. The following coupling and deprotection steps were conducted exactly the same way as we did for the correspondent 4-amino-CPA derivative. Compared to that, only the final ring closure proved troublesome as it provided, by ^{1}H NMR and HPLC analysis, besides compound **19**, a complex mixture of other compounds which we were unable to identify but which likely contained the minor diastereoisomer deriving from *ent*-**14** (as reported in chapter 5, our 5-NH_2-CPA are obtained with 94% *ee*), the dimers of both **19** and its aforementioned minor diasteroisomer and probably other heavier oligomers. This result was unexpected, since even in this case we conducted the cyclization in a slightly high-diluted solution (3.5 mM), demonstrating how the different position and orientation of the amino group and the minor size of the macrocycle can make the ring closure more complicated and cause the formation of undesired side products. Anyway, at this stage of the study, we were not interested in optimizing the procedure, so we did not carry out any other experiment by using other coupling methodologies and we proceeded, instead, with the exhaustive deprotection of the mixture containing compound **19**. As we hoped, we managed to obtain, by semipreparative HPLC, an almost pure fraction (see appendix 2 for analytical

HPLC of **4**) of major cyclopeptide **4** (molecular ion at m/z 525 $[M+1]^+$, see appendix 2) with 12% yield over the last two steps that provided sufficient material for the biological tests and the NMR analysis (see appendix 2 for full characterization).[3]

6.4 Biological Evaluation

The candidate RGD compounds **2** and **4** were firstly tested for their binding affinity towards M21 human melanoma cells that expressed high levels of $\alpha_v\beta_3$ heterodimer and low levels of $\alpha_v\beta_5$ heterodimer as shown by flow cytometry analysis (see appendix 3). The test were carried out in agreement with similar cell-based screening methods by using the same $\alpha_v\beta_3$ expressing cell line M21 as reported in the literature, in which the reference RGD ligand *c*[RGDf(Me)V] (cilengitide) showed an IC_{50} value of 0.4 nM (see section 3.3).[4,5] Tests were performed in the presence of 2 mM $MnCl_2$ to switch integrins of tumour cells into an activated form. We were pleased to find that both compounds **2** and **4**, when used at concentrations that ranged from 3 μM down to 0.3 nM, demonstrated the capacity to inhibit the binding of M21 melanoma cells to vitronectin as an RGD-containing substrate in the nanomolar range (IC_{50} = 34 ± 12 nM for compound **2**[2] and IC_{50} = 2.4 ± 1.5 nM for compound **4**[3], table 1, see appendix 3 for percentage of inhibition plots), with compound **4** resulting ten times more active than **2** and comparable to the most potent antagonists reported so far (see section 3.3). As will be discussed later, such evidence of CPA-containing peptides as good ligands for $\alpha_v\beta_3$ integrin suggests that the rigid structure of CPA induces a significant conformational asset towards optimal presentation of the pharmacophoric groups of the ligands.

Table 1.

Entry	Compound	IC_{50} (nM)	
		$\alpha_v\beta_3$ [a]	$\alpha_5\beta_1$ [b]
1	**2**	34 ± 12 [c]	15 ± 10 [c]
2	**4**	2.4 ± 1.5 [c]	26 ± 16 [c]

[a] Test carried out on M21 human melanoma cells expressing high levels of $\alpha_v\beta_3$ heterodimer. The percentage of inhibition of the integrin-mediated M21 cell adhesion to vitronectin was measured.

[b] Test carried out on human erythroleukemia cell line K562 expressing high levels of $\alpha_5\beta_1$ heterodimer. The percentage of inhibition of the integrin-mediated K562 cell adhesion to fibronectin was measured.

[c] The error was propagated from the original logarithmic value by applying the formula $\Delta x = x \cdot \ln(10) \cdot \Delta[\log(x)]$.

Both peptidomimetics were also screened for their capacity to compete with fibronectin for the binding to $\alpha_5\beta_1$ integrin expressed by human erythroleukemia cell line K562 (see appendix 4 for flow cytometry analysis). Interestingly, when both compounds were tested as $\alpha_5\beta_1$ integrin ligands, for 5-amino-CPA derivative **4** we measured an IC_{50} value of 26 ± 16 nM close to the IC_{50} value obtained for the **2** isomer (IC_{50} = 15 ± 10 nM) (table 1, see appendix 4 for percentage of inhibition plots).[3]

Since the activity of both compounds was in the nanomolar range towards both integrins, **2** and **4** can be considered as dual ligands, which could be an advantage in these compounds to find applications in cancer therapy. These results are encouraging in further employing these CPA templates for the synthesis of new integrin binders and, by exploiting the scaffold N atom as an anchoring point, for targeted delivery of drugs and diagnostics.

6.5 Conformational Analysis

Cyclopeptides **2** and **4** were subjected to conformational analysis to assess the structural determinants that lead to binding activity towards $\alpha_v\beta_3$ and $\alpha_5\beta_1$.

Diluted solutions of **2** and **4** in DMSO-d_6 were used for the NMR spectroscopic analysis to prevent aggregation. Total correlation spectroscopy (TOCSY), rotating-frame Overhauser effect spectroscopy (ROESY), and variable-temperature ^{1}H NMR spectroscopic experiments were carried out for the NMR spectroscopic analysis.

Furthermore, molecular modelling calculations were carried out to get more insight into the conformational preferences of peptides **2** and **4**.

Analysis of *Cyclo*[RGD-4-NHCPA] (2)

^{1}H NMR spectroscopic data of **2** showed two sets of signals in a 2:1 ratio as a consequence of the existence of rotamers around the N-CO_2Me bond. The major rotamer showed chemical shift values between δ=7.6 and 8.1 ppm (table 2) for the amide protons.

Variable-temperature ^{1}H NMR experiments indicated low $\Delta\delta/\Delta T$ coefficients (table 2) for Gly and Asp amide protons, which suggested the existence of intramolecular hydrogen bonds, whereas relatively high chemical shift and $\Delta\delta/\Delta T$ values suggested that CPA and Arg amide protons were interacting with the solvent with equilibrating intermolecular hydrogen bonds.

Table 2. Chemical shifts and temperature-dependent ^{1}H NMR spectroscopic data for amide protons of peptide **2**.[a]

NH	δ	$\Delta\delta/\Delta T$
CPA	7.91	– 4.8
Arg	8.11	– 4.2
Gly	7.39	– 0.5
Asp	7.61	– 2.8

[a] ^{1}H NMR spectra for determining temperature coefficients were obtained at 298–323 K with increments of 5 K. Chemical shift (δ) values are obtained at 298 K and are in ppm. $\Delta\delta/\Delta T$ coefficients were retrieved from linear regression analysis and are expressed in ppbK^{-1}.

ROESY analysis of **2** showed sequential ROESY peaks between Asp NH and Gly H-α, between Gly NH and Arg H-α, and between CPA NH and Asp H-α, which allowed us to propose the preferred conformation of **2** in solution, as shown in figure 2.

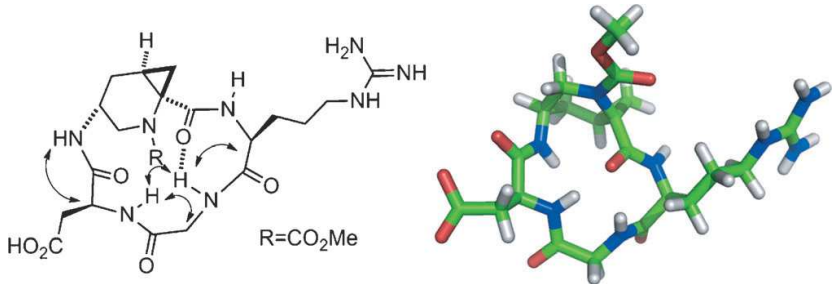


Figure 2. Selected ROESY peaks (left), and global minimum conformer (right) for **2**

Moreover, ROESY peaks between Asp and Gly amide protons suggested that these atoms were oriented inside the cyclopeptide and in agreement with a hydrogen-bonded structure (figure 2). Although temperature coefficient values suggested such hydrogen-bonded states to be in equilibrium with non-hydrogen-bonded states, the existence of a doubly hydrogen-bonded structure, and specifically of a conformation that displayed a γ-turn structure further stabilized by an equilibrating β-turn, suggested CPA nucleating a compact structure, ultimately resulting in a rigid conformation. As further proof, in the ^{1}H NMR spectrum of **2** in D_2O, the Gly CH_2 protons resonate as two highly separate doublets, one at δ=4.18 ppm and the other at δ=3.41 ppm, as similarly reported for the corresponding protons in potent aminoproline-containing integrin antagonist (see section 3.3),[1] in accordance with a rigid conformation for **2**.

Afterward, the conformational preferences of compound **2** were investigated by molecular mechanics calculations within the framework of Macromodel v6.5[6] by using Amber*[7] as a force field and the implicit water GB/SA solvation model of Still et al.[8] Monte Carlo energy minimization (MCEM)[9] conformational searches of the peptide analogues that contained methyl groups instead of the Arg and Asp side chains were performed as the first step and using conformational constraints according to ROESY data.
The global minimum conformer for compound **2** was in agreement with NMR spectroscopic data, which indicated the role of Gly and Asp amide protons in establishing intramolecular hydrogen bonds (figure 2). Specifically, Gly NH took part in a hydrogen bond with CPA C=O and likewise for Asp NH, although with minor strength, thus generating an inverse γ-turn (Gly NH), further stabilized with an equilibrating β-turn (Asp NH), within the cyclopeptide.
The results from the conformational analysis were consistent with the IC_{50} value, which suggested a close connection between conformation and ligand-binding affinity, as the role of CPA in nucleating the correct conformation was due to the existence of the rigid *cis* stereochemistry of the amino and carboxylic groups, which allowed it to maintain the required torsional angles between Asp and Arg for a good binding to the integrin. Nevertheless, the calculation of the distance between the C-β atoms of Asp and Arg in the global minimum conformer of **2** resulted in a value of 7.2 Å, which is shorter than the optimal value of around 8.9 Å.[10] This could be a possible factor responsible for the lower $\alpha_v\beta_3$ binding activity of **2** relative to the reference *c*[RGDf(Me)V] ligand (cilengitide) (see section 3.3).[4,5,10]

Finally, a docking simulation was carried out using compound **2** with the aim of gaining further insight into the possible binding modes of the cyclopeptide to the $\alpha_v\beta_3$ receptor at the molecular level (figure 3). The crystal structure of the complex formed by *c*[RGDf(Me)V] and the extracellular fragment of the $\alpha_v\beta_3$ receptor (PDB code: 1L5G) provides a general mode of interaction between the integrin and its ligands.[10] Specifically, as already illustrated (see section 3.3), Asp carboxylate and Arg guanidinium moiety of RGD-based ligands are the two key structural elements in the receptor recognition. In fact, the carboxylate group interacts with the metal-ion-dependent adhesion site (MIDAS) that consists of divalent metal ions and Ser121/Ser123, whereas the Arg guanidinium group is responsible for salt bridge interactions with the side chains of Asp218 and Asp150.
According to similar docking calculations reported in the literature for RGD ligands,[11] the docking program Autodock 4.0.1[12] was used to evaluate the binding energies of selected conformations of **2** as potential ligands for the $\alpha_v\beta_3$ receptor, and the docked conformations were analysed by taking into account the binding interactions observed in the crystal structure of the bound ligand–protein complex.

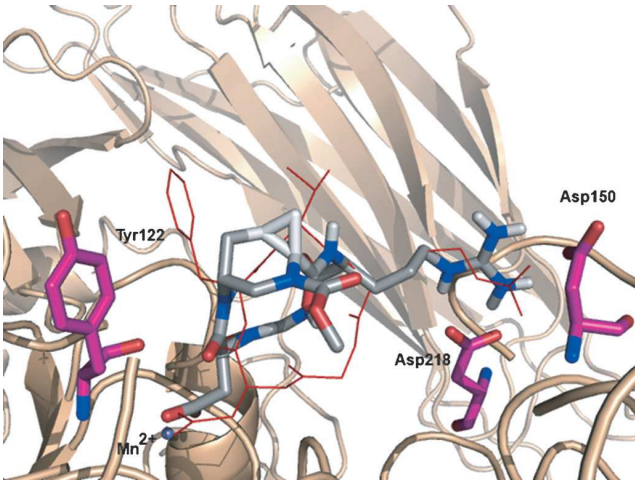


Figure 3. RGD ligand **2** (gray) docked into the binding region of $\alpha_v\beta_3$ integrin highlighting the protein residues (magenta) that form the key interactions: Asp218/Asp150 versus Arg, and Mn^{2+} versus Asp. The reference ligand c[RGDf(Me)V] (coordinated from PDB: 1L5G) is shown in red. Non polar hydrogen atoms are omitted for clarity

The docking results for ligand **2** revealed the key interactions with Asp218 and the MIDAS site of the receptor, which suggests that all these interactions are necessary for the molecular recognition of the Arg-Gly-Asp-containing ligands. Specifically, the global minimum conformer of peptide **2** displayed a low-energy cluster that showed the canonical binding mode that consisted of bidentate Asp218/Arg and MIDAS/Ser121/Asp interactions (figure 3).[2]

Analysis of *Cyclo*[RGD-5-NHCPA] (4)

^{1}H NMR data of diluted DMSO-d_6 solution of **4** showed two sets of signals in a 5:1 ratio, as a consequence of the existence of rotamers around the N-CO_2Me bond. The major rotamer showed amide proton chemical shift values between 7.2 and 8.9 ppm, with Asp amide proton being the more deshielded, thus suggesting its hydrogen-bonded status (table 3).

Table 3. Chemical shifts and temperature-dependent ^{1}H NMR spectroscopic data for amide protons of peptide **4**.[a]

NH	δ	Δδ/ΔT
CPA	7.78	– 2.9
Arg	7.51	– 2.6
Gly	7.22	– 1.3
Asp	8.90	– 3.4

[a] ^{1}H NMR spectra for determining temperature coefficients were obtained at 293–328 K with increments of 5 K or 10 K. Chemical shift (δ) values are obtained at 298 K and are in ppm. Δδ/ΔT coefficients were retrieved from linear regression analysis and are expressed in ppbK^{-1}.

Variable-temperature ^{1}H NMR experiments (figure 4) indicated low Δδ/ΔT coefficients for all amide protons (table 3), suggesting the existence of intramolecular hydrogen-bonds.

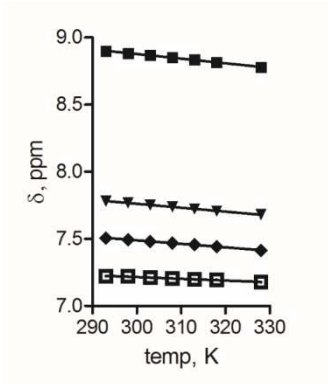


Figure 4. Temperature-dependent ^{1}H chemical shifts for amide protons of **4**; ▼: CPA NH; ◆: Arg NH; ■: Asp, NH; ◘: Gly, NH.

In contrast to **2**, Asp and Arg amide protons in **4** showed an inverted profile in terms of chemical shift and temperature coefficients, as Asp NH possessed a more deshielded chemical shift value and higher $\Delta\delta/\Delta T$ coefficient, possibly resulting from equilibrating hydrogen bonding species. Gly NH showed the lowest $\Delta\delta/\Delta T$ coefficient, indicating the involvement in strong intramolecular hydrogen bonds. Such data suggest a different intramolecular hydrogen bonding network within the two cyclic peptides **2** and **4**, contributing to a diverse arrangement of the cyclic frameworks induced by the two isomeric CPAs (figure 5).

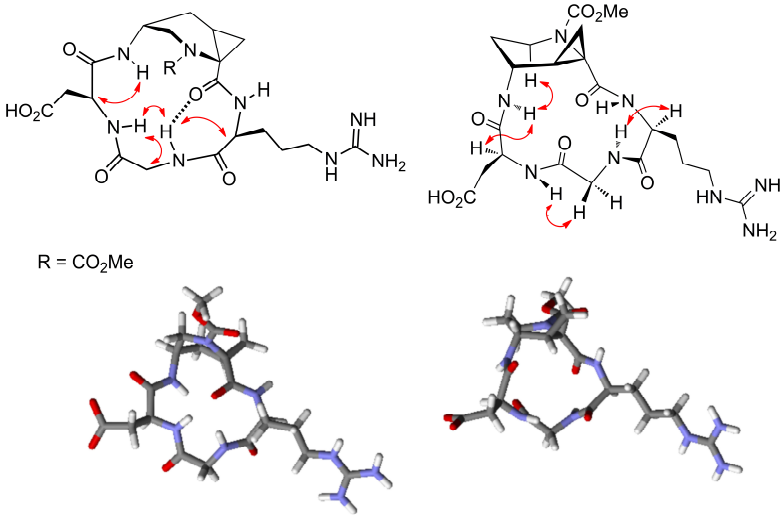


Figure 5. ROESY selected peaks and global minimum conformer for **2** (left) and **4** (right).

Molecular modelling analysis resulted in a global minimum conformer for compound **4** which indicates the role of Gly, CPA and Asp amide protons in establishing equilibrating intramolecular hydrogen-bonds (figure 5, right). Specifically, Gly NH experiences a hydrogen-

bond with CPA C=O, Asp NH with Arg C=O, and CPA NH with Gly C=O, although with variable strength, thus generating two γ-turns within the cyclopeptide. This is in contrast to the conformational asset of **2**, which displayed a similarly Arg-centered γ-turn although stabilized by a β-turn having a hydrogen bond experienced by Asp NH with CPA C=O (figure 5, left). ROESY analysis of **4** showed a ROESY peak between CPA NH and CPA 3-H_{ax}, and sequential ROESY peaks between Asp NH and Gly H-α, between CPA NH and Asp H-α, and between Gly NH and Arg H-α, consistent with the preferred conformation of **4** found by the modelling (figure 5, right), and in which the CPA NH points "inwards" below the scaffold's six-membered ring. Thus, ROESY peaks together with the variable temperature experiments suggest a conformation of the cyclopeptide characterized by the presentation of Asp and Arg side chains as showed in Figure 5, right. Although temperature coefficient values indicate that the hydrogen-bonded states could be in equilibrium with non hydrogen-bonded states, the existence of a conformation displaying γ-turns suggests CPA nucleating a compact structure in all equilibrating conformations.[3]

6.6 Structure-Activity Relationship

The results of the conformational analysis and the IC_{50} values for **2** and **4** suggest a close connection between conformational preferences and ligand binding affinity. Specifically, higher ligand binding affinity of **4** for $\alpha_v\beta_3$ resulted from equilibrium between correct conformations for the RGD recognition site of the integrin, whereas **2** showed more rigid structure though not as optimal as the isomeric **4**, producing lower affinity towards $\alpha_v\beta_3$. Such differences in binding affinity was not evinced for $\alpha_5\beta_1$ integrin, suggesting that this integrin best accommodates both RGD peptidomimetics in its recognition site. In all cases, the role of CPA in nucleating the correct conformation is due to the existence of the rigid *cis* orientation of amino and carboxylic groups, which allows maintaining the required torsional angles between Asp and Arg for an optimal binding to the integrin.

Based on NMR and conformational studies on integrin ligands, some prerequisites for a robust binding have been established amongst which the distance between Arg and Asp beta carbons (see section 3.3). In potent $\alpha_v\beta_3$ integrin antagonist cilengitide *c*(RGDf[NMe]V), for instance, this distance has an optimal value of 8.9 Å[10] as measured in the X-ray crystal structure of the complex with the $\alpha_v\beta_3$[10,13] and which leads to an extended conformation of the RGD motif and a stretched arrangement of the charged side chains.
Based on our modelling, the measured distance between the two beta C atoms in **4** is 7.9 Å,[3] that is longer than that calculated for **2** (7.1 Å)[2] and closer to the optimal value, which could account for, or contribute to, the higher activity of **4**. Additionally, the potency of both **2** and **4** towards $\alpha_5\beta_1$ integrin is justified by the fact that the binding mode is the same as in $\alpha_v\beta_3$ and that the requisite distance between the beta C atoms for an optimal binding with $\alpha_5\beta_1$ is in the same range.[14-18]

It is worth to notice that $\alpha_v\beta_3$ vs. $\alpha_5\beta_1$ selectivity is observed in many RGD-containing cyclic peptides and peptidomimetics. For example *cyclo*-(-Arg-Gly-Asp-D-Phe-Val-),[19] bicyclic lactam-containing ligand ST1646,[20] and *cyclo*-(-Arg-Gly-Asp-(+)-β-Acc-Val-)[21] (see section 3.3) are all $\alpha_v\beta_3$ selective antagonists, and the same Cilengitide *cyclo*-[-Arg-Gly-Asp-D-Phe-(N-Me)Val-] shows sub-nanomolar (0.65 nM) antagonistic activity for the $\alpha_v\beta_3$ receptor and nanomolar (13.2 nM) affinity for $\alpha_5\beta_1$. Extra N-methylation of Cilengitide further increases the $\alpha_v\beta_3/\alpha_5\beta_1$

selectivity by 2-3 orders of magnitudes.[22] In all these cases the selectivity has been shown to depend on the overall conformation of the cyclopeptide.
Our compounds are instead not selective, as they display a nanomolar potency toward both integrins.
For cyclopeptides like *cyclo*-(-Arg-Gly-Asp-(+)-β-Acc-Val-) incorporating a β-aminocyclopropane amino acid, $\alpha_V\beta_3$ selectivity has been explained on the basis of a less stretched conformation of the RGD sequence as suggested by a shorter distance between the C-α atoms of Arg and Asp (from 5.25 to 6.50 Å). In the non-selective isomer *cyclo*-(-Arg-Gly-Asp-(–)-β-Acc-Val-), this distance is 6.00-7.00 Å corresponding to a more stretched conformation for the RGD sequence.[21] On the other hand, for **2** and **4** the distances between the C-α atoms of Arg and Asp are 6.01 and 6.07 Å which are at the borderline of the two ranges above mentioned and therefore the lack of selectivity cannot be correlated with such a parameter.

6.7 Experimental

Chemistry
Chromatographic separations were performed under pressure on silica gel 60 (Merck, 70-230 mesh) using flash column techniques; R_f values refer to TLC carried out on 0.25 mm silica gel plates with the same eluent indicated for column chromatography. ^{1}H NMR (500, 400 and 200 MHz) and ^{13}C NMR (50.33 and 100.4 MHz) spectra were recorded on Bruker Avance II 500 MHz Ultrashield, and Varian Inova and Mercury spectrometers in $CDCl_3$ at 25 °C unless otherwise stated. Mass spectra were carried out by direct inlet on a LCQ Fleet™ Ion Trap LC/MS system (Thermo Fisher Scientific) with an ESI interface in the positive mode. Ligand **2** and **4** was purified by Beckman-Gold HPLC system equipped with a reverse-phase semi-preparative column (Alltima C18 10 μm, 250 mm × 10 mm, Alltech). Analytical HPLC analyses were performed on Dionex Ulltimate 3000 system equipped with a reverse-phase column (Acclaim 120, C18, 5 μm , 4.6-250 mm). Anhydrous solvents were either commercial or prepared according to standard techniques.

Biological Assays
Cell Lines and Culture Conditions: The M21 human melanoma cell line (for $\alpha_v\beta_3$ binding assays) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Melanoma cells were grown in Dulbecco's modified Eagle medium, containing 4500 mg/L glucose (DMEM 4500, GIBCO) supplemented with 10% foetal calf serum (FCS) at 37 °C in a humidified incubator containing 10% CO_2. $5.0x10^5$ melanoma cells were seeded in 100 mm Sarstedt dishes and propagated every 3 days by incubation with a trypsin−EDTA solution. The human erythroleukemia cell line K562 (for $\alpha_5\beta_1$ binding assays) was maintained in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO) supplemented with 10% FCS in T25 culture flasks (Sarstedt) in humidified incubator at 37°C, 5% CO_2. When cultures reached a cell density between $1x10^5$ and $1x10^6$ cells/ml cells were re-suspended in warm fresh media at a volume to yield a density of $2x10^5$ cells/ml. Both M21 and K562 cultures were periodically monitored for mycoplasma contamination.
Citofluorimetric Assays: M21 cells were detached by gentle treatment with Accutase (Lonza), a 0.5 mM EDTA solution. K562 cells were removed from culture flasks and re-suspended in fresh medium. Cells were then washed, and incubated for 1 h at 4°C in the presence of anti-$\alpha_v\beta_3$ monoclonal antibody (1 μg/50 μL, anti-integrin $\alpha_v\beta_3$, clone LM609, Millipore), anti-$\alpha_v\beta_5$ monoclonal antibody (1 μg/50 μL, anti-integrin $\alpha_v\beta_5$, Santa Cruz 13588) and anti-$\alpha_5\beta_1$

monoclonal antibody (1 μg/50 μL, anti-integrin $\alpha_5\beta_1$, Abcam ab75472) . Cells were then washed and incubated for 1 h at 4 °C with a specific secondary antibody, 5 μg/mL goat anti-mouse IgG conjugated with FITC (Santa Cruz Biotecnology, Inc., Santa Cruz, CA). Integrin-Positive cells were analysed at 488 nm on the flow cytometer FACScan system (BD-FACS Canto).

Cell Adhesion Assay: Plates (96 wells) were coated with vitronectin (for $\alpha_v\beta_3$ binding assays, 10 μg/mL) or fibronectin (for $\alpha_5\beta_1$ binding assays, 10 μg/mL) (both from Sigma) by overnight incubation at 4 °C. Plates were washed with PBS and then incubated at 37 °C for 1 h with PBS−1% BSA. After being washed cells were counted and re-suspended in serum free medium, and exposed to compound (final concentration was 10 nM, 0.1, 1, or 10 μM) at 37 °C for 30 min to allow the ligand-receptor equilibrium to be reached. Assays were performed in the presence of 2 mmol/L $MnCl_2$. Cells were then plated (4−5x10^4 cells/well) and incubated at 37 °C for 1 h. All the wells were washed with PBS to remove the non-adherent cells, and 0.5% crystal violet solution in 20% methanol was added. After 2 h of incubation at 4 °C, plates were examined at 540 nm in a counter ELX800 (Bio TEK Instruments). Experiments were conducted in triplicate and were repeated at least three times. The values are expressed as % inhibition ± SEM of cell adhesion relative to untreated cells.

Data Analysis: The IC_{50} values were determined by fitting binding inhibition data by non-linear regression using GraphPad Prism 4.0 Software Package (GraphPad Prism, San Diego, CA).

NMR Methods

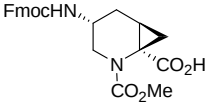
NMR experiments on diluted DMSO-d_6 solutions of **2** and **4** were performed at a temperature of 298 K on a Varian Mercury 400 MHz NMR spectrometer and on a Bruker Avance II 500 MHz Ultrashield. All proton chemical shifts were assigned unambiguously for **2** and **4**. Variable temperature 1D, and 2D experiments (TOCSY, gCOSY, ROESY) were carried out at the sample concentration of 3 mM for **2** and **4**. One-dimensional ^{1}H NMR spectra for determining temperature coefficients were obtained at 298-323 K with increments of 5 K for **2** and at 293–328 K with increments of 5 K or 10 K for **4**. Sample temperatures were controlled with the variable-temperature unit of the instrument. Proton signals were assigned via TOCSY spectra, and ROESY spectra provided the data discussed in the conformational analysis. TOCSY spectra were recorded with 2048 points in t1, 200 points or 256 points in t2, and 16 scans per t2 increment and using a mixing time of 80 ms. ROESY spectra were recorded with a similar number of t1 and t2 points unless otherwise noted, and 64 scans per t2 increment, and using a spinlock of 0.5 s or 0.2 s.

Molecular Modelling

Molecular modelling calculations were carried out within the framework of Macromodel v6.5,[6] using Amber*[7] as a force field and the implicit water GB/SA solvation model of Still et al.[8] Monte Carlo energy minimization (MCEM)[9] conformational searches of the peptide analogue containing methyl groups instead of the Arg and Asp side chains were performed as the first step. The torsional space of each AGA cyclopeptide was randomly varied with the usage-directed Monte Carlo conformational search. Ring-closure bonds were defined in the CPA ring and in the cyclopeptide ring. Amide bonds were included among the rotatable bonds. For each search, at least 1000 starting structures for each variable torsion angle were generated and minimized until the gradient was less than 0.05 kJ/Åmol using the truncated Newton-Raphson method implemented in Macromodel. Duplicate conformations and those with an energy greater than 6 kcal/mol above the global minimum were discarded.

Docking Calculations

Automated docking studies were carried out by the Autodock 4.0.1 program[12] by using the Lamarckian genetic algorithm (LGA) as a search engine. The AutoDockTools 1.4.5 (ADT) graphical interface[23] was used to prepare the receptor and the ligands PDBQT files. The coordinates of the ligand **2** were retrieved from the lowest energy conformers that resulted from calculation using NMR spectroscopic data, whereas the coordinates of $\alpha_v\beta_3$ receptor was retrieved from the Protein Data Bank (PDB code: 1L5G), and the ligand–protein complex was unmerged for achieving free receptor structure. Water molecules were removed. For the protein receptor and ligand **2**, all hydrogen atoms were added, Gasteiger charges were computed, and non-polar hydrogen atoms were merged. A charge value of +2.0 to each Mn atom of the protein receptor was successively added. Three-dimensional energy scoring grids of 0.375 Å resolution and 40 Å x 40 Å x 40 Å dimensions were computed. The centre of the grid was set to be coincident with the mass centre of the ligands preliminary fitted on the X-ray structure of c[RGDf(Me)V] in the $\alpha_v\beta_3$ complex (1L5G). A total of 50 runs with a maximum of 2 500000 energy evaluations were carried out for each ligand, using the default parameters for LGA. Cluster analysis was performed on the docked results using a root-meansquare (rms) tolerance of 1.5 Å. The analysis of the binding mode, the calculation of the binding energy and the prediction of the binding activity of the docked conformations were carried out using PyMol Autodock Tools plugin within PyMol software.[24]

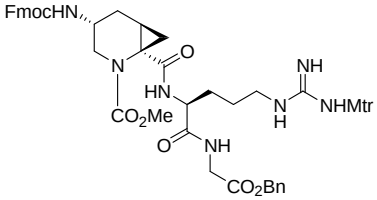


(1*R*,4*R*,6*S*)-4-(9-Fluorenylmethoxycarbonylamino)-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [(–)-5]

A 10% solution of Na_2CO_3 (2.5 mL) was added to a suspension of amino acid **1**[chap. 4] in THF (2 mL). The resulting mixture was cooled to 0 °C and a solution of Fmoc-OSu (297 mg, 0.88 mmol) in THF (6 mL) was added. The reaction mixture was vigorously stirred at room temperature for 18 h. Afterward, the solvent was evaporated under *vacuum* and the residue was taken up in EtOAc (10 mL), a satd. solution of NH_4Cl (10 mL) was then added and the mixture was extracted with EtOAc (5 x 10 mL). The aqueous layer was acidified to pH 2 by adding a 1 N solution of HCl and extracted again with EtOAc (5 x 10 mL). The combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent the crude was purified by flash chromatography (pure EtOAc; R_f 0.14) to afford compound **5** (282 mg, 74%) as a white solid.

M.p. 95-100 °C (dec.). $[\alpha]_D^{24}$ –6.5 (*c* 0.40, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of rotamers) δ (ppm): 7.75 (d, *J* = 7.5 Hz, 2 H, Fmoc), 7.56-7.55 (m, 2 H, Fmoc), 7.39 (t, *J* = 7.5 Hz, 2 H, Fmoc), 7.30 (t, *J* = 7.5 Hz, 2 H, Fmoc), 4.96 (bs, 1 H, NH), 4.58-4.30 (m, 2 H, CH_2 Fmoc), 4.25-4.15 (m, 2 H, CH Fmoc + 3-H_{eq} major rotamer), 4.10-3.98 (m, 1 H, 3-H_{eq}, minor rotamer), 3.73 (s, 3 H, OCH_3, minor rotamer), 3.71 (s, 3 H, OCH_3, major rotamer), 3.61-3.48 (m, 1 H, 4-H, major rotamer), 3.46-3.40 (m, 1 H, 4-H, minor rotamer), 2.88-2.60 (m, 1 H, 3-H_{ax}, both rotamers), 2.10-1.55 (m, 3 H, 5-H + 5-H' + 7-H), 1.42-1.10 (m, 1 H, 6-H), 1.0-0.78 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 177.3 (COOH), 157.0 (NCO), 155.7 (NCO), 143.7 (2 C, C_{arom}), 141.3 (2 C, C_{arom}), 127.7 (2 C, C_{arom}), 127.1 (2 C, C_{arom}), 125.0 (2 C, C_{arom}), 120.0 (2 C, C_{arom}), 66.7 (CH_2 Fmoc), 53.1 (OCH_3), 47.2 (CH Fmoc), 46.4, 45.7, 33.6, 29.7,

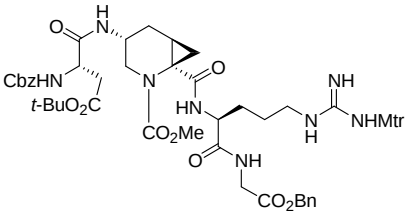
24.8, 21.0. MS (ESI) *m/z* (%): 895 (100) $[2M + Na]^+$, 459 (80) $[M + Na]^+$. Elemental analysis calcd (%) for $C_{24}H_{24}N_2O_6$ (436.46): C, 66.04; H, 5.54; N, 6.42. Found: C, 66.27; H, 5.29; N, 6.08.



4-Fmoc-[2-(methoxycarbonyl)-4-NHCPA]-Arg(Mtr)-Gly-OBn [(–)-6]

DEPBT (108 mg, 0.36 mmol) and DIPEA (63 µL, 0.36 mmol) were added under an N_2 atmosphere to a solution of **5** (79 mg, 0.18 mmol) in anhydrous THF (1.5 mL), cooled at 0 °C, and the resulting mixture was allowed to warm to room temperature. After 15 min the reaction was cooled again to 0 °C and a solution of H-Arg(Mtr)-Gly-OBn (144 mg, 0.27 mmol) in anhydrous THF (1.5 mL) was added. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (30 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (2 x 7.5 mL), a satd. solution of $NaHCO_3$ (2 x 7.5 mL) and H_2O (2 x 7.5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (pure EtOAc; R_f 0.40) affording pure **6** (122 mg, 71 %) as a white solid.

M.p. 165.6-169.9 °C (dec.). $[\alpha]_D^{26}$ –6.4 (*c* 0.70, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (major rotamer) δ (ppm): 7.85-7.72 (m, 3 H, NH + Fmoc), 7.57-7.55 (m, 2 H, Fmoc), 7.40-7.36 (m, 2 H, Fmoc), 7.34-7.27 (m, 7 H, Fmoc + Ph), 7.22-7.05 (m, 1 H, NH), 6.50 (s, 1 H, CH Mtr), 6.33 (bs, 3 H, NH_ε Arg), 5.22 (bs, 1 H, NH), 5.11-5.09 (m, 2 H, C*H*$_2$Ph), 4.65-4.55 (m, 1 H, H_α Arg), 4.42-4.25 (m, 2 H, CH_2 Fmoc), 4.20-4.18 (m, 1 H, CH Fmoc), 4.13-4.11 (m, 1 H, 3-H_{eq}), 4.06 (dd, *J* = 17.7, 5.3 Hz, 1 H, H_α Gly), 3.93 (dd, *J* = 17.7, 5.3 Hz, 1 H, H_α Gly), 3.79 (s, 3 H, OCH_3 Mtr), 3.67 (bs, 3 H, OCH_3), 3.46-3.40 (m, 1 H, 4-H), 3.31-3.15 (m, 2 H, H_δ Arg), 2.82-2.40 (m, 7 H, 3-H_{ax} + 2 x CH_3 Mtr), 2.10 (s, 3 H, CH_3 Mtr), 1.89-1.42 (m, 7 H, 5-H + 5-H' + 7-H + H_β Arg + H_γ Arg), 1.28-1.22 (m, 1 H, 6-H), 0.80-0.60 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.5 (C_q), 169.8 (C_q), 158.5 (C_q), 157.3 (C_q), 156.4 (C_q), 155.8 (C_q), 155.6 (C_q), 143.8 (2 C, $C_{q,arom}$), 141.2 (2 C, $C_{q,arom}$), 138.5 ($C_{q,arom}$), 136.6 ($C_{q,arom}$), 135.2 ($C_{q,arom}$), 133.2 ($C_{q,arom}$), 128.5 (2 C, CH_{arom}), 128.4 (CH_{arom}), 128.2 (2 C, CH_{arom}), 127.7 (2 C, C_{arom}), 127.0 (2 C, C_{arom}), 125.0 (2 C, C_{arom}), 124.8 ($C_{q,arom}$), 119.9 (2 C, C_{arom}), 111.7 (CH_{arom}), 67.1 (*C*H_2Ph), 66.8 (CH_2 Fmoc), 55.4 (OCH_3 Mtr), 53.4 (OCH_3), 52.5 (C_α Arg), 47.1 (CH Fmoc), 45.7 (C-3), 45.5 (C-4), 41.2 (C_α Gly), 40.3 (C_δ Arg), 30.0 (CH_2), 29.6 (CH_2), 27.7 (CH_2), 24.9 (CH_2), 24.8, 24.1 (CH_3 Mtr), 21.0, 18.3 (CH_3 Mtr), 11.9 (CH_3 Mtr). MS (ESI) *m/z* (%): 954 (23), 953 (51), 952 (100) $[M + 1]^+$. Elemental analysis calcd (%) for $C_{49}H_{57}N_7O_{11}S$ (952.08): C, 61.81; H, 6.03; N, 10.30. Found: C, 61.66; H, 6.28; N, 10.02.



4-[Z-Asp(O*t*Bu)]-2-(methoxycarbonyl)-4-NHCPA-Arg(Mtr)-Gly-OBn [(–)-8]

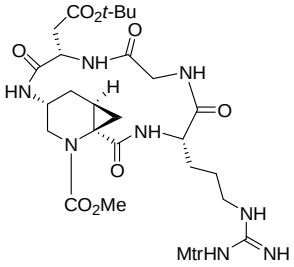
Compound **6** (122 mg, 0.13 mmol) was dissolved in a 1:1 CH_2Cl_2/diethylamine mixture (1.5 mL) under an N_2 atmosphere. The resulting solution was stirred at room temperature for 3 h, meanwhile additional 1:1 CH_2Cl_2/diethylamine mixture (1 mL) was added. Afterward, the solution was concentrated under *vacuum*, the residue was taken up in CH_2Cl_2 (4 mL) and then concentrated again. The crude was purified by flash chromatography (pure EtOAc, then MeOH/EtOAc 1:1; R_f 0.38), affording pure **7** (88 mg, 94 %) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) (major rotamer) δ (ppm): 7.76-7.73 (m, 1 H, NH), 7.32-7.28 (m, 5 H, Ph), 7.20-7.16 (m, 1 H, NH), 6.50 (s, 1 H, CH Mtr), 6.38-6.10 (m, 3 H, NH_ε Arg), 5.13-5.11 (m, 2 H, CH_2Ph), 4.62-4.50 (m, 1 H, H_α Arg), 4.25-3.86 (m, 5 H, NH_2 CPA + 3-H_{eq} + H_α Gly), 3.80 (s, 3 H, OCH_3 Mtr), 3.72-3.58 (m, 4 H, 4-H + OCH_3), 3.31-3.12 (m, 2 H, H_δ Arg), 2.85-2.70 (m, 1 H, 3-H_{ax}), 2.66 (s, 3 H, CH_3 Mtr), 2.59 (s, 3 H, CH_3 Mtr), 2.11 (s, 3 H, CH_3 Mtr), 1.96-1.45 (m, 7 H, 5-H + 5-H' + 7-H + H_β Arg + H_γ Arg), 1.28-1.22 (m, 1 H, 6-H), 0.72-0.65 (m, 1 H, 7-H'). MS (ESI) *m/z* (%): 753 (43), 752 (100) $[M + Na]^+$, 731 (38), 730 (83) $[M + 1]^+$.

DEPBT (79 mg, 0.27 mmol) and DIPEA (47 µL, 0.27 mmol) were added under an N_2 atmosphere to a solution of Z-Asp(O*t*Bu)-OH monohydrate (62 mg, 0.18 mmol) in anhydrous THF (1.5 mL), cooled at 0 °C, and the resulting mixture was allowed to warm to room temperature. After 15 min this solution was slowly added to a solution of compound **7** (88 mg, 0.12 mmol) in anhydrous THF (500 µL) precooled at 0 °C. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (20 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (2 x 5 mL), a satd. solution of $NaHCO_3$ (2 x 5 mL) and H_2O (2 x 5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (pure EtOAc; R_f 0.38) affording pure **8** (74 mg, 59 %) as a white solid.

M.p. 102-109 °C (dec.). $[\alpha]_D^{25}$ –4.4 (*c* 0.75, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (major rotamer) δ (ppm): 7.65 (bs, 1 H, NH), 7.32-7.28 (m, 10 H, 2 x Ph), 7.05 (bs, 1 H, NH), 6.75 (bs, 1 H, NH), 6.50 (s, 1 H, CH Mtr), 6.31 (bs, 3 H, NH_ε Arg), 6.06 (bs, 1 H, NH), 5.10-5.02 (m, 4 H, 2 x CH_2Ph), 4.60-4.57 (m, 1 H, H_α Asp), 4.49-4.40 (m, 1 H, H_α Arg), 4.06 (dd, *J* = 17.8, 5.6 Hz, 1 H, H_α Gly), 3.93 (dd, *J* = 17.8, 4.9 Hz, 1 H, H_α Gly), 3.90-3.82 (m, 1 H, 4-H), 3.84-3.72 (m, 4 H, 3-H_{eq} + OCH_3 Mtr), 3.66 (bs, 3 H, OCH_3), 3.60-3.58 (m, 1 H, 3-H_{ax}), 3.31-3.17 (m, 2 H, H_δ Arg), 2.82-2.68 (m, 2 H, H_β Asp), 2.66 (s, 3 H, CH_3 Mtr), 2.59 (s, 3 H, CH_3 Mtr), 2.10 (s, 3 H, CH_3 Mtr), 2.00-1.46 (m, 7 H, 5-H + 5-H' + 7-H + H_β Arg + H_γ Arg), 1.40 [s, 9 H, $OC(CH_3)_3$], 1.28-1.22 (m, 1 H, 6-H), 0.80-0.60 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.5, 170.5, 170.4, 169.8, 158.4, 157.3, 156.4, 156.1, 138.5 (C_{arom}), 136.5 (C_{arom}), 136.4 (C_{arom}), 136.0 (C_{arom}), 135.2 (C_{arom}), 133.4 (C_{arom}), 128.6 (2 C, C_{arom}), 128.5 (2 C, C_{arom}), 128.4 (C_{arom}), 128.3 (2 C, C_{arom}), 128.2 (2 C, C_{arom}), 128.1 (C_{arom}), 124.8 (C_{arom}), 111.7 (C_{arom}), 81.7 [O*C*$(CH_3)_3$], 67.2 (*C*H_2Ph), 67.1 (*C*H_2Ph), 55.4 (OCH_3 Mtr), 53.4 (OCH_3), 52.4 (C_α Arg), 51.3 (C_α Asp), 46.1 (C-3), 43.8 (C-4), 41.2 (C_α Gly), 40.3 (C_δ Arg), 37.7 (C_β Asp), 30.2, 29.6, 28.0 [3 C, OC(*C*$H_3)_3$], 27.1, 25.7, 24.8, 24.1 (CH_3 Mtr), 22.6, 18.3 (CH_3 Mtr), 11.9 (CH_3 Mtr). MS (ESI) m/z (%): 1057 (55) $[M + Na]^+$, 1036 (58),

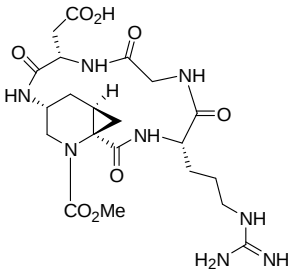
1035 (100) $[M + 1]^+$. Elemental analysis calcd (%) for $C_{50}H_{66}N_8O_{14}S$ (1035.17): C, 58.01; H, 6.43; N, 10.82. Found: C, 57.77; H, 6.35; N, 10.59.



***Cyclo*[Arg(Mtr)-Gly-Asp(O*t*Bu)-2-(methoxycarbonyl)-4-NHCPA] [(–)-10]**

Pd/C (35 mg, 10%) was added to a solution of **8** (74 mg, 0.07 mmol) in ethanol (4 mL), under an N_2 atmosphere. The resulting suspension was stirred under a H_2 atmosphere (balloon) at room temperature for 24 h. After filtration over a celite layer and evaporation of the solvent, compound **9** (51 mg, 88%) was obtained as a white solid. This crude was suspended in THF (20 mL) under an N_2 atmosphere. The suspension was cooled to 0 °C and DEPBT (63 mg, 0.21 mmol) and DIPEA (37 µL, 0.21 mmol) were added. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (10 mL) was added and the mixture washed with a satd. solution of NH_4Cl (5 mL), a satd. solution of $NaHCO_3$ (5 mL) and H_2O (5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (pure EtOAc, then MeOH/EtOAc, 1:10; R_f 0.48) affording pure **10** (27 mg, 55%) as a white solid.

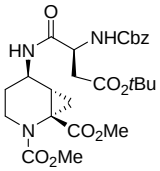
$[\alpha]_D^{23}$ –7.6 (*c* 1.35, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (major rotamer) δ (ppm): 7.82 (bs, 1 H, NH), 7.52 (bs, 1 H, NH), 7.40 (bs, 1 H, NH), 7.06-6.88 (bs, 1 H, NH), 6.51 (s, 1 H, CH Mtr), 6.40-6.15 (m, 3 H, NH_ε Arg), 4.71-4.59 (m, 1 H, H_α Asp), 4.56-4.46 (m, 1 H, H_α Arg), 4.34-4.18 (m, 2 H, 4-H + H_α Gly), 3.81 (s, 3 H, OCH_3 Mtr), 3.76-3.58 (m, 4 H, 3-H_{eq} + OCH_3), 3.45-3.35 (m, 2 H, 3-H_{ax} + H_α Gly), 3.31-3.17 (m, 2 H, H_δ Arg), 2.80-2.38 (m, 9 H, H_β Asp + 5-H + 2 x CH_3 Mtr), 2.11 (s, 3 H, CH_3 Mtr), 2.04-1.46 (m, 6 H, 5-H' + 7-H + H_β Arg + H_γ Arg), 1.41 (s, 9 H, $OC(CH_3)_3$), 1.15-1.04 (m, 1 H, 6-H), 0.82-0.71 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 173.1 (C_q), 171.5 (C_q), 170.6 (C_q), 170.1 (C_q), 158.4 (C_q), 157.3 (C_q), 156.5 (C_q), 156.4 (C_q), 138.5 ($C_{q,arom}$), 136.5 ($C_{q,arom}$), 133.4 ($C_{q,arom}$), 124.8 (C_{arom}), 111.7 (CH_{arom}), 81.7 [$OC(CH_3)_3$], 55.4 (OCH_3 Mtr), 53.4 (OCH_3), 52.6 (C_α Arg), 49.3 (C_α Asp), 47.4 (C-3), 44.1 (C_α Gly), 43.6 (C-4), 40.3 (C_δ Arg), 37.4 (C_β Asp), 30.3, 29.7, 29.3, 28.0 [3 C, $OC(CH_3)_3$], 26.4, 25.4, 24.1 (CH_3 Mtr), 22.7, 18.3 (CH_3 Mtr), 11.9 (CH_3 Mtr). MS (ESI) *m/z* (%): 815 (20) $[M + Na]^+$, 794 (46), 793 (100) $[M + 1]^+$. Elemental analysis calcd (%) for $C_{35}H_{52}N_8O_{11}S$ (792.90): C, 53.02; H, 6.61; N, 14.13. Found: C, 52.87; H, 6.54; N, 13.97.



***Cyclo*[Arg-Gly-Asp-2-(methoxycarbonyl)-4-NHCPA]·TFA [(–)-2]**

Protected tetrapeptide **10** (27 mg, 0.034 mmol) was dissolved in a 95:2.5:2.5 trifluoroacetic acid/triisopropylsilane/H_2O mixture (2 mL) and the resulting solution was stirred at room temperature for 18 h. Afterward, the mixture was evaporated under *vacuum* and the residue was taken up in H_2O (3 mL) and washed with Et_2O (4 x 1 mL). The aqueous layer was then concentrated under *vacuum*, affording the deprotected cyclic tetrapeptide as a trifluoroacetate salt. This crude was purified by semi-preparative HPLC (C18 column, 10 µm, 250 mm × 10 mm i.d.) using acetonitrile (0.1% TFA) in H_2O (0.1% TFA), 5-35% linear gradient over 22.5 min at room temperature with a flow rate of 2 mL/min (λ = 223 nm; R_t = 20.6 min). The HPLC sample was concentrated under *vacuum* and lyophilized, affording pure **2** (9.6 mg, 44%) as a colourless glassy solid. Purity was checked by HPLC analysis (C18 column, 5 µm , 250 mm x 4.6-mm i.d.), using acetonitrile (0.1% TFA) in water (0.1% TFA) as eluant, 5-35% linear gradient over 35 min at room temperature.

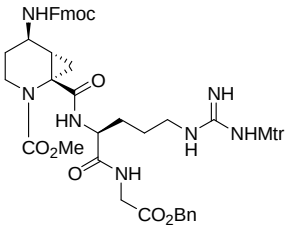
$[\alpha]_D^{23}$ –16.0 (*c* 0.25, D_2O). ^{1}H NMR (400 MHz, DMSO-d_6) (2:1 mixture of rotamers) (major rotamer) δ (ppm): 12.14 (bs, 1 H, COOH), 8.16-8.10 (m, NH Arg), 7.96-7.88 (m, NH CPA), 7.66-7.60 (m, NH Asp), 7.58-7.46 (m, 1 H, NH_ε Arg), 7.39 (d, *J* = 9.3 Hz, 1 H, NH Gly), 7.32-6.64 (m, 3 H, NH_ε Arg), 4.56-4.51 (m, 1 H, H_α Asp), 4.42-4.38 (m, 1 H, H_α Arg), 4,31-4.24 (m, 1 H, H_α Gly), 4.05-3.92 (m, 1 H, 4-H), 3.62-3.48 (m, 4 H, 3-H_{eq} + OCH_3), 3.21-3.17 (m, 1 H, H_α Gly), 3.15-3.02 (m, 2 H, H_δ Arg), 2.98-2.90 (m, 1 H, 3-H_{ax}), 2.65-2.51 (m, 1 H, H_β Asp), 2.32-2.21 (m, 2 H, 5-H + H_β Asp), 1.92-1.80 (m, 2 H, 7-H + H_β Arg), 1.73-1.55 (m, 2 H, 6-H + H_β Arg), 1.48-1.25 (m, 3 H, 5-H' + H_γ Arg), 0.60-0.46 (m, 1 H, 7-H'). ^{1}H NMR (400 MHz, D_2O) (3:1 mixture of rotamers) (major rotamer) δ (ppm): 4.65-4.60 (m, 1 H, H_α Asp), 4.42-4.35 (m, 1 H, H_α Arg), 4.18 (d, *J* = 15.6 Hz, 1 H, H_α Gly), 4.12-4.04 (m, 1 H, 4-H), 3.61 (s, 3 H, OCH_3), 3.50-3.44 (m, 1 H, 3-H_{eq}), 3.41 (d, *J* = 15.6 Hz, 1 H, H_α Gly), 3.34-3.26 (m, 1 H, 3-H_{ax}), 3.12-3.04 (m, 2 H, H_δ Arg), 2.78-2.66 (m, 2 H, H_β Asp), 2.38-2.30 (m, 1 H, 5-H), 1.88-1.84 (m, 1 H, 7-H), 1.82-1.65 (m, 3 H, 6-H + H_β Arg), 1.58-1.41 (m, 2 H, H_γ Arg), 1.18-1.09 (m, 1 H, 5-H'), 0.89-0.80 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, D_2O) (major rotamer) δ (ppm): 184.4 (C_q), 174.9 (C_q), 172.0 (C_q), 171.6 (C_q), 171.1 (C_q), 163.1 (C_q), 162.8 (C_q), 156.7 (C_q), 117.8 (C_q), 53.4 (OCH_3), 52.7 (C_α Arg), 49.5 (C_α Asp), 46.7 (C-3), 44.1 (C_α Gly), 43.2 (C-4), 40.5 (C_δ Arg), 36.6 (C_β Asp), 28.1, 26.0, 25.1, 24.3, 22.4, 22.2. MS (ESI) *m/z* (%): 526 (29), 525 (100) $[M + 1]^+$.



5-[Z-Asp(O*t*Bu)]-2-(methoxycarbonyl)-5-NHCPA-OMe [(+)-13]

DEPBT (347 mg, 1.16 mmol) and DIPEA (202 µL, 1.16 mmol) were added to a solution of Z-Asp(O*t*Bu)-OH·H_2O (273 mg, 0.80 mmol) in anhydrous THF (3.5 mL) cooled at 0 °C, under an N_2 atmosphere, and the resulting mixture was allowed to warm to room temperature. After 15 min this solution was slowly added to a solution of compound **12**[chap. 5] (92 mg, 0.40 mmol) in anhydrous THF (3 mL) precooled at 0 °C. The resulting reaction mixture was stirred at 35 °C for 6 days. Afterward, EtOAc (20 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (10 mL), a satd. solution of $NaHCO_3$ (10 mL) and H_2O (10 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (*n*-hexane/EtOAc 1:1, R_f 0.23) affording pure **13** (178 mg, 84 %) as a white solid.

M.p. 62.7-66.0 °C. $[\alpha]_D^{21}$ +16.6 (*c* 1.40, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of rotamers) δ (ppm): 7.38-7.30 (m, 5 H, Ph, both rotamers), 7.00-6.90 (m, 1 H, NH CPA, both rotamers), 6.02-5.90 (m, 1 H, NH Asp, both rotamers), 5.11 (s, 2 H, CH_2Ph, both rotamers), 4.55-4.44 (m, 1 H, H_α Asp, both rotamers), 4.18-4.05 (m, 1 H, 5-H, both rotamers), 3.88-3.81 (m, 1 H, 3-H_{eq}, major rotamer), 3.75-3.62 (m, 7 H, 3-H_{eq} minor rotamer + 2 x OCH_3 both rotamers), 3.06-2.98 (m, 1 H, 3-H_{ax}, minor rotamer), 2.94-2.84 (m, 2 H, 3-H_{ax} major rotamer + H_β Asp both rotamers), 2.59 (dd, *J* = 17.2, 7.4 Hz, 1 H, H_β' Asp, both rotamers), 1.97 (dd, *J* = 10.0, 5.7 Hz, 1 H, 7-H, minor rotamer), 1.90 (dd, *J* = 10.1, 5.4 Hz, 1 H, 7-H, major rotamer), 1.68-1.56 (m, 3 H, 4-H + 4-H' + 6-H, both rotamers), 1.42 [s, 9 H, $OC(CH_3)_3$], 0.92-0.84 (m, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.2, 171.2, 169.9, 156.9, 156.1, 135.9 (C_{arom}), 128.6 (2 C, C_{arom}), 128.3 (2 C, C_{arom}), 128.1 (C_{arom}), 82.0 [O*C*$(CH_3)_3$], 67.3 (*C*H_2Ph), 53.0 (OCH_3), 52.5 (OCH_3), 50.9 (C_α Asp), 43.1, 38.3, 37.9, 37.7 (C_β Asp), 29.1, 28.0 [3 C, OC(*C*$H_3)_3$], 27.8, 21.3. MS (ESI) m/z (%): 1089 (22) $[2M + Na]^+$, 556 (100) $[M + Na]^+$. Elemental analysis calcd (%) for $C_{26}H_{35}N_3O_9$ (533.57): C, 58.53; H, 6.61; N, 7.88. Found: C, 58.64; H, 6.33; N, 7.62.

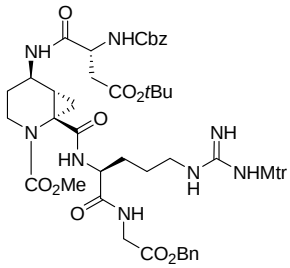


5-Fmoc-[2-(Methoxycarbonyl)-5-NHCPA]-Arg(Mtr)-Gly-OBn [(–)-15]

Prepared as reported for **6**, starting from **14**[chap. 5] (100 mg, 0.23 mmol). After flash chromatography (pure EtOAc, R_f 0.59), pure **15** (172 mg, 79 %) was obtained as a white solid.

M.p. 110-130 °C (dec.). $[\alpha]_D^{21}$ –5.8 (*c* 0.80, $CHCl_3$). ^{1}H NMR (400 MHz, CD_3OD) (2:1 mixture of rotamers) δ (ppm): 8.41-8.36 (m, 1 H, NH Gly, minor rotamer), 8.15 (bs, 2 H, NH Gly + NH Arg, major rotamer), 7.90-7.86 (m, 1 H, NH Arg, minor rotamer), 7.76 (d, *J* = 7.4 Hz, 2 H, Fmoc, both

rotamers), 7.60 (t, *J* = 7.1 Hz, 2 H, Fmoc, both rotamers), 7.37-7.25 (m, 9 H, Fmoc + Ph, both rotamers), 6.65 (s, 1 H, CH Mtr, minor rotamer), 6.60 (s, 1 H, CH Mtr, major rotamer), 5.17-5.12 (m, 2 H, C*H*$_2$Ph, both rotamers), 4.41-4.35 (m, 3 H, H$_\alpha$ Arg + CH$_2$ Fmoc, both rotamers), 4.17-4.05 (m, 2 H, H$_\alpha$ Gly + CH Fmoc, both rotamers), 4.02-3.84 (m, 2 H, H$_\alpha$ Gly + 5-H, both rotamers), 3.81 (s, 3 H, OCH$_3$ Mtr, minor rotamer), 3.79-3.74 (m, 4 H, 3-H$_{eq}$ both rotamers + OCH$_3$ Mtr major rotamer), 3.66 (bs, 3 H, OCH$_3$, both rotamers), 3.23-2.90 (m, 3 H, 3-H$_{ax}$ + H$_\delta$ Arg, both rotamers), 2.67 (s, 3 H, CH$_3$ Mtr, minor rotamer), 2.66 (s, 3 H, CH$_3$ Mtr, major rotamer), 2.61 (s, 3 H, CH$_3$ Mtr, minor rotamer), 2.59 (s, 3 H, CH$_3$ Mtr, major rotamer), 2.11 (s, 3 H, CH$_3$ Mtr, minor rotamer), 2.07 (s, 3 H, CH$_3$ Mtr, major rotamer), 1.99-1.42 (m, 8 H, 4-H + 4-H' + 6-H + 7-H + H$_\beta$ Arg + H$_\gamma$ Arg, both rotamers), 0.81-0.72 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, CD$_3$OD) (major rotamer) δ (ppm): 174.8, 173.8, 171.7, 159.8, 159.1, 158.1, 157.9, 145.3 (2 C, C$_{arom}$), 142.6 (2 C, C$_{arom}$), 139.5 (C$_{arom}$), 137.8 (C$_{arom}$), 137.1 (C$_{arom}$), 134.8 (C$_{arom}$), 129.6 (2 C, C$_{arom}$), 129.3 (C$_{arom}$), 128.7 (2 C, C$_{arom}$), 128.3 (C$_{arom}$), 128.1 (C$_{arom}$), 128.0 (2 C, C$_{arom}$), 126.1 (2 C, C$_{arom}$), 125.7 (C$_{arom}$), 120.9 (2 C, C$_{arom}$), 112.7 (C$_{arom}$), 68.0 (*C*H$_2$Ph), 67.5 (CH$_2$ Fmoc), 55.9 (OCH$_3$ Mtr), 54.6, 53.9 (OCH$_3$), 48.5 (CH Fmoc), 45.2, 44.8, 42.0 (C$_\alpha$ Gly), 41.7, 39.5, 29.8, 29.3, 28.9, 27.1, 24.4 (CH$_3$ Mtr), 20.8, 18.9(CH$_3$ Mtr), 12.1 (CH$_3$ Mtr). MS (ESI) m/z (%): 1926 (9) [2M + Na]$^+$, 974 (100) [M + Na]$^+$.



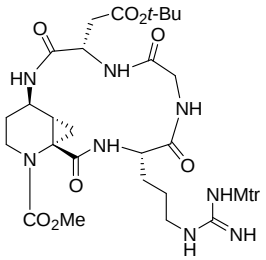
5-[Z-Asp(O*t*Bu)]-2-(methoxycarbonyl)-5-NHCPA-Arg(Mtr)-Gly-OBn [(+)-17]

Prepared in two steps as reported for **8**, starting from **15** (170 mg, 0.18 mmol). After flash chromatography (pure EtOAc, R$_f$ 0.40), pure **17** (84 mg, 49 % over two steps) was obtained as a white solid.

16: M.p. 160 °C (dec.). [α]$_D^{19}$ –3.6 (*c* 0.92, CH$_3$OH). ^{1}H NMR (400 MHz, CD$_3$OD) (major rotamer) δ (ppm): 7.35-7.30 (m, 5 H, Ph), 6.65 (s, 1 H, CH Mtr), 5.16 (s, 2 H, C*H*$_2$Ph), 4.42-4.39 (m, 1 H, H$_\alpha$ Arg), 4.12-3.90 (m, 2 H, H$_\alpha$ Gly), 3.82 (s, 3 H, OCH$_3$ Mtr), 3.71-3.60 (m, 4 H, 3-H$_{eq}$ + OCH$_3$), 3.35-3.10 (m, 4 H, 3-H$_{ax}$ + 5-H + H$_\delta$ Arg), 2.67 (s, 3 H, CH$_3$ Mtr), 2.61 (s, 3 H, CH$_3$ Mtr), 2.12 (s, 3 H, CH$_3$ Mtr), 1.98-1.82 (m, 3 H, 7-H + H$_\gamma$ Arg), 1.71-1.48 (m, 7 H, 5-H, 4-H + 4-H' + 6-H + H$_\beta$ Arg), 0.80-0.72 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, CD$_3$OD) (major rotamer) δ (ppm): 174.8, 174.0, 171.9, 159.8, 158.2, 142.7 (C$_{arom}$), 139.4 (C$_{arom}$), 137.8 (C$_{arom}$), 137.1 (C$_{arom}$),, 134.8 (C$_{arom}$), 129.5 (2 C, C$_{arom}$), 129.3 (C$_{arom}$), 128.3 (C$_{arom}$), 125.7 (2 C, C$_{arom}$), 112.7 (C$_{arom}$), 68.0 (*C*H$_2$Ph), 56.0 (OCH$_3$ Mtr), 54.5, 53.9 (OCH$_3$), 45.0, 42.0, 41.8, 39.0, 31.4, 30.7, 30.3, 29.3, 27.0, 24.3 (CH$_3$ Mtr), 20.9, 18.8 (CH$_3$ Mtr), 12.1 (CH$_3$ Mtr). MS (ESI) m/z (%): 1459 (18) [2M + 1]$^+$, 752 (17) [M + Na]$^+$, 731 (42), 730 (100) [M + 1]$^+$.

17: M.p. 110 °C (dec.). [α]$_D^{21}$ +22.4 (*c* 0.84, CHCl$_3$). ^{1}H NMR (400 MHz, CDCl$_3$) (major rotamer) δ (ppm): 7.78 (bs, 1 H, NH Gly), 7.68-7.40 (m, 1 H, NH CPA), 7.36-7.12 (m, 10 H, 2 x Ph), 7.18-7.05 (m, 1 H, NH Arg), 6.50 (s, 1 H, CH Mtr), 6.28-5.96 (m, 4 H, NH$_\varepsilon$ Arg + NH Asp), 5.16-4.96 (m, 4 H, 2 x C*H*$_2$Ph), 4.61-4.40 (m, 2 H, H$_\alpha$ Asp + H$_\alpha$ Arg), 4.26 (bs, 1 H, 5-H), 4.16-3.98 (m, 2 H, H$_\alpha$ Gly), 3.90-3.74 (m, 4 H, 3-H$_{eq}$ + OCH$_3$ Mtr), 3.64 (s, 3 H, OCH$_3$), 3.28-2.90 (m, 3 H, 3-H$_{ax}$ + H$_\delta$

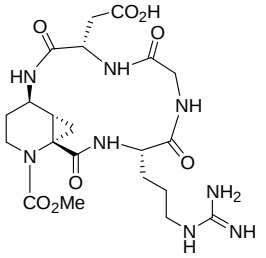
Arg), 2.75-2.65 (m, 5 H, H_β Asp + CH_3 Mtr), 2.60 (s, 3 H, CH_3 Mtr), 2.11 (s, 3 H, CH_3 Mtr), 2.04-1.86 (m, 3 H, 7-H + H_β Arg), 1.78-1.50 (m, 5 H, 4-H + 4-H' + 6-H + H_γ Arg), 1.37 (s, 9 H, $OC(CH_3)_3$), 0.72-0.68 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.4, 171.6, 170.2, 169.8, 158.3, 157.5, 156.3, 156.0, 138.4 (C_{arom}), 136.4 (C_{arom}), 136.1 (C_{arom}), 135.1 (C_{arom}), 135.0 (C_{arom}), 133.3 (C_{arom}), 128.5 (2 C, C_{arom}), 128.4 (2 C, C_{arom}), 128.3 (C_{arom}), 128.2 (2 C, C_{arom}), 128.1 (2 C, C_{arom}), 127.8 (C_{arom}), 124.7 (C_{arom}), 111.6 (CH_{arom}), 81.4 [*O*$C(CH_3)_3$], 67.0 (*C*H_2Ph), 66.8 (*C*H_2Ph), 55.3 (OCH_3 Mtr), 53.3 (OCH_3), 52.8 (C_α Arg), 51.3 (C_α Asp), 42.7 (C-5), 41.1 (C_α Gly), 40.4 (C_δ Arg), 38.3 (C-3), 37.9 (C_β Asp), 29.6 (C_β Arg), 29.1, 28.3 (C-6), 27.8 [3 C, $OC(CH_3)_3$], 27.4 (C-4), 25.1 (C_γ Arg), 24.0 (CH_3 Mtr), 20.0 (C-7), 18.3 (CH_3 Mtr), 11.8 (CH_3 Mtr). MS (ESI) m/z (%): 1057 (100) $[M + Na]^+$, 1035 (16) $[M + 1]^+$.



***Cyclo*[Arg(Mtr)-Gly-Asp(O*t*Bu)-2-(methoxycarbonyl)-5-NHCPA] (19)**

Prepared in two steps as reported for **10**, starting from **17** (84 mg, 0.08 mmol). After flash chromatography (EtOAc, then MeOH/EtOAc 1:4, R_f 0.60), **19** (21 mg) was obtained in mixture with other unidentified products and as a white solid.

^{1}H NMR (400 MHz, CD_3OD) (major product, major rotamer) δ (ppm): 6.66 (s, 1 H, CH Mtr), 4.69-4.61 (m, 1 H, H_α Asp), 4.53-4.43 (m, 1 H, 5-H), 4.30-4.23 (m, 1 H, H_α Arg), 4.07-3.96 (m, 1 H, H_α Gly), 3.93-3.80 (m, 4 H, 3-H_{eq} + OCH_3 Mtr), 3.78-3.63 (m, 3 H, OCH_3), 3.46 (d, *J* = 13.6 Hz, 1 H, H_α Gly), 3.23-3.07 (m, 2 H, H_δ Arg), 2.94-2.76 (m, 3 H, 3-H_{ax} + H_β Asp), 2.65 (s, 3 H, CH_3 Mtr), 2.62-2.45 (m, 4 H, H_β Asp + CH_3 Mtr), 2.11 (s, 3 H, CH_3 Mtr), 1.96-1.47 (m, 7 H, 4-H + 4-H' + 7-H + H_β Arg + H_γ Arg), 1.41 (s, 9 H, $OC(CH_3)_3$), 1.33-1.22 (m, 1 H, 6-H), 0.87-0.66 (m, 1 H, 7-H'). MS (ESI) m/z (%): 815 (36), 794 (37), 793 (100) $[M + 1]^+$.



***Cyclo*[Arg-Gly-Asp-2-(methoxycarbonyl)-5-NHCPA]·TFA (4)**

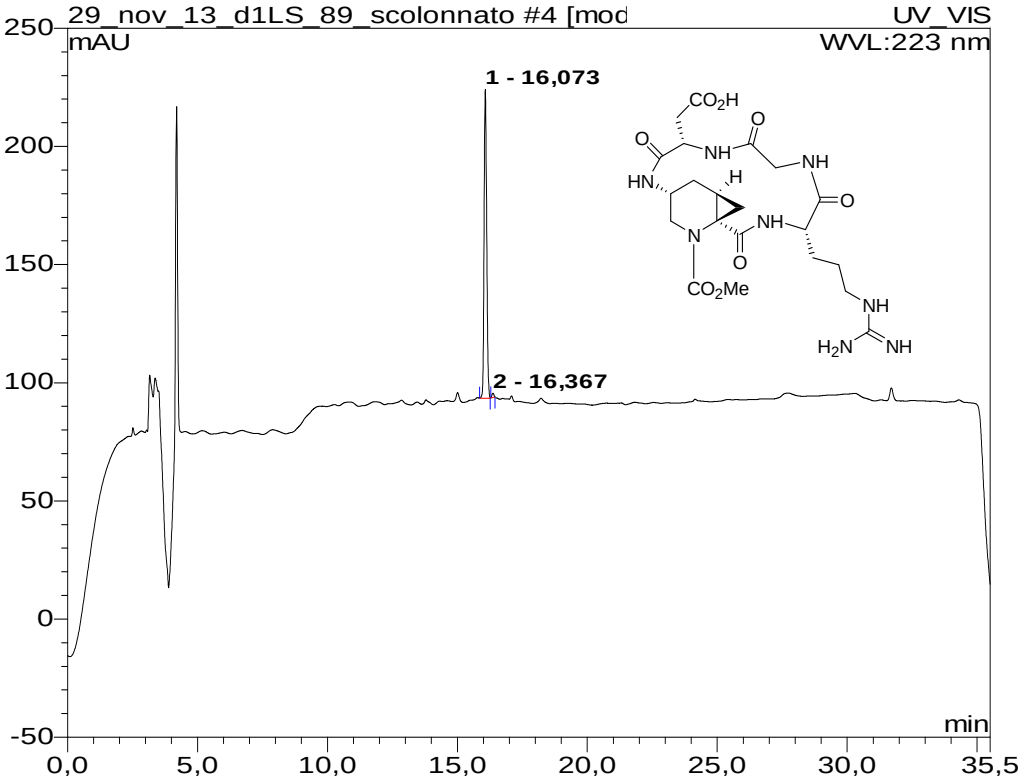
Prepared as reported for **2**, starting from **19** (21 mg, 0.026 mmol). The crude was purified by semi-preparative HPLC (C18 column, 10 μm, 250 mm × 10 mm) using acetonitrile (0.1% TFA) in H_2O (0.1% TFA), 0-50% linear gradient over 35 min at room temperature with a flow rate of 2 mL/min (λ = 223 nm; R_t = 25.4 min). The HPLC sample was concentrated under vacuum and

lyophilized, affording pure **4** (5.6 mg, 12%, calculated from **18**) as a colourless glassy solid. Purity checked by HPLC analysis (C18 column, 5 µm , 4.6-250 mm), using acetonitrile (0.1% TFA) in water (0.1% TFA) as eluant, 5-35% linear gradient over 35 min at room temperature.
^{1}H NMR (500 MHz, DMSO) (5:1 mixture of rotamers) (major rotamer) δ (ppm): 8.88 (d, *J* = 7.6 Hz, NH Asp), 7.77 (d, *J* = 8.1 Hz, NH CPA), 7.53-7.47 (m, NH Arg), 7.24-7.20 (m, NH Gly), 7.19-6.64 (m, 4 H, NH_{ε} Arg), 4.52-4.46 (m, 1 H, H_{α} Asp), 4.16-4.11 (m, 1 H, 5-H), 4.04-3.95 (m, 1 H, H_{α} Gly), 3.86-3.79 (m, 1 H, 3-H_{eq}), 3.66-3.59 (m, 4 H, H_{α} Arg + OCH_3), 3.34-3.26 (m, 1 H, H_{α} Gly), 3.12-3.02 (m, 2 H, H_{δ} Arg), 2.99-2.87 (m, 1 H, 3-H_{ax}), 2.82 (dd, *J* = 16.5, 9.0 Hz, 1 H, H_{β} Asp), 2.45 (dd, *J* = 16.5, 6.1 Hz, 1 H, H_{β} Asp), 1.99-1.81 (m, 2 H, H_{β} Arg), 1.67-1.60 (m, 1 H, 7-H), 1.55-1.46 (m, 2 H, 4-H), 1.44-1.36 (m, 2 H, H_{γ} Arg), 1.36-1.29 (m, 1 H, 6-H), 0.83-0.71 (m, 1 H, 7-H'). MS (ESI) m/z (%): 526 (26), 525 (100) $[M + 1]^{+}$. Elemental analysis calcd (%) for $C_{23}H_{33}F_3N_8O_{10}$ (638.55): C, 43.26; H, 5.21; N, 17.55. Found: C, 43.48; H, 5.02; N, 17.23.

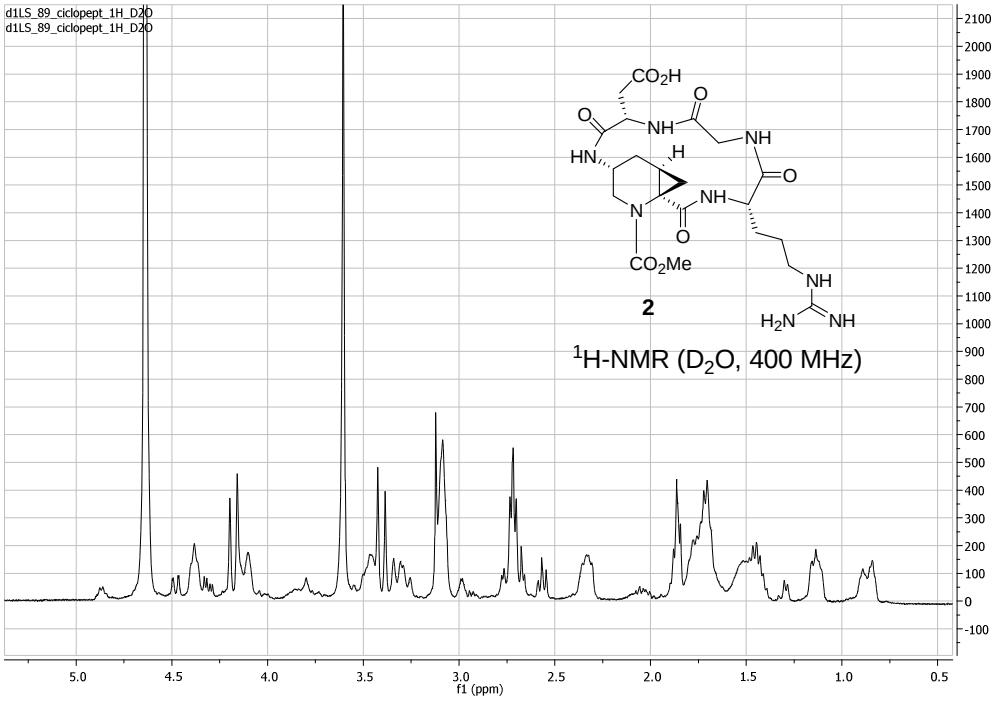
6.8 Appendix

Appendix 1: Analytical HPLC and Full NMR Characterisation of Ligand 2

HPLC analysis of purified cyclopeptide **2**. Column: Acclaim 120 (C18, 5 µ, 4.6-250 mm), acetonitrile (0.1% TFA) in water (0.1% TFA), 5-35% linear gradient over 35 min at room temperature.

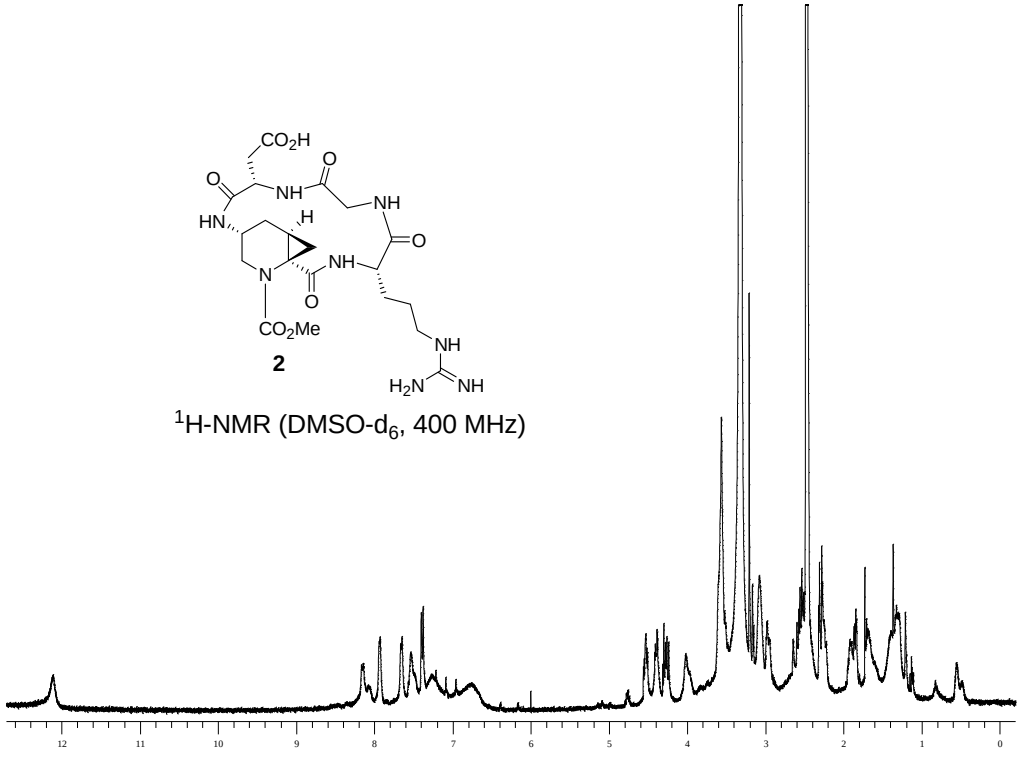


No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area (%)
1	16.07	130.677	14.976	98.94
2	16.37	1.808	0.160	1.06
Total		132.485	15.136	100.00



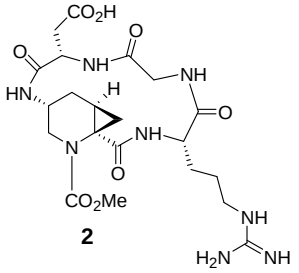
2

^{1}H-NMR (D_2O, 400 MHz)



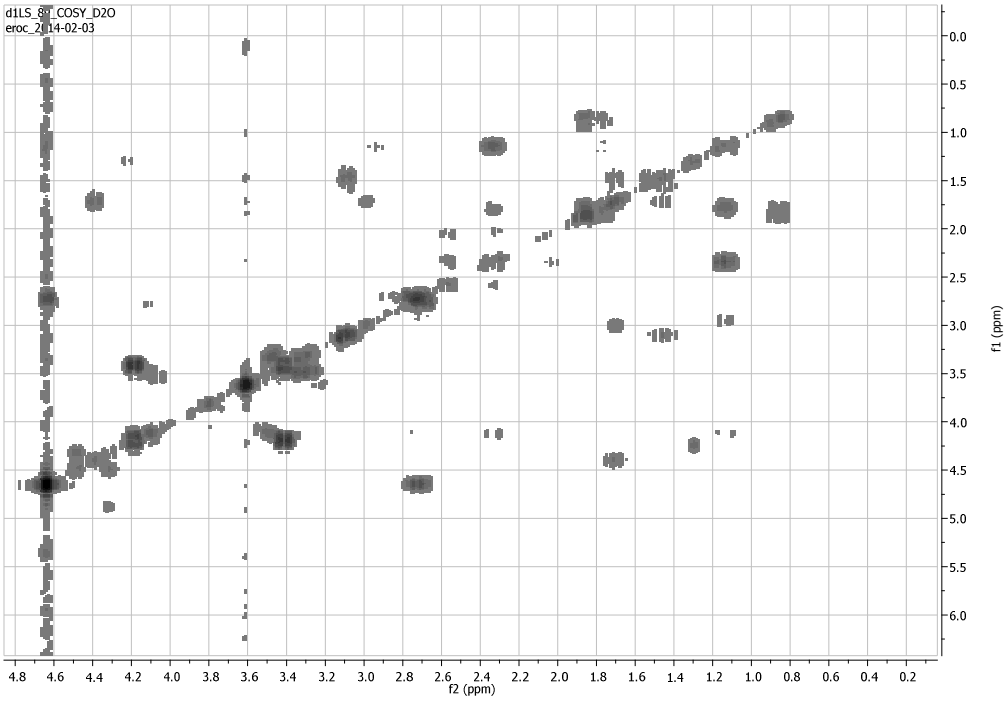
2

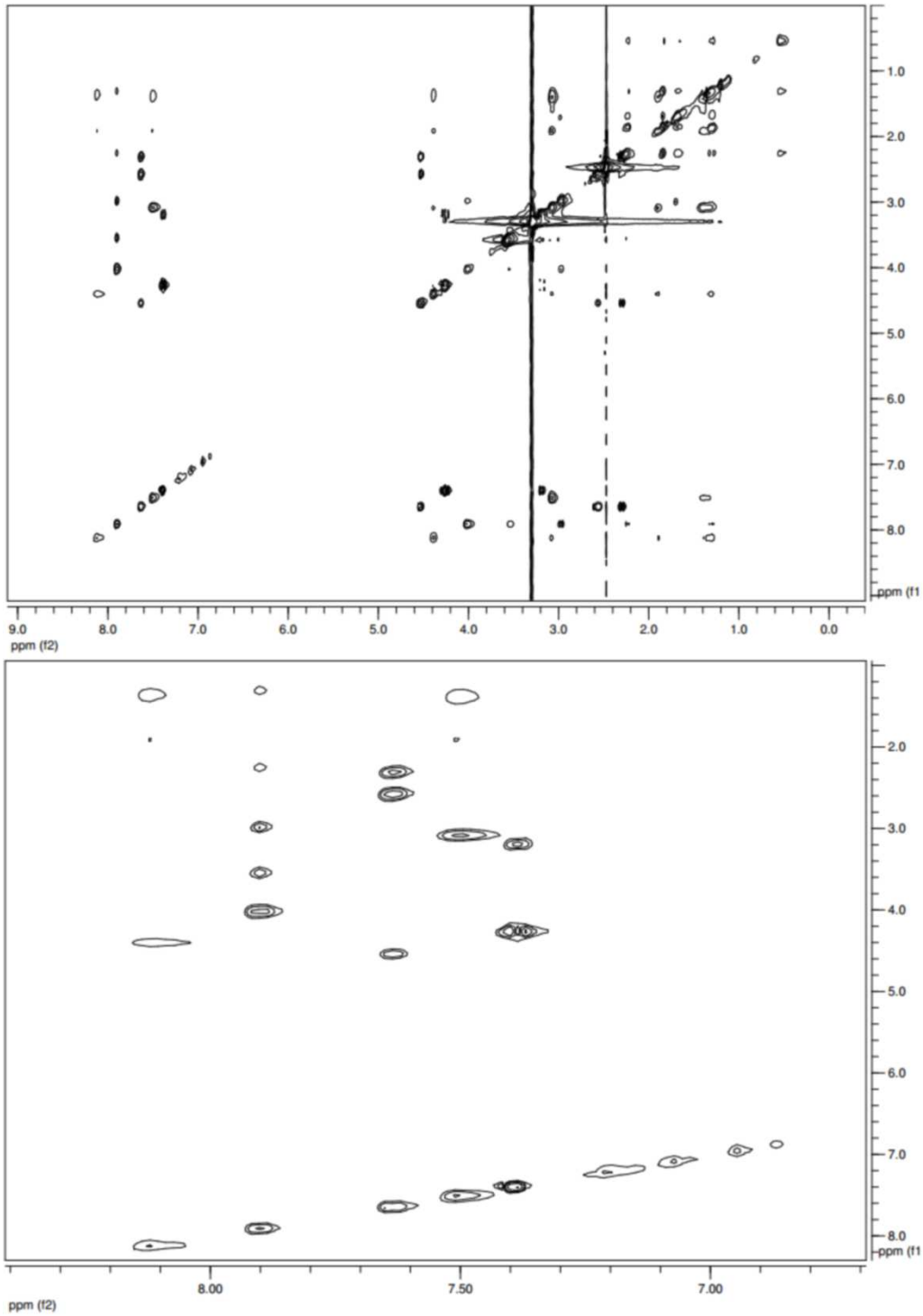
^{1}H-NMR (DMSO-d_6, 400 MHz)



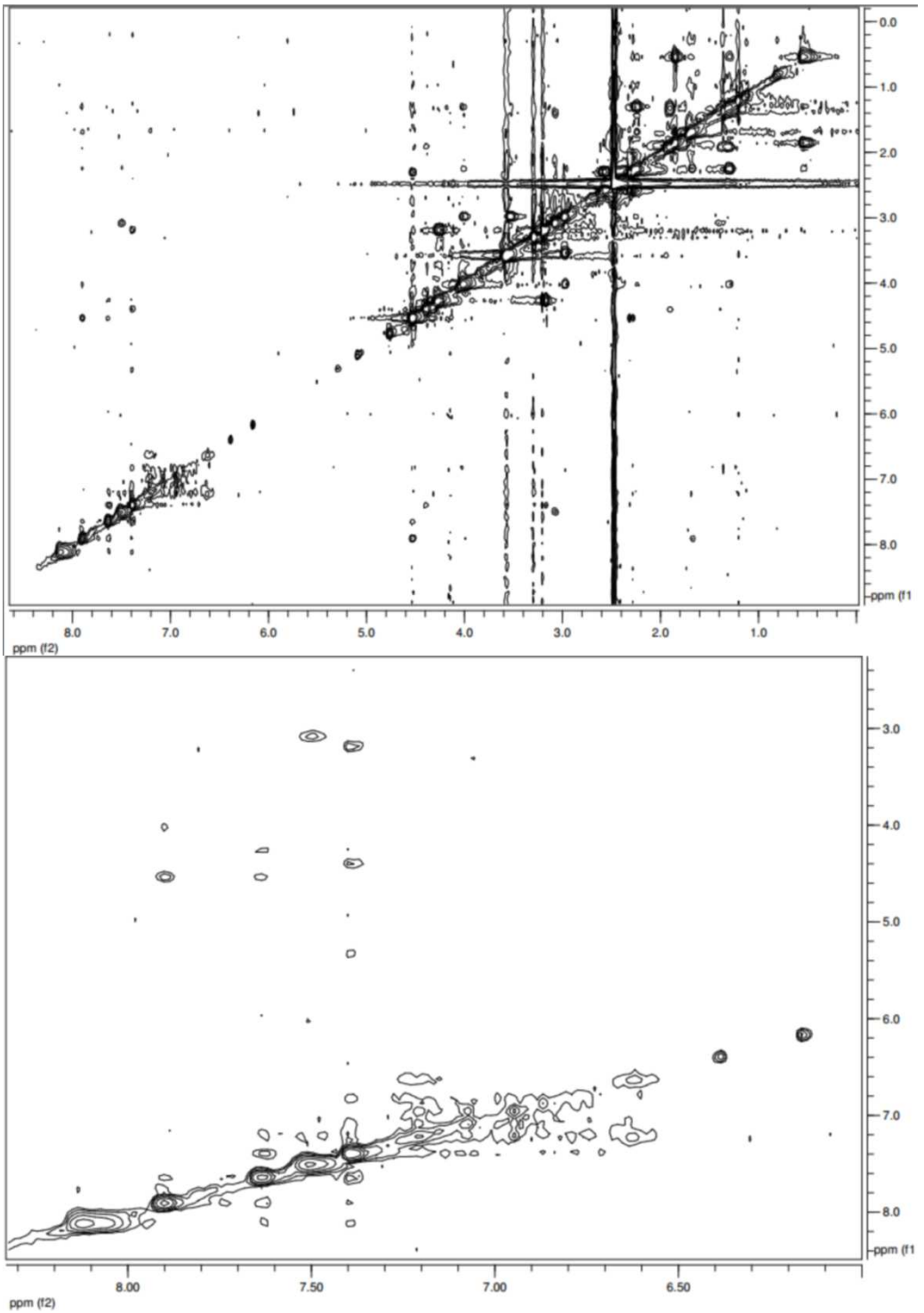
2

gCOSY (D_2O, 400 MHz)





TOCSY full spectrum (top) and TOCSY NH-α region (bottom) of ligand **2** (DMSO-d_6, 400 MHz)



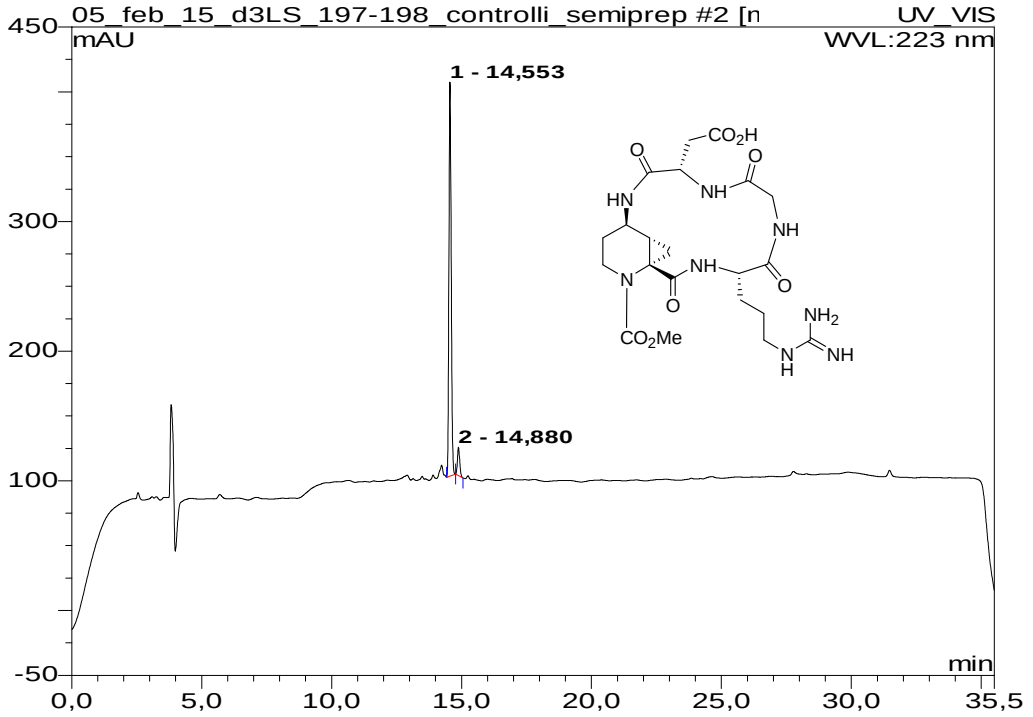
ROESY full spectrum (top) and ROESY NH-α region (bottom) of ligand **2** (DMSO-d_6, 400 MHz)

^{1}H and ^{13}C NMR chemical shifts of **2** at 298 K

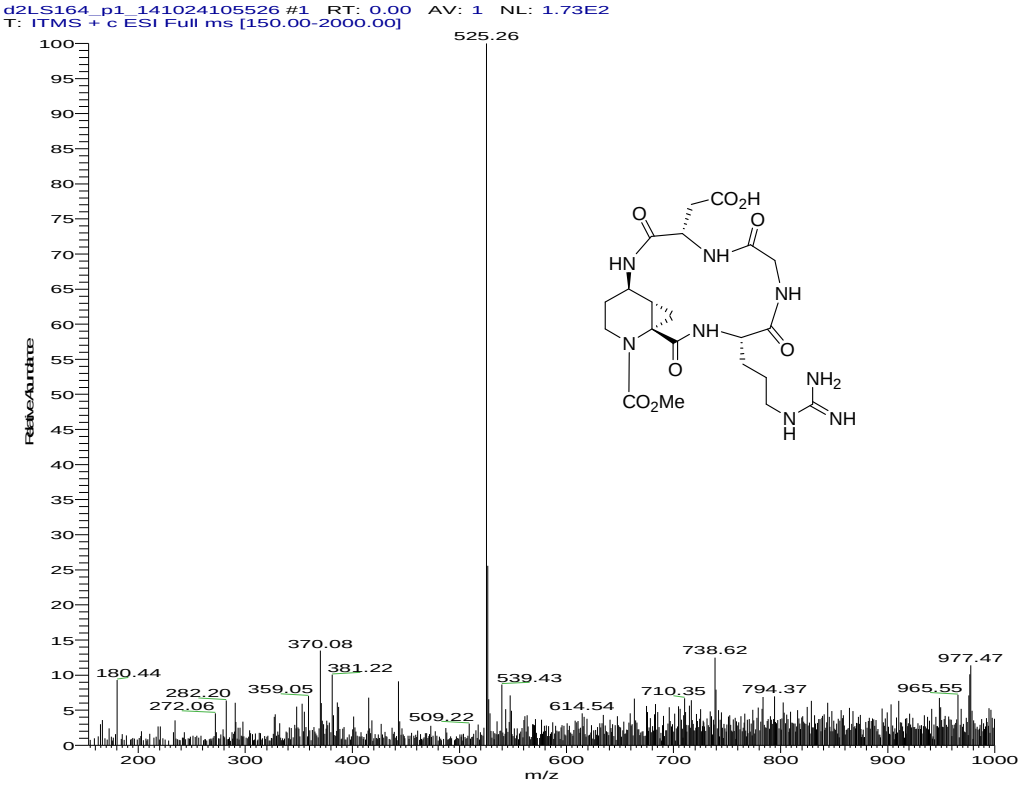
2 δ, ppm			
^{1}H (DMSO-d_6)		^{13}C (D_2O)	
-	-	C-1	28.1
H-3	3.62-3.48, 2.98-2.90	C-3	46.7
H-4	4.05-3.92	C-4	43.2
H-5	2.32-2.21, 1.48-1.25	C-5	25.1
H-6	1.73-1.55	C-6	22.4
H-7	1.92-1.80, 0.60-0.46	C-7	22.2
OCH_3	3.62-3.48	OCH_3	53.4
CPA NH	7.96-7.88	-	-
Gly NH	7.39	-	-
Arg NH	8.16-8.10	-	-
Arg NH_ε	7.58-7.46, 7.32-6.64	-	-
Asp NH	7.66-7.60	-	-
Gly H-α	4.31-4.24, 3.21-3.17	Gly C-α	44.1
Arg H-α	4.42-4.38	Arg C-α	52.7
Arg H-β	1.92-1.80, 1.73-1.55	Arg C-β	26.0
Arg H-γ	1.48-1.25	Arg C-γ	24.3
Arg H-δ	3.15-3.02	Arg C-δ	40.5
Asp H-α	4.56-4.51	Asp C-α	49.5
Asp H-β	2.65-2.51, 2.32-2.21	Asp C-β	36.6

Appendix 2: Analytical HPLC, MS-ESI and Full NMR Characterisation of Ligand 4

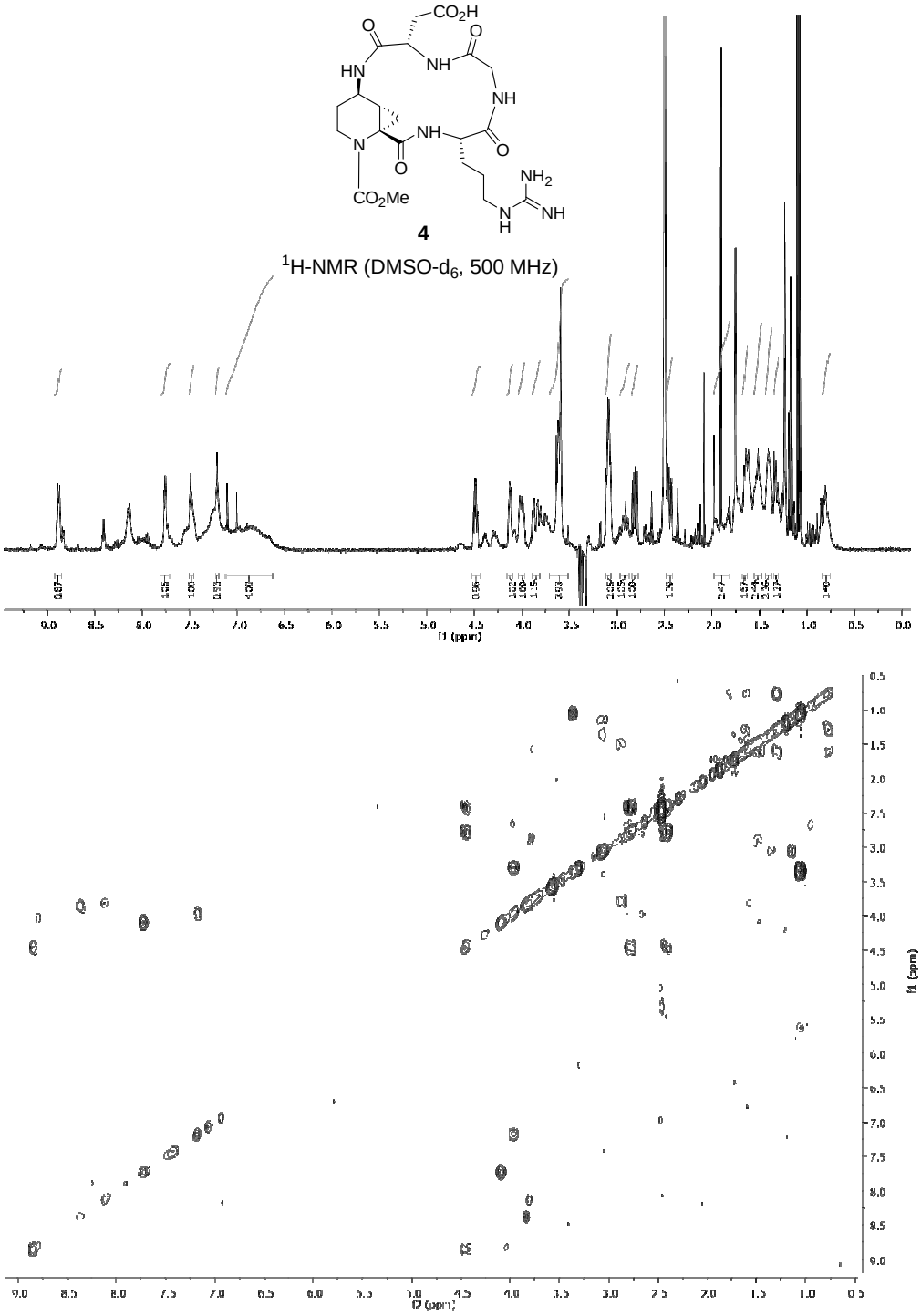
HPLC analysis of purified cyclopeptide **4**. Column: Acclaim 120 (C18, 5 µ, 4.6-250 mm), acetonitrile (0.1% TFA) in water (0.1% TFA), 5-35% linear gradient over 35 min at room temperature.



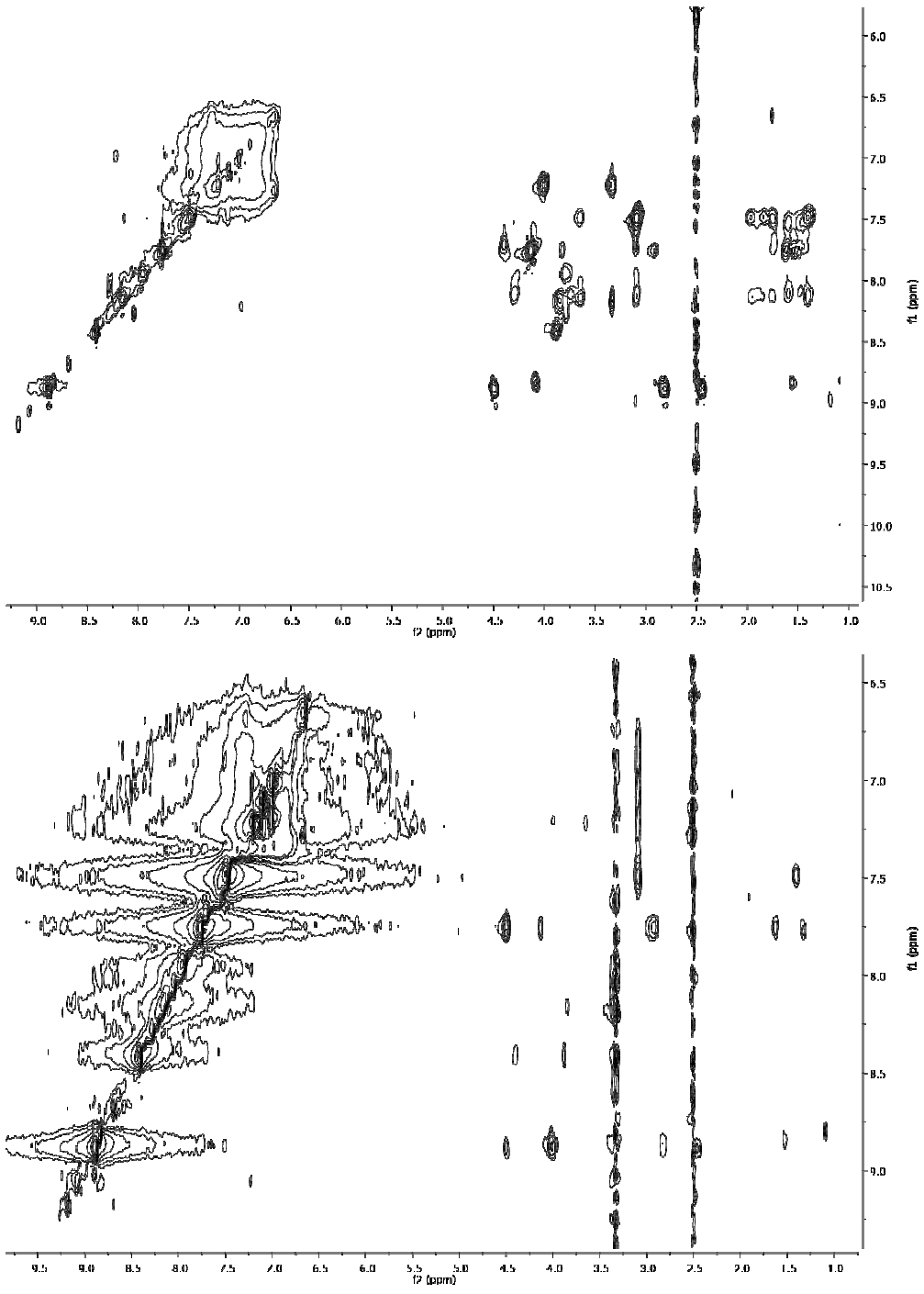
No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area (%)
1	14.55	303.910	30.711	93.24
2	14.88	21.836	2.226	6.76
Total		325.746	32.936	100.00



MS (ESI) m/z (%) of **4**: 526 (26), 525 (100) $[M + 1]^+$.



^{1}H-NMR (DMSO-d_6, 500 MHz) (top) and gCOSY (DMSO-d_6, 400 MHz) (bottom) of ligand **4**



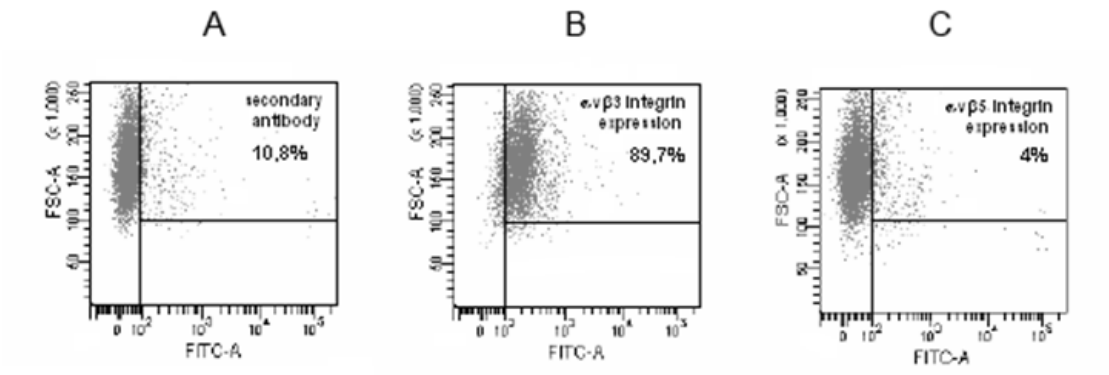
TOCSY NH-α region (top) and ROESY NH-α region (bottom) of ligand **4** (DMSO-d_6, 500 MHz)

^{1}H chemical shifts of **4** in DMSO-d_6 solutions at 298 K

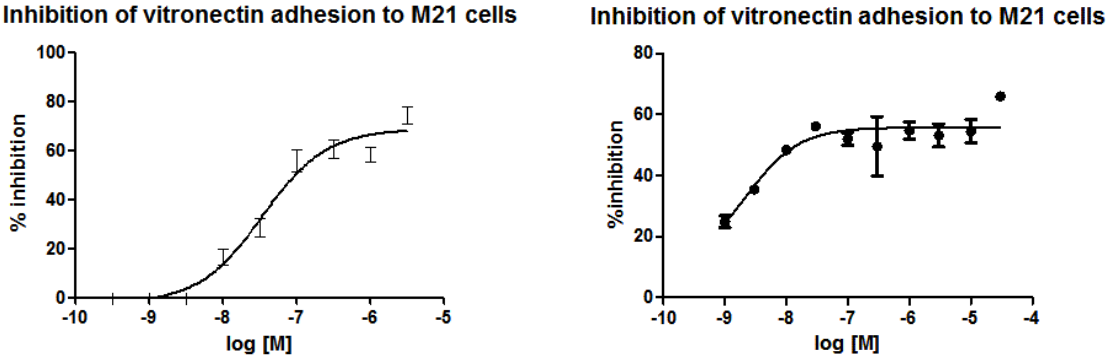
4 δ, ppm			
Proton	^{1}H	Proton	^{1}H
CPA NH	7.78	Arg NH	7.51
CPA 5-H	4.14	Asp NH	8.90
CPA 3-H_{eq}	3.81	Gly H-α	3.96; 3.29
CPA 3-H_{ax}	2.91	Arg H-α	3.63
CPA 7-H	1.62	Arg H-β,γ	1.93-1.39
CPA 4-H (2H)	1.49	Arg H-δ	3.04
CPA 6-H	1.31	Asp H-α	4.47
CPA 7-H'	0.75	Asp H-β	2.80; 2.40
Gly NH	7.22		

Appendix 3: Cell Biology Assays ($\alpha_v\beta_3$)

The RGD peptidomimetic ligands **2** and **4** were tested for their integrin binding affinity towards M21 human melanoma cells expressing high levels of $\alpha_v\beta_3$ heterodimer and low levels of $\alpha_v\beta_5$ heterodimer, as shown by flow cytometry analysis below.



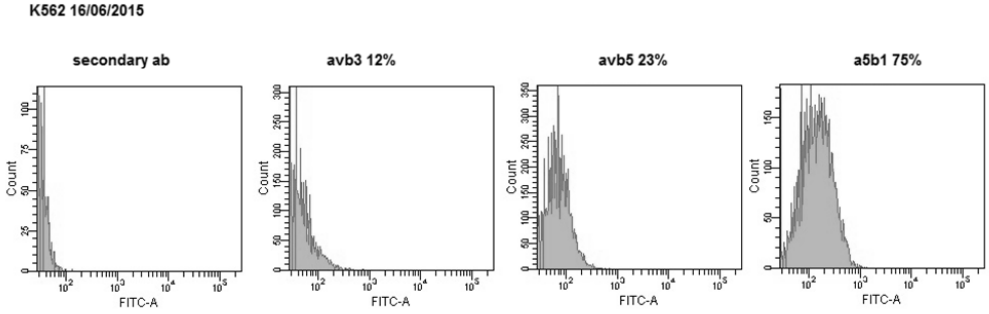
$\alpha_v\beta_3$ (B) and $\alpha_v\beta_5$ (C) integrin expression in M21 human melanoma cells by flow cytometry analysis



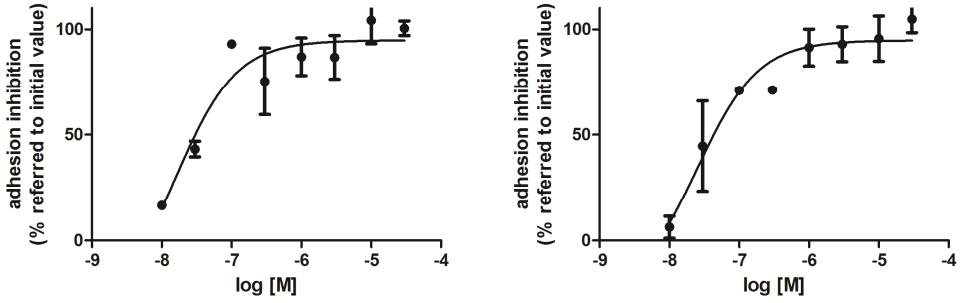
Percentage of inhibition of integrin-mediated M21 cell adhesion to vitronectin by compound **2** (left) and **4** (right). Experiments were conducted in triplicate and repeated at least three times. Data are presented as means ±SEM from three independent experiments.

Appendix 4: Cell Biology Assays ($\alpha_5\beta_1$)

The RGD peptidomimetic ligands **2** and **4** were tested for their integrin binding affinity towards K562 human erythroleukemia cells expressing high levels of $\alpha_5\beta_1$ heterodimer, as shown by flow cytometry analysis below.



$\alpha_5\beta_1$ integrin expression (right) in K562 human erythroleukemia cells by flow cytometry analysis



Percentage of inhibition of integrin-mediated K562 cell adhesion to fibronectin by compounds **2** (left) and **4** (right). Experiments were conducted in triplicate and repeated at least three times. Data are presented as means ±SEM from three independent experiments.

6.9 References

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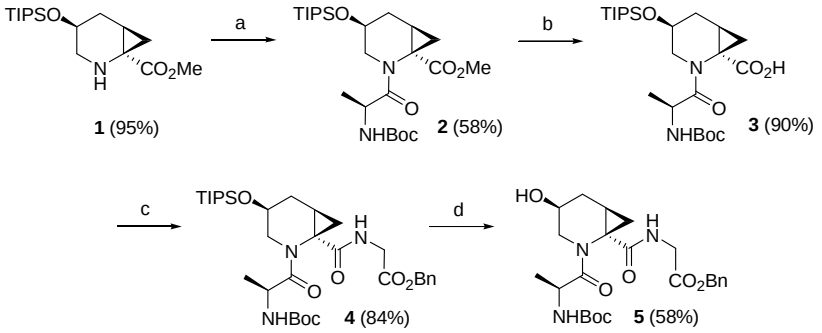
Chapter 7: Conformational Evaluation of a CPA-containing Linear Peptide

7.1 Synthesis of a Model Tripeptide Including a 4-OH-CPA

Eventually, we wanted to investigate the possibility to employ CPAs to form peptide bonds by exploiting the piperidine nitrogen atom, by the same way natural pipecolic acid is usually employed in peptide chemistry.

At the same time, we took the opportunity to assess the conformational features offered by CPAs, when used in this way. Specifically, we evaluated the *cis*/*trans* isomerism about the pipecolic acid peptide bond and we compared it with that of selected published proline- or pipecolic acid-containing peptides (see Scope of the work).

Thus, we chose 4-OH-CPA **1** (see section 4.6) as the central template to build the model linear tripeptide Boc-Ala-4-OH-CPA-Gly-OBn (**5**) (scheme 1).



Scheme 1. Reagents and conditions: a) Boc-Ala-OH, DEPBT, DIPEA, THF, RT, 4 d; b) NaOH, MeOH, 24 h; c) H-Gly-OBn hydrochloride, DEPBT, DIPEA, THF, 35 °C, 4 d; d) TBAF, THF, RT.

Amino ester **1** was coupled with an excess amount of N-Boc-protected alanine (1.5 eq.) by using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one) (DEPBT) as the coupling reagent in THF at 25 °C. As usual, the reaction resulted very slow and required 4 days to reach completion, but to our delight it afforded the desired dipeptide **2** in 58% yield. Once again (see sections 4.2 and 5.3), treatment of **2** with 1 N NaOH for 24 h at room temperature was sufficient for the hydrolysis of the ester group, providing acid **3**, which was coupled to O-benzyl-protected glycine by using DEPBT as the coupling reagent. In such a way, desired tripeptide **4** was obtained in 84% yield and cleavage of the TIPS protecting group by TBAF in THF afforded final product **5**.

For the preliminary conformational investigation we intended to conduct, we preferred to work in organic solvent, so we did not proceed with the exhaustive deprotection of the peptide and we performed our NMR studies directly on compound **5**.

7.2 NMR Investigation of the *Cis*/*Trans* Isomerism

To determine the *cis*/*trans* isomeric ratio, we carried out ^{1}H NMR experiments, which showed, as expected, the existence of two parallel sets of signals for every compound from intermediate **2** to final product **5**. Such sets are correlated to the two possible *cis* and *trans* rotational isomers, so that the isomeric ratio can be evaluated confronting the integration values of two signals (one from each the *cis* and the *trans* set) originated by the same selected

proton. On the other hand, we employed NOE correlation to assign each set to the appropriate rotational isomer.

At first, we evaluated the change in the *cis*/*trans* isomerism along the synthetic path by comparing the behaviour of intermediates **2** and **4** with that of the final product **5**.

In the proton NMR spectrum of compound **2** the signals assigned to corresponding protons in the two rotamers are markedly separated (by almost 1 ppm). 3-H_{ax} in the major rotamer (69%, figure 1, A) resonates at δ=2.7 ppm, whereas in the minor one (31%, figure 1, A) at δ=3.4 ppm. The signal of 3-H_{eq} is found at δ=4.4 ppm for the major rotamer, whereas the same proton resonates at δ=3.7 ppm in the minor rotamer. In the major rotamer, we do not observe any NOE correlation between any of 3-H protons with the α-CH of alanine resonating at δ=4.8 ppm. Instead, we do observe NOE correlations between the latter and both protons on the cyclopropane ring resonating at δ=1.4 and 2.1 ppm, as well as with the ester methyl group. Unfortunately, we were unable to record any NOE for the minor rotamer. Nonetheless, we could conclude that for intermediate **2** the major rotamer was the *cis* one (figure 1, A).

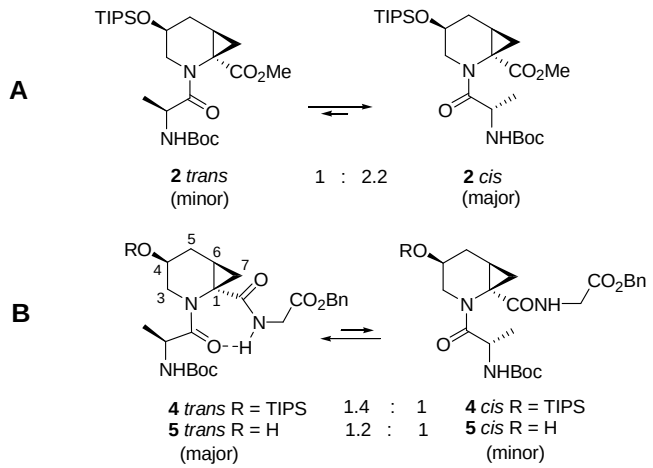


Figure 1. The cis/trans isomerism about the CPA peptide bond

Interestingly, after the second coupling, the ratio between the *cis* and *trans* isomers changed to 1:1.4 for intermediate **4** (figure 1, B) with an increase in the relative amount of the *trans* isomer. In **4** the α-CH of alanine resonate at δ=4.42 ppm in the major rotamer and at δ=4.9 ppm in the minor rotamer. The NOESY 1D study revealed a correlation between the α-CH of alanine resonating at δ=4.42 ppm and the 3-H_{eq} in the major rotamer, thereby confirming that the major isomer in compound **4** is *trans*.

This increment of *trans* concentration can be explained by the augmented steric hindrance with the bigger substituent at C-1 in the *cis*-peptide isomer. However, a hydrogen bond between the glycine NH and the alanine CO that forms a seven-membered cycle should stabilize the *trans* isomer, too (figure 1, B).[1] The deshielded signal assigned to the glycine NH, which resonates at δ=7.2 ppm, is consistent with the presence of this hydrogen bond, whereas in the *cis* isomer the NH resonates at δ=6.6 ppm.

Final removal of the TIPS group provided final product **5**, in which the *cis*/*trans* isomeric ratio, despite our expectations (owing to the lack of the sterically encumbered 4-OH protecting

group) remained practically unchanged (1:1.2) (figure 1, B). In *trans* isomer the glycine NH proton is still engaged in a hydrogen bond with the CO of the alanine, as suggested by its chemical shift at δ=7.43 ppm, so the relatively high amount of the *cis* isomer must be due to increased steric effects with the protons at C-3 caused by the presence of the cyclopropane ring.

7.3 Cis/Trans Isomerism in CPA-, pipecolic acid- and proline-containing model peptides

NMR spectroscopic experiments in water have shown that substitution of a proline residue (figure 2, A) with a pipecolic acid residue (figure 2, B) leads to a significant increase in population of the *cis* conformer (although the *trans* form still predominates) because the larger size of the pipecolic acid ring causes unfavourable interactions that are larger in the *trans* conformer.[2]

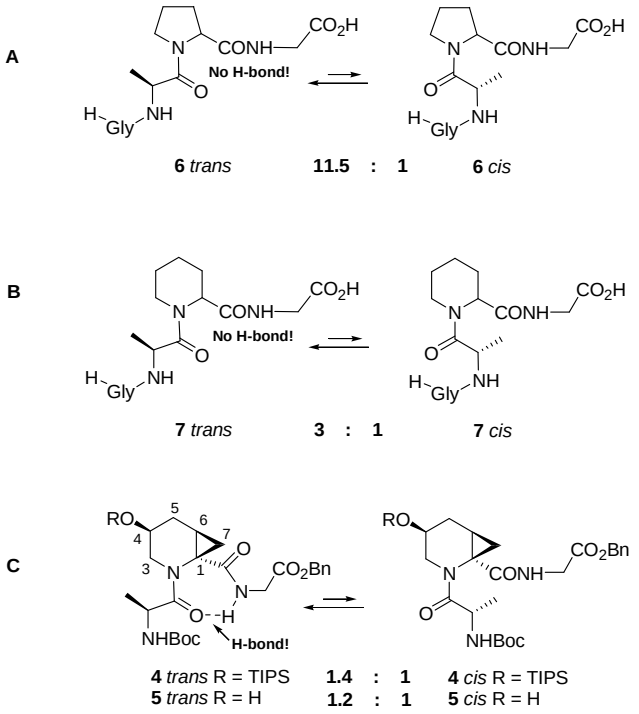


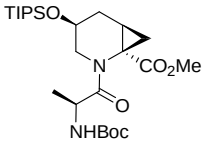
Figure 2. *Cis*/*trans* isomerism in proline-, pipecolic acid- and CPA-containing model peptides

Although our spectra were recorded in $CDCl_3$ and some solvent effect cannot be completely ruled out, we can still compare our NMR data with those of tetrapeptides **6** and **7**.

It is immediately clear that substitution of natural pipecolic acid with CPA leads to a further increment in *cis* population (figure 2), following the same trend already observed for the substitution of proline with pipecolic acid. On the other hand, the absence of any stabilizing hydrogen bond in the *trans* rotamer of **6** and **7** is in contradiction with the previous observation, since peptides **4** and **5** are those with the lower concentration of *trans* isomer, although their *trans* isomers are the only ones stabilized by hydrogen bonds. Therefore, the

intrinsic tendency of CPA to give *cis* rotamer has to be higher enough to balance and overcome the hydrogen bond stabilization. As mentioned above, we speculated that this effect could be due to further unfavourable interactions present in the *trans* rotamer between the α-CH of the alanine and the protons at C-3 of the CPA due to piperidine ring distortion imposed by the cyclopropane moiety. This is in accordance with data reported for *N*-acetyl-*N*'-methyl pipecolinamide (in water) in which there is an increase in the *cis* isomer from 28 to 43% when the ε position of the pipecolic amino acid (which corresponds to C-3 in CPA) bears a *tert*-butyl group.[1]

7.4 Experimental

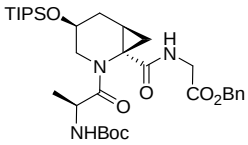


2-(Boc-Ala)-4-(triisopropylsilanyloxy)-CPA-OMe [(+)-2]

DEPBT (105 mg, 0.35 mmol) and DIPEA (61 μL, 0.35 mmol) were added under an N_2 atmosphere to a solution of Boc-Ala-OH (45 mg, 0.24 mmol) in anhydrous THF (1 mL), cooled at 0 °C, and the resulting mixture was allowed to warm to room temperature. After 15 min this solution was slowly added to a solution of compound **1**[chap. 4] (52 mg, 0.16 mmol) in anhydrous THF (500 μL) precooled at 0 °C. The resulting reaction mixture was stirred at room temperature for 4 days. Afterward, EtOAc (5 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (2.5 mL), a satd. solution of $NaHCO_3$ (2 x 2.5 mL), and H_2O (2.5 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under vacuum. The residue was purified by flash chromatography (*n*-hexane/EtOAc, 4:1; R_f 0.37) affording pure **2** (46 mg, 58%) as a colourless oil.

$[\alpha]_D^{21}$ +1.7 (*c* 0.9, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (2:1 mixture of rotamers) δ (ppm): 5.41 (d, *J* = 8.0 Hz, 1 H, NH Ala, minor rotamer), 5.03 (d, *J* = 9.4 Hz, 1 H, NH Ala, major rotamer), 4.84-4.76 (m, 1 H, H_α Ala, major rotamer), 4.68-4.58 (m, 1 H, H_α Ala, minor rotamer), 4.36 (dd, *J* = 13.1, 4.2 Hz, 1 H, 3-H_{eq}, major rotamer), 4.17-4.15 (m, 1 H, 4-H, minor rotamer), 4.12-4.08 (m, 1 H, 4-H, major rotamer), 3.75-3.63 (m, 4 H, 3-H_{eq} minor rotamer + OCH_3 both rotamers), 3.35 (dd, *J* = 13.1, 2.1 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.76 (d, *J* = 13.1 Hz, 1 H, 3-H_{ax}, major rotamer), 2.20-2.08 (m, 2 H, 5-H + 7-H, major rotamers), 2.04-1.98 (m, 1 H, 5-H, minor rotamer), 1.96 (dd, *J* = 9.6, 4.9 Hz, 1 H, 7-H, minor rotamer), 1.83-1.67 (m, 2 H, 5-H' both rotamers + 6-H major rotamer), 1.64-1.56 (m, 1 H, 6-H, minor rotamer), 1.48 (dd, *J* = 7.9, 4.8 Hz, 1 H, 7-H', major rotamer), 1.44 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.40 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.31 (d, *J* = 6.8 Hz, 3 H, CH_3 Ala, minor rotamer), 1.27 (d, *J* = 6.4 Hz, 3 H, CH_3 Ala, major rotamer), 1.20-1.18 (m, 1 H, 7-H', minor rotamer), 1.04 {s, 21 H, $Si[CH(CH_3)_2]_3$ + $Si[CH(CH_3)_2]_3$, minor rotamer}, 1.03 {s, 21 H, $Si[CH(CH_3)_2]_3$ + $Si[CH(CH_3)_2]_3$, major rotamer}. ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 175.8, 172.5, 154.6 (NCO_2tBu), 79.2 [$OC(CH_3)_3$], 65.4 (C-4), 52.5 (OCH_3), 46.2, 45.7, 38.5 (C-1), 30.0 (C-5), 28.3 [3 C, $OC(CH_3)_3$], 24.0, 21.9, 19.2 (CH_3 Ala), 18.0 {6 C, $Si[CH(CH_3)_2]_3$}, 12.0 {3 C, $Si[CH(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 1019 (100) [2M +

$Na]^+$, 521 (42) $[M + Na]^+$, 499 (29) $[M + 1]^+$. Elemental analysis calcd (%) for $C_{25}H_{46}N_2O_6Si$ (498.73): C, 60.21; H, 9.30; N, 5.62. Found: C, 59.93; H, 9.31; N, 5.34.



2-(Boc-Ala)-4-(triisopropylsilanyloxy)-CPA-Gly-OBn [(+)-4]

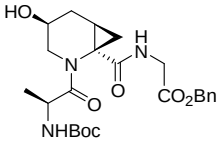
A 1 N solution of NaOH (140 µL) was added to a solution of **2** (46 mg, 0.092 mmol) in methanol (210 µL) and the resulting mixture was vigorously stirred for 24 h at room temperature. Afterward, the methanol was evaporated and the remaining aqueous layer was diluted with H_2O (3 mL). The resulting solution was acidified to pH 6 by adding dropwise a 1 N solution of HCl, and the product was extracted with CH_2Cl_2 (3 x 3 mL). Then the aqueous layer was further acidified to pH 3 and the product was extracted again with CH_2Cl_2 (3 x 3 mL). The combined organic layers were dried over Na_2SO_4 and after filtration and evaporation of the solvent, compound **3** (40 mg, 90%) was obtained as a thick colourless oil.

^{1}H NMR (400 MHz, $CDCl_3$) (2.5:1 mixture of rotamers) δ (ppm): 8.86 (bs, 1 H, COOH), 5.60 (d, *J* = 8.6 Hz, 1 H, NH Ala, minor rotamer), 5.27 (d, *J* = 9.4 Hz, 1 H, NH Ala, major rotamer), 4.84-4.77 (m, 1 H, H_α Ala, major rotamer), 4.65-4.61 (m, 1 H, H_α Ala, minor rotamer), 4.38 (dd, *J* = 13.0, 4.3 Hz, 1 H, 3-H_{eq}, major rotamer), 4.19-4.16 (m, 1 H, 4-H, minor rotamer), 4.13-4.10 (m, 1 H, 4-H, major rotamer), 3.75 (dd, *J* = 13.2, 3.9 Hz, 1 H, 3-H_{eq}, minor rotamer), 3.29 (dd, *J* = 13.2, 2.3 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.72 (dd, *J* = 13.0, 2.2 Hz, 1 H, 3-H_{ax}, major rotamer), 2.16-2.08 (m, 2 H, 5-H + 7-H, major rotamers), 2.04-1.99 (m, 1 H, 5-H, minor rotamer), 1.96 (dd, *J* = 9.7, 4.7 Hz, 1 H, 7-H, minor rotamer), 1.92-1.78 (m, 2 H, 5-H' both rotamers + 6-H major rotamer), 1.76-1.68 (m, 1 H, 6-H, minor rotamer), 1.45 (dd, *J* = 8.0, 4.9 Hz, 1 H, 7-H', major rotamer), 1.41 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.40 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.32 (d, *J* = 7.1 Hz, 3 H, CH_3 Ala, major rotamer), 1.29-1.24 (m, 3 H, CH_3 Ala, minor rotamer), 1.18 (dd, *J* = 7.6, 4.7 Hz, 1 H, 7-H', minor rotamer), 1.04 {s, 21 H, $Si[CH(CH_3)_2]_3$ + $Si[CH(CH_3)_2]_3$, minor rotamer}, 1.02 {s, 21 H, $Si[CH(CH_3)_2]_3$ + $Si[CH(CH_3)_2]_3$, major rotamer}. ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 175.9 (NCO Ala), 175.8, 155.2 (NCO_2tBu), 80.0 [$OC(CH_3)_3$], 65.5 (C-4), 46.7, 45.7, 38.6 (C-1), 30.0 (C-5), 28.5 [3 C, $OC(CH_3)_3$], 26.2, 24.0, 19.6 (CH_3 Ala), 18.1 {6 C, $Si[CH(CH_3)_2]_3$}, 12.1 {3 C, $Si[CH(CH_3)_2]_3$}.

DEPBT (50 mg, 0.166 mmol) and DIPEA (58 µL, 0.332 mmol) were added under an N_2 atmosphere to a solution of intermediate **3** (40 mg, 0.083 mmol) in anhydrous THF (1.5 mL) cooled at 0 °C, and the resulting mixture was allowed to warm to room temperature. After 15 min the mixture was cooled again at 0 °C and H-Gly-OBn·HCl (25 mg, 0.125 mmol) was added. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (2 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (1 mL), a satd. solution of $NaHCO_3$ (1 mL) and H_2O (1 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (*n*-hexane/EtOAc, 2:1; R_f 0.24) affording pure **4** (44 mg, 84 %) as a colourless gummy solid.

$[\alpha]_D^{22}$ +2.7 (*c* 1.25, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (1.4:1 mixture of rotamers) δ (ppm): 7.38-7.29 (m, 6 H, NH Gly major rotamer + Ph both rotamers), 6.59-6.56 (m, 1 H, NH Gly, minor rotamer), 5.18-5.10 (m, 3 H, NH Ala major rotamer + CH_2Ph both rotamers), 4.99-4.96 (m, 1 H,

NH Ala, minor rotamer), 4.91-4.85 (m, 1 H, H_α Ala, minor rotamer), 4.51 (dd, *J* = 12.7, 3.9 Hz, 1 H, 3-H_{eq}, minor rotamer), 4.46-4.41 (m, 1 H, H_α Ala, major rotamer), 4.28 (dd, *J* = 17.9, 6.2 Hz, 1 H, H_α Gly, major rotamer), 4.20-4.10 (m, 2 H, 4-H both rotamers + H_α Gly, minor rotamer), 4.02 (dd, *J* = 18.5, 4.8 Hz, 1 H, H_α Gly, minor rotamer), 3.78-3.65 (m, 2 H, H_α Gly major rotamer + 3-H_{eq} major rotamer), 3.42 (dd, *J* = 13.2, 3.1 Hz, 3-H_{ax}, major rotamer), 2.68 (d, *J* = 12.7 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.23-2.16 (m, 2 H, 5-H major rotamer + 6-H minor rotamer), 2.08-2.03 (m, 1 H, 5-H, minor rotamer), 1.94-1.86 (m, 2 H, 5-H' minor rotamer + 7-H major rotamer), 1.76-1.70 (m, 1 H, 6-H major rotamer), 1.66-1.60 (m, 2 H, 5-H' major rotamer + 7-H minor rotamer), 1.44-1.33 [m, 10 H, 7-H' minor rotamer + $OC(CH_3)_3$ both rotamers], 1.31 (d, *J* = 7.0 Hz, 3 H, CH_3 Ala, major rotamer), 1.28 (d, *J* = 6.6 Hz, 3 H, CH_3 Ala, minor rotamer), 1.11-0.96 {m, 22 H, 7-H' major rotamer + $Si[CH(CH_3)_2]_3$ + $Si[CH(CH_3)_2]_3$ both rotamers}. ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 174.8 (C_q), 171.3 (C_q), 169.9 (C_q), 154.5 (NCO_2tBu), 135.2 ($C_{q,arom}$), 128.8 (2 C, CH_{arom}), 128.7 (2 C, CH_{arom}), 128.4 (CH_{arom}), 80.3 [$OC(CH_3)_3$], 67.4 (CH_2Ph), 66.4 (C-4), 50.5 (C-3), 46.9 (C_α Ala), 41.8 (C_α Gly), 39.8 (C-1), 30.6 (C-5), 28.4 [3 C, $OC(CH_3)_3$], 23.5, 23.4, 21.2 (CH_3 Ala), 18.1 {6 C, $Si[CH(CH_3)_2]_3$}, 12.3 {3 C, $Si[CH(CH_3)_2]_3$}. MS (ESI) *m/z* (%): 1285 (79) $[2M + Na]^+$, 654 (100) $[M + Na]^+$, 632 (9) $[M + 1]^+$. Elemental analysis calcd (%) for $C_{33}H_{53}N_3O_7Si$ (631.88): C, 62.73; H, 8.45; N, 6.65. Found: C, 62.54; H, 8.32; N, 6.61.



2-(Boc-Ala)-4-(hydroxy)-CPA-Gly-OBn [(+)-5]

TBAF (80 µL of a 1 M solution in THF, 0.080 mmol) was slowly added to a solution of **4** (44 mg, 0.070 mmol) in anhydrous THF (3 mL), under an N_2 atmosphere. After 1 h the solvent was evaporated under *vacuum* and the residue was taken up in Et_2O (3 mL), then the solvent was evaporated again. After purification by flash chromatography (EtOAc; R_f 0.36), pure **5** (19 mg, 58%) was obtained as a colourless gummy solid.

$[\alpha]_D^{22}$ +18.8 (*c* 0.75, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (1.2:1 mixture of rotamers) δ (ppm): 7.43-7.40 (m, 1 H, NH Gly, major rotamer), 7.39-7.29 (m, 5 H, Ph, both rotamers), 6.73-6.68 (m, 1 H, NH Gly, minor rotamer), 5.18-5.10 (m, 3 H, NH Ala, major rotamer + CH_2Ph, both rotamers), 4.98 (d, *J* = 7.5 Hz, 1 H, NH Ala, minor rotamer), 4.78-4.72 (m, 1 H, H_α Ala, minor rotamer), 4.56 (d, *J* = 13.0 Hz, 1 H, 3-H_{eq}, minor rotamer), 4.48-4.40 (m, 1 H, H_α Ala, major rotamer), 4.31 (dd, *J* = 17.9, 7.1 Hz, 1 H, H_α Gly, major rotamer), 4.22-4.10 (m, 1 H, H_α Gly, minor rotamer), 4.08-4.02 (m, 2 H, H_α Gly minor rotamer, 4-H both rotamers), 3.77 (dd, *J* = 13.2, 3.9 Hz, 1 H, 3-H_{eq}, major rotamer), 3.70 (dd, *J* = 17.9, 4.7 Hz, 1 H, H_α Gly, major rotamer), 3.29 (dd, *J* = 13.2, 2.1 Hz, 1 H, 3-H_{ax}, major rotamer), 2.72 (d, *J* = 13.0 Hz, 1 H, 3-H_{ax}, minor rotamer), 2.30-1.98 (m, 2 H, 5-H both rotamers + OH both rotamers), 1.90 (dd, *J* = 10.0, 4.7 Hz, 1 H, 7-H, both rotamers), 1.80-1.72 (m, 2 H, 5-H' minor rotamer + 6-H both rotamers), 1.66-1.59 (m, 1 H, 5-H', major rotamer), 1.40 [s, 9 H, $OC(CH_3)_3$, minor rotamer], 1.37 [s, 9 H, $OC(CH_3)_3$, major rotamer], 1.35-1.25 (m, 3 H, CH_3 Ala, both rotamers), 1.04 (dd, *J* = 7.8, 4.7 Hz, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 175.5, 171.3, 169.8, 156.6 (NCO_2tBu), 135.6 (C_{arom}), 128.7 (2 C, CH_{arom}), 128.6 (2 C, CH_{arom}), 128.4 (CH_{arom}), 80.2 [$OC(CH_3)_3$], 67.4 (CH_2Ph), 64.8 (C-4), 48.6 (C-3), 47.0 (C_α Ala), 41.8 (C_α Gly), 40.2 (C-1), 29.7 (C-5), 28.3 [3 C, $OC(CH_3)_3$], 23.3, 20.8 (CH_3 Ala), 16.5. MS (ESI) *m/z* (%): 973 (100) $[2M + Na]^+$,

498 (41) $[M + Na]^+$, 476 (17) $[M + 1]^+$. Elemental analysis calcd (%) for $C_{24}H_{33}N_3O_7$ (475.53): C, 60.62; H, 6.99; N, 8.84. Found: C, 60.50; H, 6.76; N, 8.55.

7.5 References

[1] M. E. Swarbrick, F. Gosselin, W. D. Lubell, *J. Org. Chem.* **1999**, 64, 1993
[2] W.-J. Wu, D. P. Raleigh, *J. Org. Chem.* **1998**, 63, 6689

Chapter 8: Synthesis of Both Enantiomers of *Trans* 3-Hydroxypipecolic Acid

8.1 General Synthetic Approach for Obtaining Hydroxypipecolic Acids

Owing to their widespread presence in nature and their significant medicinal potential, there has been much interest in the development of new methods for the asymmetric synthesis of substituted pipecolic acids.[1] In this context, *trans*- and *cis*-3-hydroxypipecolic acids **1** and **2** (figure 1) are no exceptions because a number of syntheses of both compounds have been reported, also owing to the fact that 3-hydroxypiperidine is a privileged scaffold in nature.[2] Moreover, 3-hydroxypipecolic acids are further examples of constrained amino acids and can be either employed directly as rigid serine analogues for the construction of peptidomimetics, or converted into the corresponding 3-aminopipecolic acids. The latter compounds could be embedded in cyclic RGD-based ligands similarly to what we reported for CPAs (see chapter 6).

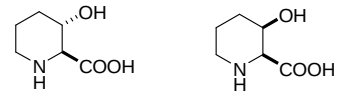
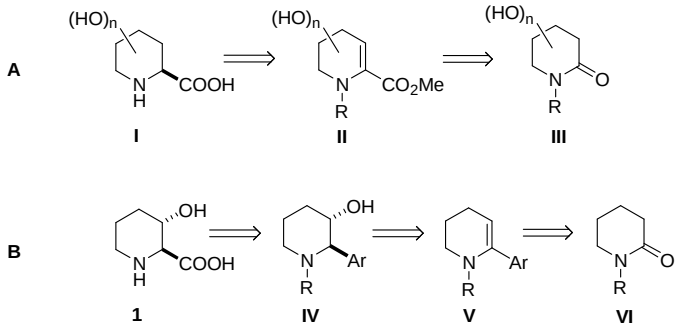


Figure 1. 3-Hydroxypipecolic acids.

As recently reviewed, methods based on the use of starting material from the chiral pool or that involve enantiomerically enriched reagents or catalysts have been mainly exploited, with a few additional tactics centered on the enzymatic resolution of racemic compounds.[3] Our group recently demonstrated that enantiopure 4-hydroxy-, 5-hydroxy- and 4,5-dihydroxypipecolic acids can be efficiently prepared from the same enecarbamate esters **II** (scheme 1, A) that we already repeatedly described as intermediates in the synthesis of CPAs (see chapters 4 and 5).[4-7] By this strategy, racemic or enantiopure lactams **III** are converted into enecarbamate esters **II**, which are then reduced and deprotected to give target compounds **I**.

We decided to investigate if an analogous approach, but which entails a Suzuki–Miyaura reaction instead of a carbonylative process to form the C–C bond at C-2 (Scheme 1, B), could efficiently provide *trans*-3-hydroxypipecolic acid **1**.

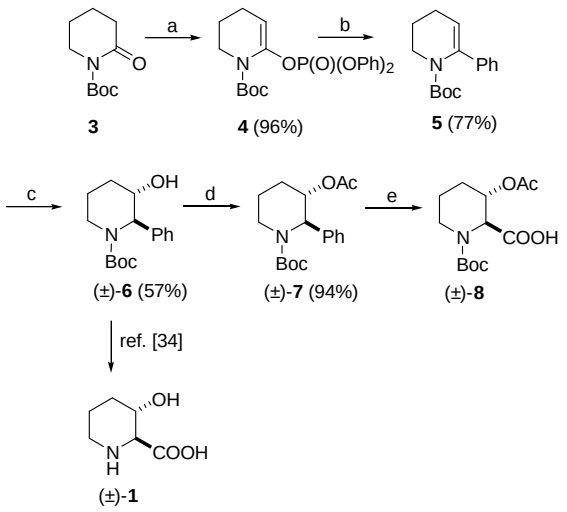


Scheme 1. Retrosynthetic analysis.

After hydroboration/oxidation of coupling product **V**, an enzymatic kinetic resolution should provide enantiopure intermediate **IV**, which is finally oxidized to target compound **1**.
Lipase-catalyzed resolutions have successfully been exploited for the synthesis of 3-hydroxypipecolic acids (both *cis* and *trans*) on only a few occasions, which involved piperidin-3-ols with the 2-carboxyxlic group already installed on the piperidine ring.[8-12] In our case, the resolution will be attempted on a 2-aryl- or heteroaryl-substituted piperidin-3-ol, with the advantage of obtaining enantiopure intermediates potentially useful for preparing biologically relevant 2-aryl-substituted piperidines.[13,14]

8.2 Synthesis of Racemic *Trans* 3-Hydroxypipecolic acid

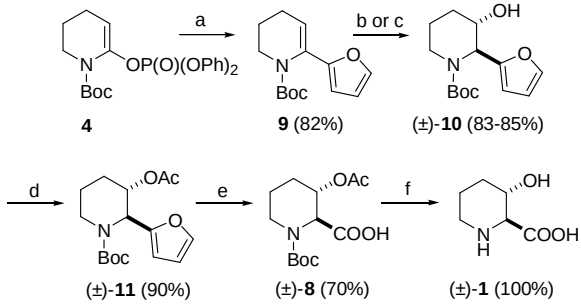
The Suzuki–Miyaura coupling of lactam-derived vinyl phosphates and triflates has been extensively surveyed in the past mainly by the group of Coudert and Gillaizeau[15-19] and by our own group,[20-27] and has found many applications in the synthesis of natural and biologically active heterocyclic compounds.[28-33] We decided to use this reaction to install a masked carboxylic group at C-2 mainly because α,β-unsaturated esters such as **II** are not suitable to install the 3-OH group by hydroboration. Moreover, we desired to establish a new method to introduce the carboxylic function at C-2 alternative to the usual carbonylative approach, which requires the availability and the manipulation of a toxic and expensive gas. Of course, this strategy involves a further oxidative step not required previously.
To evaluate the feasibility of our approach, we first coupled vinyl phosphate **4** (scheme 2), obtained in 96% yield from commercial N-tert-butoxycarbonyl(Boc)-protected δ-valerolactam (**3**), with phenylboronic acid under conditions that worked best for the corresponding vinyl triflate.[21]



Scheme 2. First attempt of synthesis of racemic *trans* 3-hydroxypipecolic acid. Reagents and conditions: a) $(PhO)_2POCl$, KHMDS, THF, –78 °C; b) phenylboronic acid, $(Ph_3P)_2PdCl_2$, Na_2CO_3 (2 M), THF, 40 °C; c) BH_3·DMS, THF; then H_2O_2 (35 %), EtOH, NaOH (3 N); d) Et_3N, DMAP, Ac_2O, CH_2Cl_2; e) $NaIO_4$, $RuCl_3$·xH_2O, CCl_4/MeCN/H_2O, 0 °C.

The reaction was carried out at 40 °C in the presence of $(Ph_3P)_2PdCl_2$ (5 mol-%) in a tetrahydrofuran (THF) and Na_2CO_3 (2 M) mixture and it was complete in 24 h to provide known dehydropiperidine **5** in 77% yield after chromatography. Hydroboration of **5** was carried out with BH_3·DMS at room temperature followed by oxidation with H_2O_2 in alkaline medium,[35] which provided racemic alcohol **6** in moderate yield (57 %). The expected *trans* stereochemistry in **6** (and similarly in **10**, see later) is easily assigned on the basis of very low coupling constant values for protons at C-2 and C-3, which appear as broad singlets at δ = 4.5 and 5.4 ppm, respectively, in the ^{1}H NMR spectrum because of their equatorial orientation. Both the aryl and OH groups are thus axially oriented, the former to remove the $A^{(1,3)}$ strain with the N-protecting group.[36]

Before attempting the enzymatic kinetic resolution of this alcohol we wanted to test the $RuCl_3$ catalysed oxidation reaction of the phenyl group and so, after acetylation reaction of the OH group, we treated compound **7** under standard oxidative conditions.[37] To our disappointment, the reaction carried out with a catalytic amount of $RuCl_3$·xH_2O and in the presence of $NaIO_4$ (4 eq.) in a CCl_4/acetonitrile/water mixture was sluggish, required the addition of further oxidant, and yielded carboxylic acid **8** in a very low yield and in mixture with by-products derived from over-oxidation at position 6. The oxidative degradation of unsubstituted phenyl rings on similar systems has been reported for N-trifluoroacetyl-protected piperidines only,[14,34,38,39] and never with N-Boc-protected analogues, which are probably not compatible with the reaction conditions. Although pipecolic acid **1** could formally be obtained from **6** after N deprotection and trifluoroacetylation,[34] to keep our synthesis as short as possible we decided to introduce a more easily oxidized substituent at C2 from the beginning. So, we opted for a more electron-rich furan, which was introduceed by the Pd-catalyzed coupling of N-Boc protected vinyl phosphate **4** with 2-furanylboronic acid (Scheme 3). Also in this case the coupling was successful and provided **9** in 82% yield after chromatography. Hydroboration of **9** was carried out both as reported above and with BH_3·THF at 0 °C for 22 h, followed by oxidation with trimethylamine N-oxide.[40] In both cases (±)-**10** was obtained in good yield (83 and 85%, respectively), although with the latter method purity of the crude reaction mixture was higher.



Scheme 3. Optimised synthesis of racemic *trans* 3-hydroxypipecolic acid. Reagents and conditions: a) furanylboronic acid, $(Ph_3P)_2PdCl_2$, Na_2CO_3 (2 M), THF, 40 °C; b) BH_3·THF, 0 °C, THF; then Me_3NO, THF, 65 °C; c) BH_3·DMS, THF; then H_2O_2 (35 %), NaOH (3 N), EtOH; d) Et_3N, DMAP, Ac_2O, CH_2Cl_2; e) $NaIO_4$, $RuCl_3$·xH_2O, EtOAc/MeCN/H_2O, 0 °C; f) HCl (6 N), reflux, 3 h.

After acetylation, we tested the oxidative conditions on compound (±)-**11** and were pleased to obtain acid (±)-**8** in good yield (70% after chromatography) after 10 min at room temperature. After exhaustive hydrolysis in HCl (6 N) at reflux temperature, an appropriate work-up allowed us to obtain racemic 3-hydroxypipecolic acid (±)-**1** in quantitative yield and excellent purity.[41] Despite a further oxidative step being required, the Suzuki–Miyaura coupling strategy proved an excellent alternative to the alkoxycarbonylative process on lactam-derived vinyl phosphates for the introduction of a C-2 substituent in the synthesis of pipecolic acids.

8.3 Enzymatic Resolution of the Racemic Mixture

Once established an optimised synthetic procedure, we tackled the enzymatic kinetic resolution of racemic intermediate (±)-**10**, since we were interested in the obtaining of the two separated enantiomers of the final product (+)-**1** and (–)-**1**.

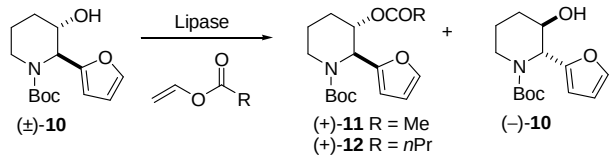
We opted to test three commercially available immobilized lipases, which our group have successfully employed in the past for the enantioselective acylation reaction of 4-hydroxypipecolic acid precursors (see sections 5.1 and 5.2),[5] CAL-B (*Candida antarctica* lipase B, supported on acrylic resin, trade name Novozym 435),[42-44] CAL-A (*C. antarctica* lipase A on Immobead 150),[45] and PS-AMANO-IM (*Burkholderia cepacia* lipase, immobilized diatomaceous earth).[46,47] As reported in table 1, only CAL-B proved useful giving an enantiomeric ratio (see table 1, note *f*) around 50 when the resolution was carried out in THF and with vinyl butyrate as the acylant (table 1, entry 3).

CAL-A proved less efficient but immobilized *B. cepacia* lipase did not appreciably convert **10** into any of the products even with an excess of acylant. This enzyme was the best in the kinetic resolution of 4-hydroxypipecolic acid derivatives (see section 5.2)[5] and the same enzyme (lipase PS-C, but immobilized on ceramic particles)[46,47] provided the best results in the transesterification reaction of a methyl 3-hydroxypipecolate.[9,12] So, although the different immobilization forms resulted in differences, the 2-heteroaryl group could play a role in the inefficacy of this enzyme.

The absolute configuration of residual substrate (–)-**10** was determined by converting it, on a small scale, into corresponding pipecolic acid (–)-**1** (see later) and, by comparison of the optical rotation of its HCl salt ($[\alpha]_D^{21}$ = –14.9, *c* = 1.4, H_2O) with the one reported in the literature ($[\alpha]_D^{23}$ = –13.5, *c* = 0.3, H_2O),[49] we found that CAL-B catalyses the acylation of the (2*S*,3*S*) substrate. Interestingly, CAL-B is known to preferentially catalyse the acylation of the *R* alcohol in compounds like simple secondary and cyclic allylic alcohols,[42-44,50,51] but clearly this general rule cannot be extended to our case owing to the presence of a second vicinal stereocenter. CAL-A similarly showed a stereopreference for the (2*S*,3*S*) enantiomer, whereas for the PS-AMANO-IM catalysed resolution, owing to the low conversions, the *ee* values nor the absolute configuration of the resolution products were calculated.

For the synthesis of (2*R*,3*R*)-3-hydroxypipecolic acid (–)-**1**, resolution of (±)-**10** was carried out on 200 mg of substrate and was stopped at a 57% of conversion to obtain required (–)-(2*R*,3*R*)-**10** in enantiopure form (99% *ee*, see appendix 2 for enantioselective HPLC analysis) in an acceptable yield (38% after chromatography). After the lipase-catalysed resolution, compound (–)-**10** was acetylated to give (–)-**11** (90%) (scheme 4) and then oxidized as usual to afford (–)-**8** in 72% yield after chromatography. Exhaustive deprotection was attained by treatment with HCl (6 N) at reflux temperature to give (–)-(2*R*,3*R*)-**1** as the HCl salt in almost quantitative yield.[41]

Table 1. Lipase-catalysed kinetic resolution of (±)-**10**.

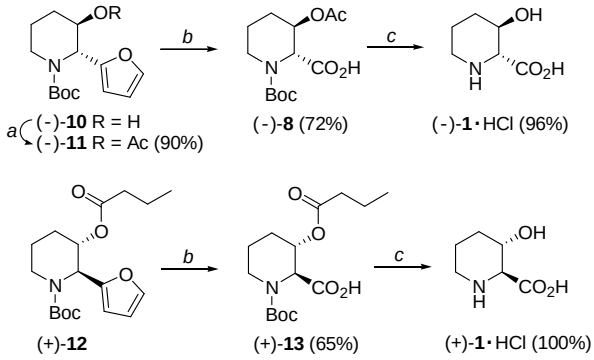


Entry	Acylant[a]	Solvent[b]	*t* (h)	Conversion (%)[c]	(*S,S*)-**11/12** *ee* (%)[d]	(*R,R*)-**10** *ee* (%)[e]	*E*[f]
				CAL-B[g]			
1	VA	DIPE	19	58	50	75	6
2	VB	DIPE	17	60	42	99	11
3	VB	THF	25	45	91	81	53
4[h]	VB	THF	19	46	89	75	39
5	VB	TBME	17	57	50	98	12
				CAL-A[g]			
6	VB	THF	24[i]	31	89	37	25
7	VA	THF	43	45	83	63	21
				PS "Amano" IM[g]			
8[h]	VA	DIPE	39[i]	10	-	-	-
9	VB	THF	96[i]	25	-	-	-
10	VB	TBME	52[i]	18	-	-	-

[a] VA: vinyl acetate, VB: vinyl butyrate. [b] Anhydrous solvents were used. DIPE: diisopropyl ether, THF: tetrahydrofuran, TBME: *tert*-butyl methyl ether. [c] Reaction monitored by GLC and conversion determined by ^{1}H NMR. [d] Determined by HPLC analysis after hydrolysis to (+)-**10** on a HPLC Lux 5µ Cellulose-4 column (250 x 4.60 mm). [e] Determined by HPLC analysis on a HPLC Lux 5µ Cellulose-4 column (250 x 4.60 mm). [f] The enantioselectivity in an enzymatic kinetic resolution process can be conveniently expressed by *E* = enantiomeric ratio. The ratio between the specificity constants (k_{cat}/K_M) of the enzyme for the competing *R* and *S* enantiomers: $E = (k_{cat}/K_M)_R/(k_{cat}/K_M)_S$. *E* was calculated by using the formula $E = \ln[(1 - ee_s)/(1 + ee_s/ee_p)]/\ln[(1 + ee_s)/(1 + ee_s/ee_p)]$, where ee_s is the enantiomeric excess of the substrate and ee_p is the enantiomeric excess of the product.[48] [g] Reaction carried out on 0.4 mmol of substrate at 30 °C; substrate concentration: 0.8 M; enzyme (mg)/substrate (mmol) ratio: 200 mg/mmol; 3.5 equivalents of acylant. [h] The reaction was carried out at 40 °C. [i] The reaction did not proceed further and it was stopped.

Its enantiomer was instead prepared by stopping the resolution at a 45% conversion, thus obtaining (+)-(2*S*,3*S*)-**12** in 91% *ee* (see appendix 3 for enantioselective HPLC analysis) (37% yield) and with a suitable protection already installed on 3-OH group. After usual oxidation and

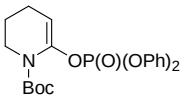
exhaustive deprotection (scheme 4), (+)-(2*S*,3*S*)-**1** was obtained in quantitative yield as the HCl salt and with optical rotation ($[\alpha]_D^{21}$ = +13.7, *c* = 0.7, H_2O) consistent to that reported for this enantiomer ($[\alpha]_D^{23}$ = +14.5, *c* = 0.4, H_2O [52] and $[\alpha]_D^{25}$ = +14.2, *c* = 0.95, H_2O [53]).[41]



Scheme 4. Syntheses of (–)-(2*R*,3*R*)-3-hydroxypipecolic acid and (+)-(2*S*,3*S*)-3-hydroxypipecolic acid. Reagents and conditions: a) Et_3N, DMAP, Ac_2O, CH_2Cl_2; b) $NaIO_4$, $RuCl_3 \cdot xH_2O$, EtOAc/MeCN/H_2O, 0 °C; c) HCl (6 N), reflux, 3 h.

By treating the residual substrate of the enzymatic kinetic resolution with further CAL-B and vinyl butyrate, and by stopping the reaction at a 18% conversion, further enantiopure (–)-(2*R*,3*R*)-**10** (32% yield) was provided, which was converted into pipecolic acid (–)-**1** as described above.[41]

8.4 Experimental



***tert*-Butyl 6-[(Diphenoxyphosphoryl)oxy]-3,4-dihydropyridine-1(2*H*)-carboxylate (4)**

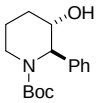
A solution of potassium bis(trimethylsilyl)amide (KHMDS) in toluene (0.5 M, 15.8 mL, 7.89 mmol) was diluted in anhydrous THF (36 mL) and cooled to –78 °C. A solution of **3** (1.26 g, 6.31 mmol) in anhydrous THF (15 mL) was then added dropwise, keeping the temperature below –70 °C, and the resulting mixture was stirred for 1.5 h. A solution of diphenylchlorophosphate (1.63 mL, 7.89 mmol) in anhydrous THF (9 mL) was then added dropwise and, after 1 h, the mixture was warmed to 0 °C. Aqueous NaOH (10%, 150 mL) was slowly added and the product extracted with Et_2O (3 x 100 mL). The combined organic extracts were washed with NaOH (10%, 50 mL) and dried over K_2CO_3 for 40 min. After filtration and evaporation of the solvent, the crude was purified with a short pad of silica gel (*n*-hexane/EtOAc, 3:1 with 1% Et_3N; R_f 0.30) to afford pure **4** (2.61 g, 96%) as a pale yellow oil.

^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 7.38–7.15 (m, 10 H, Ph), 5.10 (q, *J* = 3.3 Hz, 1 H, 5-H), 3.60–3.55 (m, 2 H, 2-H), 2.35–2.04 (m, 2 H, 4-H), 1.75–1.69 (m, 2 H, 3-H), 1.43 [s, 9 H, $C(CH_3)_3$].

***tert*-Butyl 6-Phenyl-3,4-dihydropyridine-1(2*H*)-carboxylate (5)**[21]

Aqueous Na_2CO_3 (2 M, 14.7 mL, 29.4 mmol), $(Ph_3P)_2PdCl_2$ (77.9 mg, 0.11 mmol) and phenylboronic acid (406 mg, 3.33 mmol) were added to a solution of **4** (958 mg, 2.22 mmol) in THF (30 mL). The reaction was stirred at 40 °C for 24 h. The reaction was diluted with H_2O (35 mL) and the product extracted with Et_2O (3 x 35 mL). The combined organic layers were dried over K_2CO_3. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane, then *n*-hexane/EtOAc, 20:1; R_f 0.30) to afford pure **5** (445 mg, 77%).

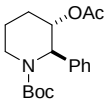
^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 7.70–7.16 (m, 5 H, Ph), 5.31 (t, *J* = 3.7 Hz, 1 H, 5-H), 3.73–3.68 (m, 2 H, 2-H), 2.26 (dt, *J* = 6.6, 3.7 Hz, 2 H, 4-H), 1.91–1.78 (m, 2 H, 3-H), 1.05 [s, 9 H, $C(CH_3)_3$].



***tert*-Butyl 3-Hydroxy-2-phenylpiperidine-1-carboxylate [(±)-6]**

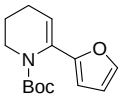
A solution of $(CH_3)_2S{\cdot}BH_3$ in THF (2 M, 375 µL, 0.75 mmol) was added to a solution of **5** (129 mg, 0.5 mmol) in anhydrous THF (1 mL) at 0 °C and under an N_2 atmosphere. The cooling bath was removed and the mixture was stirred at room temperature for 5 h. The reaction was cooled to 0 °C and EtOH (550 µL), NaOH (3 M, 440 µL) and H_2O_2 (35%, 200 µL) were added. The resulting mixture was heated to reflux for 1 h and, after cooling, it was transferred into a flask that contained ice. The product was extract with Et_2O (4 x 4 mL) and the combined organic extracts were dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography (*n*-hexane/EtOAc, 2:1; R_f 0.26) to give (±)-**6** (79 mg, 57%) as a white solid.

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.37–7.34 (m, 2 H, Ph), 7.27–7.25 (m, 1 H, Ph), 7.25–7.19 (m, 2 H, Ph), 5.37 (bs, 1 H, 2-H), 4.52 (m, 1 H, 3-H), 4.12–4.07 (d, *J* = 13.3 Hz, 1 H, 6-H_{eq}), 2.87 (td, *J* = 13.3, 3.3 Hz, 1 H, 6-H_{ax}), 1.98–1.87 (m, 2 H, 5-H and OH), 1.78–1.73 (m, 1 H, 4-H), 1.66–1.58 (m, 1 H, 4-H'), 1.48 [s, 9 H, $C(CH_3)_3$], 1.43–1.39 (m, 1 H, 5-H'). ^{13}C NMR ($CDCl_3$, 100.32 MHz) δ (ppm): 156.3 (CO), 137.8 (C_{ipso}, Ph), 128.4 (2 C, Ph), 126.5 (C, Ph), 126.0 (2 C, Ph), 79.8 [$C(CH_3)_3$], 67.2 (*C*HOH), 60.0 (*C*HPh), 39.6 (C-6), 28.1 [$C(CH_3)_3$], 25.7 (C-4), 18.6 (C-5). MS (ESI) *m/z* (%): 577 (100) $[2M + Na]^+$, 300 (20) $[M + Na]^+$. Elemental analysis calcd. (%) for $C_{16}H_{23}NO_3$ (277.36): C, 69.29; H, 8.36; N, 5.05. Found: C, 69.31; H, 8.25; N, 5.26.



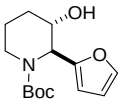
***tert*-Butyl 3-(Acetyloxy)-2-phenylpiperidine-1-carboxylate [(±)-7]**

Et_3N (70 µL, 0.5 mmol) and 4-dimethylaminopyridine (DMAP, 6.5 mg, 0.053 mmol) were added to a solution of alcohol (±)-**6** (70 mg, 0.25 mmol) in dry CH_2Cl_2 (1 mL) under an N_2 atmosphere. The mixture was cooled to 0 °C and acetic anhydride (43 µL, 0.46 mmol) was slowly added. The cooling bath was removed and the reaction was stirred at room temperature for 18 h. After dilution with CH_2Cl_2 (5 mL), a satd. solution of $NaHCO_3$ (5 mL) was added and the aqueous phase was extracted with CH_2Cl_2 (3 x 4 mL). The combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography (*n*-hexane/EtOAc, 8:1; R_f 0.24) to give (±)-**7** (75 mg, 94%) as a pale yellow oil.
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.38–7.34 (m, 2 H, Ph), 7.27–7.24 (m, 2 H, Ph), 5.53–5.51 (m, 1 H, 3-H), 5.49 (s, 1 H, 2-H), 4.17–4.11 (d, *J* = 13.3 Hz, 1 H, 6-H_{eq}), 2.88 (td, *J* = 13.3, 3.3 Hz, 1 H, 6-H_{ax}), 2.12 (s, 3 H, COCH_3), 1.93–1.87 (m, 1 H, 5-H), 1.85–1.80 (m, 1 H, 4-H), 1.69–1.60 (m, 1 H, 4-H'), 1.45 [s, 9 H, C(CH_3)$_3$], 1.43–1.39 (m, 1 H, 5-H'). ^{13}C NMR ($CDCl_3$, 100.32 MHz) δ (ppm): 170.5 (*CO*CH_3), 156.0 (N*C*O), 137.6 (C_{ipso}, Ph), 128.7 (2 C, Ph), 127 (C, Ph), 126.3 (2 C, Ph), 79.8 [*C*$(CH_3)_3$], 70.1 (C-3), 57 (C-2), 39.7 (C-6), 28.4 [C(*C*H_3)$_3$], 23.9 (C-4), 21.3 (CO*C*H_3), 19.5 (C-5). MS (ESI) *m/z* (%): 661 (100) $[2M + Na]^+$, 342 (27) $[M + Na]^+$. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 342 (3) $[M + Na]^+$, 286 (100), 242 (9), 182 (11). Elemental analysis calcd. (%) for $C_{18}H_{25}NO_4$ (319.40): C, 67.69; H, 7.89; N, 4.39. Found: C, 67.63; H, 7.66; N, 4.69.



***tert*-Butyl 6-(Furan-2-yl)-3,4-dihydropyridine-1(2*H*)-carboxylate (9)**[21]

Aqueous Na_2CO_3 (2 M, 24.4 mL, 48.8 mmol), $(Ph_3P)_2PdCl_2$ (130 mg, 0.185 mmol) and 2-furanylboronic acid (621 mg, 5.55 mmol) were added to a solution of **4** (1.6 g, 3.73 mmol) in THF (48 mL). The mixture was heated at 40 °C for 2 h, then it was diluted with H_2O (68 mL) and the product was extracted with Et_2O (3 x 68 mL). The combined organic layers were dried over K_2CO_3. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane/EtOAc, 20:1, then *n*-hexane/EtOAc, 10:1; R_f 0.48) to afford pure **9** (755 mg, 82%).
^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 7.32 (dd, *J* = 1.8, 0.7 Hz, 1 H, 5'-H), 6.35 (dd, *J* = 3.3, 1.8 Hz, 1 H, 4'-H), 6.22 (d, *J* = 3.3 Hz, 1 H, 3'-H), 5.52 (t, *J* = 4.0 Hz, 1 H, 5-H), 3.68–3.63 (m, 2 H, 2-H), 2.26 (td, *J* = 7.0, 4.0 Hz, 2 H, 4-H), 1.91–1.82 (m, 2 H, 3-H), 1.26 [s, 9 H, C(CH_3)$_3$].



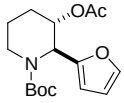
***tert*-Butyl 2-(Furan-2-yl)-3-hydroxypiperidine-1-carboxylate [(±)-10].**

Method A: A solution of $(CH_3)_2S{\cdot}BH_3$ in THF (2 M, 1 mL, 2 mmol) was added to a solution of **9** (250 mg, 1 mmol) in anhydrous THF (2 mL) at 0 °C under an N_2 atmosphere. The cooling bath

was removed and the mixture was stirred at room temperature for 4 h. The reaction was cooled to 0 °C and EtOH (1.1 mL), NaOH (3 N, 900 µL) and H_2O_2 (35%, 390 µL) were added. The resulting mixture was heated to reflux for 1 h. The solution was transferred into a flask that contained ice and the product was extracted with Et_2O (4 x 18 mL). The combined organic extracts were dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography (*n*-hexane/EtOAc, 3:1; R_f 0.20) to give (±)-**10** (222 mg, 83%) as a pale yellow oil.

Method B: A solution of BH_3·THF in THF (1 M, 2.87 mL, 2.87 mmol) was added to a stirred solution of **9** (219 mg, 0.82 mmol) in anhydrous THF (40 mL) at –78 °C and under a nitrogen atmosphere. After 15 min, the temperature was raised to 0 °C and the reaction was stirred for 20 h. Then the mixture was warmed to room temperature and Me_3NO was added. The reaction was heated at 65 °C for 2 h. After cooling, EtOAc (80 mL) was added and the organic layer was washed with brine (2 x 40 mL) and dried over anhydrous Na_2SO_4. Chromatography (*n*-hexane/EtOAc, 3:1; R_f 0.20) afforded pure (±)-**10** (187 mg, 85%) as a colourless oil.

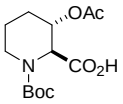
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.35 (dd, *J* = 1.8, 0.6 Hz, 1 H, 5'-H), 6.33 (dd, *J* = 3.3, 1.8 Hz, 1 H, 4'-H), 6.11 (d, *J* = 3.3 Hz, 1 H, 3'-H), 5.34 (bs, 1 H, 2-H), 4.34 (m, 1 H, 3-H), 4.04–4.00 (d, *J* = 13.1 Hz, 1 H, 6-H_{eq}), 2.87 (td, *J* = 13.1, 3.1 Hz, 1 H, 6-H_{ax}), 2.01 (bs, 1 H, OH), 1.93–1.73 (m, 3 H, 5-H + 4-H + 4-H'), 1.47 [s, 9 H, C(CH_3)$_3$], 1.45–1.42 (m, 1 H, 5-H'). ^{13}C NMR ($CDCl_3$, 100.32 MHz) δ (ppm): 156.1 (N*C*O), 151.9 (C-2'), 141.7 (C-5'), 110.2 (C-4'), 107.0 (C-3'), 80.2 [*C*(CH$_3$)$_3$], 66.1 (C-3), 56.2 (C-2), 40.0 (C-6), 28.4 [C(*C*H$_3$)$_3$], 26.8 (C-4), 18.8 (C-5). MS (ESI) *m/z* (%) = 557 (48) $[2M + Na]^+$, 290 (100) $[M + Na]^+$. Elemental analysis calcd. (%) for $C_{14}H_{21}NO_4$ (267.32): C, 62.90; H, 7.92; N, 5.24. Found: C, 62.64; H, 7.89; N, 5.37.



***tert*-Butyl 3-(Acetyloxy)-2-(furan-2-yl)piperidine-1-carboxylate [(±)-11]**

Prepared as reported for (±)-**7**, starting from alcohol (±)-**10** (178 mg, 0.67 mmol). After chromatography (*n*-hexane/EtOAc, 6:1; R_f 0.21) pure (±)-**11** (160 mg, 90%) was obtained as a colourless oil.

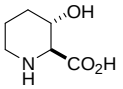
^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.36 (dd, *J* = 1.8, 1.0 Hz, 1 H, 5'-H), 6.33 (dd, *J* = 3.3, 1.8 Hz, 1 H, 4'-H), 6.15 (dd, *J* = 3.3, 1.0 Hz, 1 H, 3'-H), 5.44 (bs, 1 H, 3-H), 5.32 (bs, 1 H, 2-H), 4.07 (d, *J* = 12.7 Hz, 1 H, 6-H_{eq}), 2.88 (t, *J* = 12.7 Hz, 1 H, 6-H_{ax}), 2.08 (s, 3 H, COCH_3), 1.86–1.73 (m, 3 H, 5-H + 4-H + 4-H'), 1.48–1.42 [s, 9 H, C(CH_3)$_3$ and m, 1 H, 5-H']. ^{13}C NMR ($CDCl_3$, 100.32 MHz) δ (ppm): 170.3 (*C*OCH$_3$), 155.4 (N*C*O), 151.1 (C-2'), 141.9 (C-5'), 110.3 (C-4'), 107.4 (C-3'), 80.0 [*C*(CH$_3$)$_3$], 68.4 (C-3), 53.1 (C-2), 39.7 (C-6), 28.4 [C(*C*H$_3$)$_3$], 24.7 (C-4), 21.2 (CO*C*H$_3$), 19.5 (C-5). MS (ESI) *m/z* (%): 641 (100) $[2M + Na]^+$, 332 (69) $[M + Na]^+$. MS/MS (ESI of $[M + Na]^+$) *m/z* (%): 332 (10) $[M + Na]^+$, 276 (100), 232 (15), 172 (11). Elemental analysis calcd. (%) for $C_{16}H_{23}NO_5$ (309.36): C, 62.12; H, 7.49; N, 4.53. Found: C, 61.93; H, 7.22; N, 4.69.



3-(Acetyloxy)-1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic Acid [(±)-8]

$RuCl_3 \cdot xH_2O$ (2 mg, 0.01 mmol) was added to a solution of $NaIO_4$ (734 mg, 3.43 mmol) in EtOAc (3.5 mL)/CH_3CN (5.7 mL)/H_2O (2.8 mL) cooled to 0 °C. The reaction was stirred at 0 °C for 15 min, then a solution of (±)-**11** (153 mg, 0.49 mmol) in EtOAc (2.8 mL) was slowly added. After 5 min at 0 °C, H_2O (12 mL) was added and the product was extract with EtOAc (2 x 16 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and the crude product was purified by chromatography (CH_2Cl_2/MeOH, 40:1 + 0.1% AcOH, R_f 0.50) to give (±)-**8** (98 mg, 70%) as a yellow gummy solid.

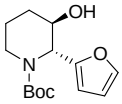
^{1}H NMR ($CDCl_3$, 400 MHz) (1:1 mixture of rotamers) δ (ppm): 10.78 (bs, 1 H, COO*H*), 5.43 (bs, 1 H, 3-H, rotamer A), 5.34 (bs, 1 H, 3-H, rotamer B), 5.07 (bs, 2-H, rotamer A), 4.99 (bs, 2-H, rotamer B), 4.11 (d, *J* = 12.5 Hz, 1 H, 6-H_{eq}, rotamer A), 3.97 (d, *J* = 12.5 Hz, 1 H, 6-H_{eq}, rotamer B), 2.97 (t, *J* = 12.5 Hz, 1 H, 6-H_{ax}, rotamer A), 2.85 (t, *J* = 12.5 Hz, 1 H, 6-H_{ax}, rotamer B), 2.06 (s, 3 H, COCH_3, rotamer A), 2.04 (s, 3 H, COCH_3, rotamer B), 1.94–1.20 (m, 4 H, 4-H + 5-H, both rotamers), 1.45 [s, 9 H, C(CH_3)$_3$, rotamer A], 1.41 [s, 9 H, C(CH_3)$_3$, rotamer B]. ^{13}C NMR ($CDCl_3$, 100.32 MHz) (1:1 mixture of rotamers) δ (ppm): 177.3, 173.8 and 170.4 (*C*OOH and *C*OCH$_3$, both rotamers), 156.3 (N*C*O, rotamer A), 155.3 (N*C*O, rotamer B), 80.7 [*C*(CH$_3$)$_3$], 67.9 (C-3, rotamer A), 67.3 (C-3, rotamer B), 57.9 (C-2, rotamer A), 57.1 (C-2, rotamer B), 41.7 (C-6, rotamer A), 40.5 (C-6, rotamer B), 28.3 [C(*C*H$_3$)$_3$], 25.4 (C-4), 21.1 (CO*C*H$_3$, rotamer A), 20.8 (CO*C*H$_3$, rotamer B), 18.9 (C-5, rotamer A), 18.8 (C-5, rotamer B). MS (ESI) *m/z* (%): 597 (100) [2M + Na]$^+$, 310 (96) [M + Na]$^+$. MS/MS (ESI of [M + Na]$^+$) *m/z* (%): 310 (3) [M + Na]$^+$, 254 (100), 210 (49). Elemental analysis calcd. (%) for $C_{13}H_{21}NO_6$ (287.31): C, 54.35; H, 7.37; N, 4.88. Found: C, 54.11; H, 7.19; N, 4.95.



3-Hydroxypiperidine-2-carboxylic Acid Hydrochloride [(±)-1·HCl]

A solution of (±)-**8** (93 mg, 0.32 mmol) in HCl (6 N, 9 mL) was heated to reflux for 2 h. The reaction was then cooled to room temperature and the solvent was evaporated under vacuum. The residue was triturated in Et_2O (2 x 5 mL), and the organic phase was discarded to obtain (±)-**1**·HCl (46 mg, 100%) as a pale yellow solid.

^{1}H NMR (D_2O, 400 MHz) δ (ppm): 3.95–3.91 (m, 1 H, 3-H), 3.66 (d, *J* = 8.2 Hz, 2-H), 3.20–3.15 (m, 1 H, 6-H), 2.91–2.85 (m, 1 H, 6-H'), 1.86–1.78 (m, 2 H, 4-H + 5-H), 1.59–1.44 (m, 2 H, 4-H' + 5-H'). ^{13}C NMR (D_2O, 100.32 MHz) δ (ppm): 169.6 (*C*OOH), 65.5 (C-3), 60.7 (C-2), 42.6 (C-6), 28.9 (C-4), 18.6 (C-5).

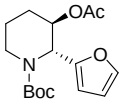


Kinetic Resolution of (±)-10 with CAL-B

***tert*-Butyl (*R*,*R*)-2-(Furan-2-yl)-3-hydroxypiperidine-1-carboxylate [(–)-10]**

CAL-B (148 mg) was added to a solution of (±)-**10** (200 mg, 0.74 mmol) in diisopropyl ether (940 µL) at 30 °C. After 10 min, vinyl butyrate (330 µL, 2.6 mmol) was added and the reaction was monitored by GLC. After 17 h, the conversion reached 57% and the reaction was stopped by filtration through a thin layer of Celite. After evaporation of the solvent, the crude was purified by chromatography (*n*-hexane/EtOAc, 6:1, then *n*-hexane/EtOAc, 3:1; R_f 0.21) to afford (–)-**10** (75 mg, 38%, 99% *ee*).

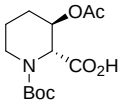
$[\alpha]_D^{18}$ = –77.3 (*c* = 1.1, $CHCl_3$). Spectroscopic data identical to those reported above for (±)-**10**.



***tert*-Butyl (*R*,*R*)-3-(Acetyloxy)-2-(furan-2-yl)piperidine-1-carboxylate [(–)-11]**

Prepared as reported above for (±)-**11**, starting from (–)-**10** (70 mg, 0.26 mmol). (–)-**11** (72 mg, 90%) was obtained after chromatography (*n*-hexane/EtOAc, 6:1, R_f 0.21) as a colourless oil.

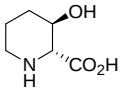
$[\alpha]_D^{21}$ = –45.9 (*c* = 1.0, $CHCl_3$). Spectroscopic data identical to those reported above for (±)-**11**.



(*R*,*R*)-3-(Acetyloxy)-1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic Acid [(–)-8]

Prepared as reported above for (±)-**8**, starting from (–)-**11** (70 mg, 0.23 mmol). (–)-**8** (49 mg, 72 %) was obtained after chromatography (CH_2Cl_2/MeOH, 20:1 with 0.1% AcOH; R_f 0.19).

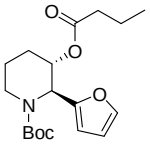
$[\alpha]_D^{22}$ = –15.7 (*c* = 1.0, $CHCl_3$). Spectroscopic data identical to those reported above for (±)-**8**.



(*R*,*R*)-3-Hydroxypiperidine-2-carboxylic Acid Hydrochloride [(–)-1·HCl][49]

Prepared as reported above for (±)-**1**·HCl, starting from (–)-**8** (49 mg, 0.17 mmol). (–)-**1**·HCl (31 mg, 96%) was obtained as a light yellow solid.

$[\alpha]_D^{21}$ = –14.9 (*c* = 1.4, H_2O). Spectroscopic data identical to those reported above for (±)-**1**·HCl.

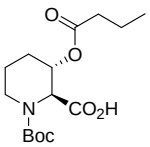


Kinetic Resolution of (±)-10 with CAL-B

***tert*-Butyl (*S*,*S*)-3-(Butyryloxy)-2-(furan-2-yl)piperidine-1-carboxylate [(+)-12]**

CAL-B (400 mg) was added to a solution of (±)-**10** (540 mg, 2 mmol) in THF (2.6 mL) at 30 °C. After 10 min, vinyl butyrate (889 µL, 7 mmol) was added and the reaction was monitored by GLC. After 25 h, the conversion reached 45% and the reaction was stopped by filtration through a thin layer of Celite. After evaporation of the solvent, the crude was purified by chromatography (*n*-hexane/EtOAc, 6:1, then *n*-hexane/EtOAc, 3:1 R_f 0.26) to afford (+)-**12** (250 mg, 37%, *ee* 91%).

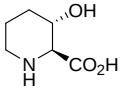
$[\alpha]_D^{23}$ = +31.7 (*c* = 0.82, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.36 (d, *J* = 1.2 Hz, 1 H, 5'-H), 6.33 (dd, *J* = 3.1, 2.0 Hz, 1 H, 4'-H), 6.15 (dd, *J* = 2.0, 1.2 Hz, 1 H, 3'-H), 5.42 (bs, 1 H, 3-H), 5.34 (bs, 1 H, 2-H), 4.08 (d, *J* = 12.5 Hz, 1 H, 6-H_{eq}), 2.88 (t, *J* = 12.5 Hz, 1 H, 6-H_{ax}), 2.32 (t, *J* = 7.4 Hz, 2 H, OCO*C*$H_2$$CH_2$), 1.86–1.76 (m, 3 H, 5-H + 4-H + 4-H'), 1.72–1.60 (m, 2 H, CH_2C$H_2$$CH_3$), 1.47–1.44 [s, 9 H, C(CH_3)$_3$ and m, 1 H, 5-H'], 0.97 (t, *J* = 7.4 Hz, 3 H, CH_2CH_2CH_3). ^{13}C NMR ($CDCl_3$, 100.32 MHz) δ (ppm): 173.0 (*C*OCH_2), 155.4 (N*C*O), 151.2 (C-2'), 142.1 (C-5'), 110.4 (C-4'), 107.4 (C-3'), 80.0 [*C*(CH_3)$_3$], 68.2 (C-3), 53.3 (C-2), 39.8 (C-6), 36.5 (CO*C*$H_2$$CH_2$), 28.5 [C(*C*$H_3$)$_3$], 24.8 (C-4), 19.7 (C-5), 18.6 (CH_2*C*$H_2$$CH_3$), 13.8 ($CH_2$*C*$H_3$). MS (ESI) *m/z* (%): 697 (100) $[2M + Na]^+$, 360 (28) $[M + Na]^+$. Elemental analysis calcd. (%) for $C_{18}H_{27}NO_5$ (337.41): C, 64.07; H, 8.07; N, 4.15. Found: C, 63.78; H, 7.93; N, 4.22.



(*S*,*S*)-3-(Butyryloxy)-1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic Acid [(+)-13]

Prepared as reported above for (±)-**8**, starting from (+)-**12** (190 mg, 0.56 mmol). Product (+)-**13** (114 mg, 65%) was obtained as a yellow gummy solid.

$[\alpha]_D^{20}$ = +12.4 (*c* = 1.1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 400 MHz) (1:1 mixture of rotamers) δ (ppm): 9.17 (bs, 1 H, COOH), 5.45 (bs, 1 H, 3-H, rotamer A), 5.36 (bs, 1 H, 3-H, rotamer B), 5.09 (bs, 2-H, rotamer A), 5.00 (bs, 2-H, rotamer B), 4.14 (d, *J* = 12.3 Hz, 1 H, 6-H_{eq}, rotamer A), 3.99 (d, *J* = 12.3 Hz, 1 H, 6-H_{eq}, rotamer B), 3.00 (t, *J* = 12.3 Hz, 1 H, 6-H_{ax}, rotamer A), 2.86 (t, *J* = 12.3 Hz, 1 H, 6-H_{ax}, rotamer B), 2.31–2.30 (m, 2 H, COC$H_2$$CH_2$, both rotamers), 1.67–1.60 (m, 2 H, CH_2C$H_2$$CH_3$, both rotamers), 1.47 [s, 9 H, C(CH_3)$_3$, rotamer A], 1.43 [s, 9 H, C(CH_3)$_3$, rotamer B], 1.94–1.20 (m, 4 H, 4-H + 5-H, both rotamers), 0.96–0.93 (m, 3 H, CH_2CH_2CH_3, both rotamers). ^{13}C NMR ($CDCl_3$, 100.32 MHz) δ (ppm): 173.8, 172.9 and 172.7 (*C*OOH and *C*OCH_2, both rotamers), 156.1 (N*C*O, rotamer A), 155.3 (N*C*O, rotamer B), 80.7 [*C*(CH_3)$_3$], 67.4 (C-3, rotamer A), 67.3 (C-3, rotamer B), 57.9 (C-2, rotamer A), 57.1 (C-2, rotamer B), 41.6 (C-6, rotamer A), 40.3 (C-6, rotamer B), 36.3 (CO*C*$H_2$$CH_2$), 28.2 [C(*C*$H_3$)$_3$], 25.4 (C-4), 18.9 (C-5, rotamer A), 18.8 (C-5, rotamer B), 18.5 (CH_2*C*$H_2$$CH_3$, rotamer A), 18.4 ($CH_2$*C*$H_2$$CH_3$, rotamer B), 13.6 ($CH_2CH_2$*C*$H_3$). MS (ESI) *m/z* (%): 984 (100) $[3M + K]^+$, 669 (70) $[2M + K]^+$, 653 (59) $[2M + Na]^+$, 338 (19) $[M + Na]^+$. Elemental analysis calcd. (%) for $C_{15}H_{25}NO_6$ (315.36): C, 57.13; H, 7.99; N, 4.44. Found: C, 57.45; H, 7.81; N, 4.28.



(*S*,*S*)-3-Hydroxypiperidine-2-carboxylic Acid Hydrochloride [(+)-1·HCl][52,53]

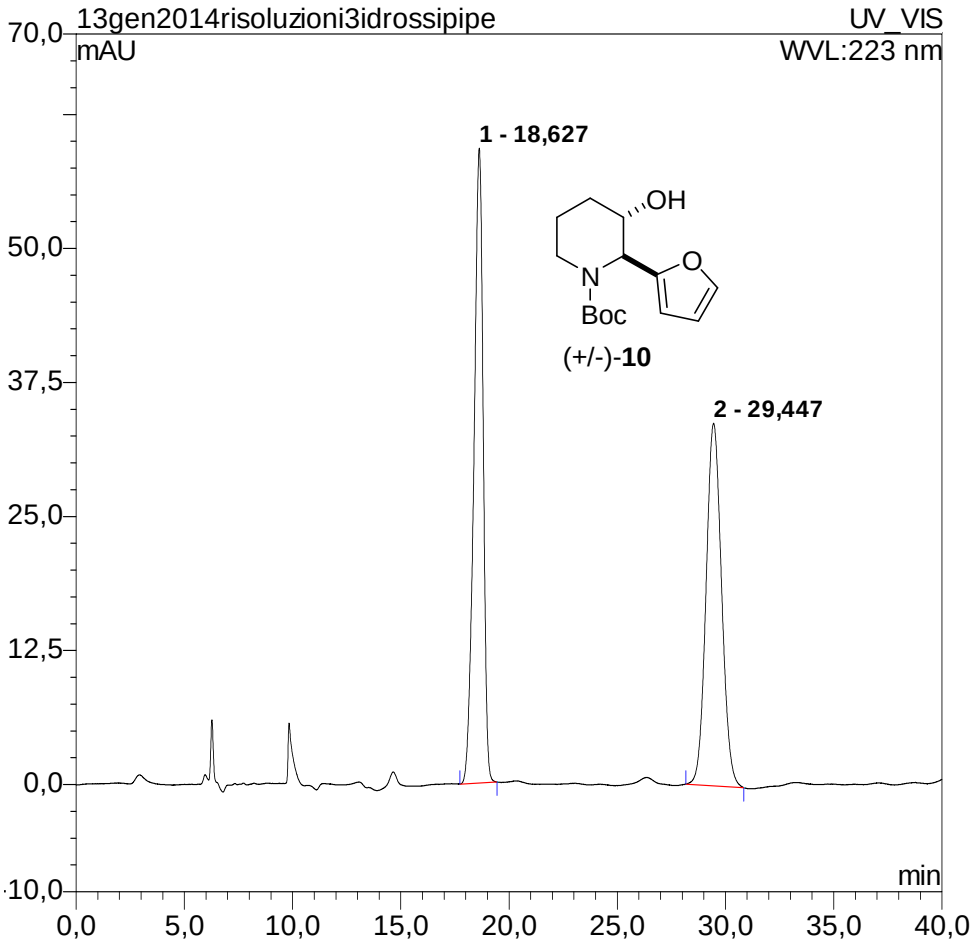
Prepared as reported above for (±)-**1**·HCl, starting from (+)-**13** (100 mg, 0.32 mmol). Compound (+)-**1**·HCl (58 mg, 100%) was obtained as a light yellow solid.

$[\alpha]_D^{21}$ = +13.7 (*c* = 0.7, H_2O). Spectroscopic data identical to those reported above for (±)-**1**·HCl.

8.5 Appendix

Appendix 1: Enantioselective Analytical HPLC of (±)-10

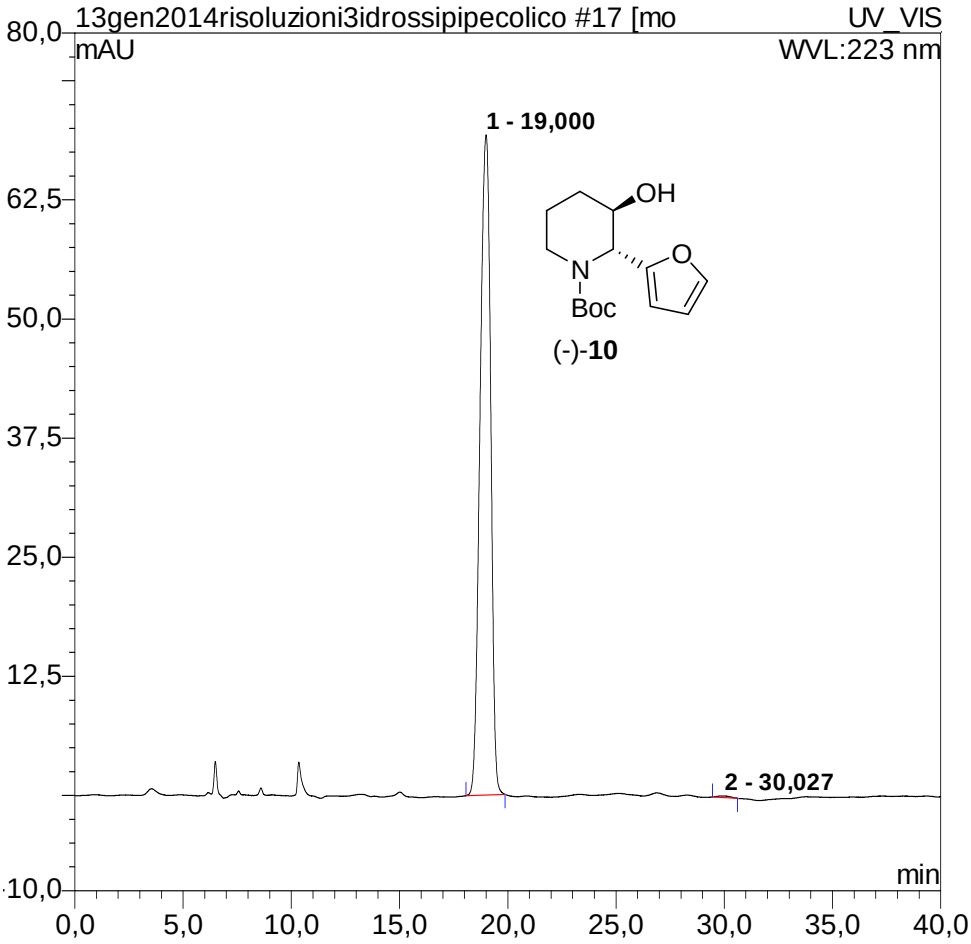
HPLC analysis of purified (±)-**10**. Column: Lux 5µ Cellulose-4 (4.6-250 mm), isocratic 10% IPA, 90% hexane at room temperature.



No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area (%)
1	18.63	59.224	29.204	50.98
2	29.45	33.830	28.078	49.02
Total		93.054	57.281	100.00

Appendix 2: Enantioselective Analytical HPLC of (–)-10

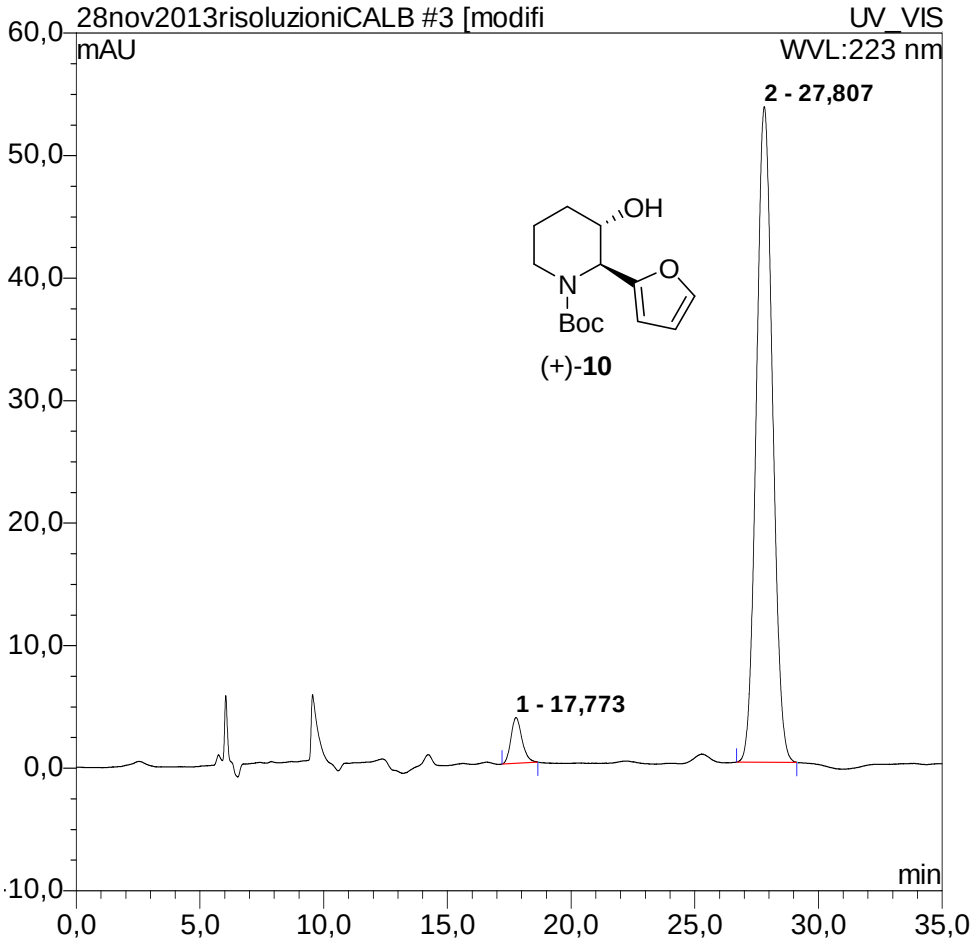
HPLC analysis of purified (–)-**10**. Column: Lux 5µ Cellulose-4 (4.6-250 mm), isocratic 10% IPA, 90% hexane at room temperature.



No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area (%)
1	19.00	69.312	39.747	99.68
2	30.03	0.193	0.126	0.32
Total		69.506	39.872	100.00

Appendix 3: Enantioselective Analytical HPLC of (+)-10

HPLC analysis of purified (+)-**10**. Column: Lux 5µ Cellulose-4 (4.6-250 mm), isocratic 10% IPA, 90% hexane at room temperature.



No.	Ret. Time (min)	Height (mAU)	Area (mAU*min)	Rel. Area (%)
1	17.77	3.734	1.929	4.53
2	27.81	53.527	40.642	95.47
Total		57.261	42.572	100.00

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Conclusions

In conclusion, we have demonstrated that substituted 2-azabicyclo[4.1.0]heptane-1-carboxylic acids (cyclopropane pipecolic acids, or CPAs) are suitable to be introduced into linear and cyclic peptides to generate a novel class of peptidomimetics for drug discovery.
First, we have shown that it is possible to efficiently prepare a number of differently hydroxy- and amino-substituted CPAs by means of the Pd-catalysed methoxycarbonylation of suitably functionalized lactam-derived vinyl phosphates followed by an OH-directed (efficient control of the relative stereochemistry of the various group) or a Michael-type cyclopropanation. By this approach, enantiopure N-Boc-, N-Cbz-, N-CO_2Me- and N-Fmoc-protected cyclopropane pipecolic acids bearing a hydroxy group at the 4- or 5-position were prepared in good yields, starting from simple commercially available materials. In addition, N-CO_2Me-protected 4- and 5-hydroxy-pipecolic acids were efficiently converted into the correspondent 4- and 5-amino derivatives, which were employed for the construction of biologically active cyclic peptidomimetics.
A combined NMR spectroscopic and modelling study on these scaffolds suggests that the cyclopropane forces the piperidine ring into a slightly distorted half-chair, with the various ring substituents projected along well-determined orientations.
4-OH-CPA was successfully introduced into a short linear peptide by using standard coupling techniques, showing that, compared to natural pipecolic acid, the presence of the cyclopropane moiety causes a remarkable shift toward the *cis* isomer about the pipecolic acid peptide bond.
Furthermore, both 4-NH_2-CPA and 5-NH_2-CPA with *cis* relative stereochemistry between the carboxylic and the amino groups proved suitable templates to prepare cyclic peptidomimetics containing the RGD sequence, which resulted potent ligands of both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors.
The RGD-containing peptidomimetic built on the 5-amino-CPA displayed an inhibition activity toward $\alpha_v\beta_3$ (IC_{50} = 2.4 nM) comparable to that of the most potent antagonists reported so far and it was also ten times more active than the corresponding ligand derived from the isomeric 4-amino-CPA. Since the activity of both compounds was in the nanomolar range towards both integrins, they can be considered as dual ligands, which could be an advantage in these compounds find applications in cancer therapy. These results are encouraging in further employing these CPA templates for the synthesis of new integrin binders and, by exploiting the scaffold N atom as an anchoring point, for targeted delivery of drugs and diagnostics.
Indeed, we can affirm that another class of artificial conformationally constrained amino acids has made its entrance in peptide synthesis. Hereafter, further peptidomimetics could be synthesised to find new biologically active compounds and to enlarge investigations concerning the conformational characteristics introduced by CPAs in peptides.

Contemporaneously, a side project led to a new efficient and facile synthesis for both enantiomers of *trans* 3-hydroxypipecolic acid, another constrained amino acid part of the pipecolic acid derivatives family. In the process, we also developed a new method for the introduction of the carboxylic functional group at the 2-position by oxidizing an aryl moiety conveniently introduced by Suzuki-Miyaura cross-coupling on the usual vinyl phosphate intermediate.

These compounds, apart from containing the interesting 3-hydroxy-piperidene moiety, can be considered as constrained serine analogues valuable for natural amino acids replacement in the construction of rigid peptidomimetics. Moreover, they could be converted into the correspondent 3-amino derivatives and employed as scaffolds for building further RGD-based integrin ligands.

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