CHAPTER SIX

Peptide and peptidomimetic tyrosinase inhibitors☆

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Abstract

Melanin, which is produced by melanocytes and spread over keratinocytes, is responsible for human skin browning. There are several processes involved in melanogenesis, mostly prompted by enzymatic activities. Tyrosinase (TYR), a copper containing metalloenzyme, is considered the main actor in melanin production, as it catalyzes two crucial steps that modify tyrosine residues in dopaquinone. For this reason, TYR inhibition has been exploited as a possible mechanism of modulation of hyper melanogenesis. There are various types of molecules used to block TYR activity, principally used as skin whitening agents in cosmetic products, e.g., tretinoin, hydroquinone, azelaic acid, kojic acid, arbutin

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and peptides. Peptides are highly valued for their versatile nature, making them promising candidates for various functions. Their specificity often leads to excellent safety, tolerability, and efficacy in humans, which can be considered their primary advantage over traditional small molecules. There are several examples of tyrosinase inhibitor peptides (TIPs) operating as possible hypo-pigmenting agents, which can be classified according to their origin: natural, hybrid or synthetically produced. Moreover, the possibility of variating their backbones, introducing non-canonical amino acids or modifying one or more peptide bond(s), to obtain peptidomimetic molecules, is an added value to avoid or delay proteolytic activity, while the possibility of conjugation with other bioactive peptides or organic moieties can bring other specific activity leading to dual-functional peptides.

Abbreviations

α-MSH	alpha-melanocyte-stimulating hormone
αS-CN	αS-casein
Ala; A	alanine
Arg; R	arginine
Asn; N	asparagine
Asp; D	aspartic acid
BLG	β-lactoglobulin
CA	caffeic acid
Cys	C: cysteine
DWMP	defatted walnut meal protein
DWMPHs	defatted walnut meal protein hydrolysates
GA	gallic acid
Gln	Q: glutamine
Glu	E: glutamic Acid
Gly	G: glycine
GSH	glutathione
GSSG	oxidized glutathione
GT	gentisic acid
His	H: histidine
HPLC	high performance/pressure liquid chromatography
IC_{50}	half maximal inhibitory concentration
Ile	I: isoleucine
L-DOPA	L-3,4-dihydroxyphenylalanine
L-ERT	L-ergothioneine
Leu	L: leucine
LMW-GH	low molecular-weight gelatin hydrolysates
Lys	K: lysine
KA	kojic acid
KAE	kojic acid equivalents
MITF	melanocyte inducing transcription factor/microphthalmia-associated transcription factor
MC1R	melanocortin-1 receptor
Met	M: methionine

MSCP	milkfish scale peptide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PA	protocatechuic acid
Phe	F: phenylalanine
PPO	polyphenol oxidase
Pro	P: proline
RA	α-resocylic acid
RBAlb	rice bran albumin
RBP	rice bran protein
ROS	reactive oxygen species
SAP	stabilized ascorbyl pentapeptide
Ser	S: serine
SPPS	solid phase peptide synthesis
Thr	T: threonine
TILI-1	tyrosinase inhibitor Leucrocin I-1
TILI-2	tyrosinase inhibitor Leucrocin I-2
TIPs	tyrosinase inhibitor peptides
TRP-1	tyrosinase related protein-1
TRP-2	tyrosinase related protein-2
TSC	thiosemicarbazone
Tyr	Y: tyrosine
TYR	tyrosinase
Trp	W: tryptophan
UV-A	ultraviolet radiation A
UV-B	ultraviolet radiation B
Val	V: valine

1. Introduction

Tyrosinase (TYR), alternatively known as polyphenol oxidase (PPO), is a copper-containing metalloenzyme found across a diverse range of organisms, including mammals. TYR is crucial to the production of melanin, the pigment responsible for coloring skin, hair, and eyes in mammals. In fact, it catalyzes the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) and subsequently to dopaquinone, which are key steps in the melanin synthesis pathway [1–3].

Due to their versatile nature, peptides are often regarded as promising compounds with ideal functions, especially as regulating and signaling molecules in stress, immunity and defense. Their specificity often results in excellent safety, tolerability, and efficacy in humans, which may be their main advantage over traditional small molecules, serving as a promising foundation for developing new therapeutics. Therefore, peptides occupy a unique position between small molecules and biopharmaceuticals in several

respects [4,5]. Moreover, peptides present reduced ability to trigger an immune response and relatively low-cost production, making them interesting as both therapeutic agents and cosmeceutical compounds. In this respect, in fact, they can be used as antioxidant, anti-aging, moisturizing ingredients as well as promoters in collagen production or wound healing processes [6].

Accordingly, there are several examples of tyrosinase inhibitor peptides (TIPs) acting as possible hypo-pigmenting agents. Their mechanism of action can be classified in three groups:

1 Anti-oxidation.

Ultraviolet radiation is responsible of low-grade oxidative stress in melanocytes, causing the production of rapidly reactive free radicals that can cause abnormal tyrosinase activation resulting in increased melanogenesis. There are three ways in which TIPs can act on the enzyme: (i) directly scavenging of free radicals, (ii) activation of anti-oxidative enzymes, and (iii) regulation of gene pathways that decrease the amount of TYR.

2 Occupation of the bioactive site TYR.

TYR is a tetrameric enzyme with two copper ions (Cu^{2+}) in the active site, characterized by a hydrophilic exterior and a hydrophobic cavity near the active site. TIPs can interact with the protein in different ways such as direct chelation of the copper ions, reactions with amino acids residues in the cavity or near the catalytic center with formations of different types of interactions such as covalent bonds, hydrogen bonds, hydrophobic interactions, Van der Waals forces or π-π stacking. Chang and coworkers identified two types of the so called "true inhibitors" that can be found in this category: (i) suicide substrate: peptides that can form a covalent bond with the enzyme thus causing its complete inactivation; (ii) reversible inhibitors: compounds that can reversibly bind to TYR causing a decrease in its catalytic capability [7].

"True inhibitors" can also be classified according to their inhibition type: (i) competitive inhibitors; (ii) uncompetitive inhibitors; (iii) mixed type, competitive/uncompetitive inhibitors and (iv) noncompetitive inhibitors.

3 Regulation of related gene expression.

There are several signaling pathways connected to the expression of tyrosinase and thus to melanogenesis production. TIPs can interact with many enzymes, mediators and other chemical entities.

Considering that peptides are composed of amide bonds that are labile in physiological condition, as they can be cleaved by specific enzymes,

several strategies can be employed to modify the peptide backbone, in order to overcome the disadvantages due to possible low bioavailability. These strategies led to the so-called peptidomimetics. In this context, chemical synthesis plays a particularly important role, as it offers access to a much wider chemical diversity than peptide derivatives produced by recombinant technologies. Peptidomimetic compounds are very similar to parent peptides, and they are intended to mimic their biological effects. Very often they contain nonpeptide structural elements, such as peptide bond surrogates that resist cleavage from peptidases, a feature also found in some natural peptidase inhibitors. In addition to permanent alterations of the structure, it is also possible to introduce temporary changes using a prodrug approach. A peptide prodrug can be obtained by combining a biologically active peptide with additional elements, which give the entire molecule greater resistance against enzymatic hydrolysis and/or bioavailability [8].

This chapter is dedicated to a deep literature investigation on peptides and peptidomimetics as TYR inhibitors deriving from natural sources, hybrid, or totally synthetically approach.

2. Amino acids with anti-tyrosinase properties

In 2018, Park et al. investigated the effect of D-tyrosine on melanin synthesis in melanocyte-derived cells such as human melanoma cells, human melanocytes and 3D human skin model [9]. Each amino acids, except for glycine, exist in two enantiomeric forms. However, our organism uses only L-amino acids to accomplish its functions, thus, D-amino acids could potentially act as inhibitor for L-form dependent enzymes. Indeed, the results of the experiments done by these authors evidenced that D-tyrosine, which did not affect cell proliferation, is instead able to reduce in a dose-dependent manner the amount of melanin produced by the cells whereas L-tyrosine, as previously reported, is able to induce melanogenesis [10]. The performed trials highlighted the ability of D-tyrosine to directly interfere the enzymatic activity of TYR in a dose-dependent manner, acting as competitive inhibitors. Interestingly, D-tyrosine can inhibit not only the melanogenic function of L-tyrosine but also melanogenesis induced by alpha-melanocyte-stimulating hormone (α-MSH) and UVB radiation. These results were confirmed by the use of MelanoDerm™ skin model (MEL-312-B, a 3D model in which human

melanocytes are incorporated into a well differentiated epidermal tissue): the samples treated with D-tyrosine revealed a reduced amount of melanin and tyrosinase compared to the ones treated with distilled water.

In an additional work [11], the same authors studied the effect of L-tyrosine on TYR by synthetizing four analogues of pentapeptide-18 (L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-leucine), adding both in position N- and C-terminal L- or D-tyrosine and then studying the effect of these derived peptides on melanin production in Human Melanoma MNT-1 cells, human melanocyte and 3D model of human epidermis. The results highlight that the presence of D-tyrosine, especially in C-terminal position, is able to reduce melanin synthesis and tyrosinase activity induce by both α-MSH and UVA radiation, thus suggesting that the presence of the amino acid could add additional anti-melanogenic effect to cosmetic peptides. However, this additional function is not shown when the addition of D-tyrosine occurs on longer peptides, where the higher number of side chains could interfere negatively with the binding of the peptide to the enzyme and thus on D-tyrosine inhibition efficacy.

Liao et al. studied the effect of ergothioneine (structure shown in Fig. 2), a naturally occurring amino acid found in fungi, oysters and actinobacteria, having antioxidative properties [12]. Ergothioneine is a thiourea derivative of histidine, thus the authors compared the two amino acids as tyrosine inhibitors. The o-diphenolase activity of tyrosine was tested using L-DOPA as substrate at different concentration of histidine, ergothioneine and kojic acid (used as control) on mushroom tyrosinase. Results evidence that L-ERT inhibits TYR in a dose dependent manner with an IC_{50} = 4.47 mM. The Lineweaver–Burk plot of ergothioneine inhibition demonstrates that the reaction velocity increases increasing L-DOPA concentration, suggesting a non-competitive type of inhibition activity. Taken all together, these results indicate that ergothioneine can potentially bind to a substrate site of free enzymes and can also bind to a site distinct from the substrate of an enzyme–substrate complex. On the other hand, histidine instead is not able to inhibit the enzyme.

Luisi et al. studied the antioxidant and tyrosinase inhibitory activity of sulphurated amino acids, with different oxidation state of the sulfur atom, alone or incorporated into small peptides [13]. In particular, the studied compounds were L-cysteine, glutathione and its gamma-oxa analogue, L-cystine, L-ergothioneine and Taurine. Moreover, three additional synthetic compounds containing the non-proteinogenic amino acid tert-Leucine, were synthetized in order to assess their antioxidant and inhibitory activity.

Tyrosinase inhibition activity was measured using the dopachrome method (a schematic representation of the dopachrome formation is reported in Fig. 1).

Final TYR inhibitory activity is expressed as kojic acid equivalents (mgKAE/g sample). All the tested compounds were active against TYR. The results are reported in Table 1.

The effect of cysteine on the o-dihydroxyphenolase activity of PPO has been investigated also by Kahn [14]. Indeed, in the presence of cysteine, an initial delay in dopachrome formation is observed, with higher cysteine concentrations producing longer lag periods. This can be probably attributed to the ability of cysteine to form a conjugate with dopaquinone. The

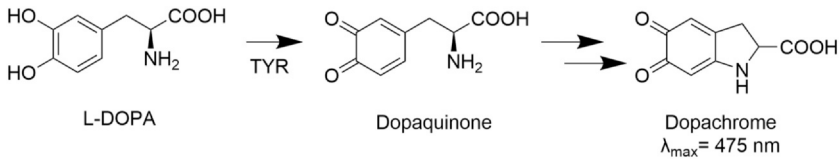
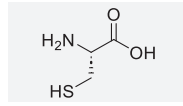
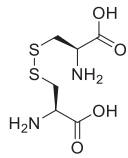
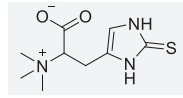
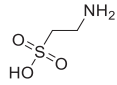


Fig. 1 Schematic representation of the conversion from L-DOPA to dopachrome in melanogenesis.

Table 1 Results of TYR inhibitory activity expressed ad kojic acid equivalents.

Compound:	Structure:	Tyrosinase inhibitory activity (mg KAE/g sample):
L-Cysteine		216.40 ± 0.17
L-Cystine		217.16 ± 0.55
L-Ergothioneine		100.97 ± 1.71
Taurine		33.87 ± 1.59

results were confirmed by performing experiments on freshly cut fruits like avocado and banana which are prone to fast browning reactions. The application of L-cysteine effectively prevented the discoloration even after 6 h after cutting, suggesting that this amino acid could be potentially use not only in the cosmetic industry but also in the food industry to prevent oxidation reactions in fruits and vegetables.

3. Anti-tyrosinase peptides from natural sources

Naturally occurring TIPs can be found in various biological sources, which include animals, plants, and microorganisms. In recent years, the pursuit of natural ingredients has become a matter of interest especially in the cosmetic field, but also in the pharmaceuticals, giving rise to new active ingredients that can potentially be included in skin care formulations and dermatological applications.

In this paragraph we will elucidate which are the most common peptides from natural sources described in literature, displaying TYR inhibiting activity and antimelanogenic effects on cells. A list of the described compounds is reported, at the end of the paragraph, in Table 2.

3.1 Peptides deriving from mushrooms

The first study reported in the literature describing peptides with anti-tyrosinases activity dates back to 1974, when Madhosingh and Sundberg identified two peptides (named *compound Ia* and *compound Ib* in their paper) from *Agaricus hortensis* mushrooms [15]. Their inhibiting activity was demonstrated by incubation with L-Dihydroxyphenylalanine (L-DOPA) as a substrate, in presence of mushrooms TYR (that has to be inhibited), and they were found to act with different mechanism of action: *compound Ia* is a competitive inhibitor, while *compound Ib* is a non-competitive inhibitor. Amino acids analysis of the extracted peptides revealed that the three most common residues were phenylalanine, aspartic and glutamic acid (ratio 1:1:1). However, the peptide sequences of these two compounds were not described.

3.2 Peptides deriving from bacteria

The first example of a well-defined peptide sequence with anti-tyrosinases activity was reported some years later, in a study based on *Lactobacillus Helveticus* [16]. The authors observed that a cyclopeptide, Cycle[Pro-Tyr-Pro-Val],

isolated from this species, was able to inhibit mushroom tyrosinase activity with a half maximal inhibitory concentration (IC_{50}) higher than arbutin that was used as a reference compound.

Some years later, a cyclic peptide capable to inhibit TYR was isolated from *Oscillatoria agardhii* [17]. This cyanobacterium produce cyclic heptapeptide hepatotoxins known as microcystins and one among these, named Oscillapeptin G (whose structure is reported in Fig. 2), were found to be a non-toxic depsipeptide [17,18]. Depsipeptides are cyclic compounds where one or more of their peptide bonds –C(O)NHR–, are substituted by the corresponding ester, –C(O)OR–; principally used as anti-cancer drugs [19,20]. Oscillapeptin G activity against TYR was studied in the presence of L-Tyr and TYR and showed a 0.1 mM IC_{50} [17].

A more recent example reports the dual activity of antioxidant and anti-TYR agent of the tetrapeptide MGRY purified from the microalga *Pavlova lutheri*. TYR-inhibiting properties were demonstrated by testing the peptide as a treatment on B16F10 melanoma cells with α-MSH-induced melanogenesis. Additionally, it showed decreased melanogenesis-related proteins, microphthalmia-associated transcription factor (MITF) and TYR protein expressions.

3.3 Peptides from plant sources

3.3.1 Pseudostellaria heterophylla

A study conducted by Morita et al. highlighted the presence of various cyclic peptides isolated from *Pseudostellaria heterophylla* roots, which were tested with the dopachrome method and exhibited mushroom TYR inhibitory activity [21–23].

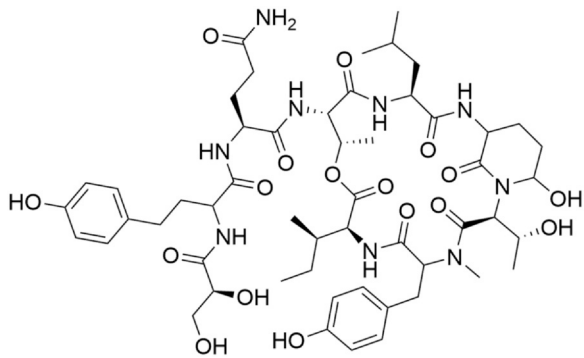


Fig. 2 Oscillapeptin G structure.

These compounds, named pseudostellarins (A,B,C,D,E,F,G) showed lower IC_{50} than the ones observed in the case of the cyclopeptide isolated in the *L. Helveticus* [16], and arbutin, both used as references. Moreover, pseudostellarins C, D and G showed antimelanogenesis effect when used as treatment from B16 melanoma cells, with a IC_{50} = 171 μM, IC_{50} = 49 μM, and IC_{50} = 102 μM, respectively. The importance of investigating the activity in vitro on cell models is due to the fact that mushrooms TYR is present in the cytoplasm, and is different from the mammals TYR present on the membrane of melanin bulks [24].

3.3.2 Rice

In 2009 Ubeid et al. described the activity of some oligopeptides of unknown origin that were able to inhibit both mushrooms and human TYR, showing in particular two highly active sequences: RADSRADC, also called peptide P3, and YRSRKYSSWY, also called peptide P4, with IC_{50} (mushroom TYR) of 123 μM and 40 μM, respectively [25]. Interestingly, P4 is still nowadays the only peptide used as a therapeutic agent for melanin-related skin disorders [26]. Further details on Ubeid and coworkers' studies describing P3 and P4 design and activity is reported below in the *Oligopeptides* paragraph.

Based on the potent activity of P4, and considering rice bran, found in rice kernel and produced as a byproduct of the rice milling process, as a new source of bioactive peptides in a view of new sustainable active ingredients, Ochiai et al. described the activity of rice bran bioactive peptides as TYR inhibitors [27,28]. Among the sequences found in each rice bran protein (RBP), MRSRERSSWY showed the highest grade of homology to P4 (YRSRKYSSWY), and this peptide was designated as TH10. Moreover, in their studies they described analogues of TH10 and P4 that revealed the functional role of tyrosine at various positions in their sequences. They observed that the TH10 variants in which the C-terminal tyrosine residue was replaced, had no effect on TYR, meaning that this position is essential for the tyrosinase inhibitory activity of TH10 and cannot be substituted with other aromatic amino acid residues. Additionally, even by reverting the sequence of TH10, therefore obtaining a tyrosine residue in N-terminal, the effect on TYR was lost. Worth of note, the same correlation was found by replacement of the tyrosine residues in peptide P4. In fact, this sequence contains three tyrosine residues, one in N-terminal, one in C-terminal and one in the middle, and it was observed that only by replacing the one in C-terminal there was a loss of activity,

that was indeed not observed by replacing one or two tyrosine in the other positions [27]. Prompted by these indications, the same research group have deepened the possibility to find other TYR inhibitors derived from RBP hydrolysates prepared by mixing chymotrypsin (that cleaves preferentially at the carboxyl sides of large hydrophobic and aromatic amino acids such as tyrosine), and trypsin (in order to obtain short peptide sequences) [28]. The resulted hydrolysate fractions were analyzed, and their amino acid sequences were determined, showing that three of them (namely CT-1, CT-2, and CT-3) contained a tyrosine residue in C-terminal. Their activity was determined both against mono- and diphenolase reaction. In fact, TYR catalyzes two different reactions in the presence of molecular oxygen: the hydroxylation of monophenol (monophenolase activity) and the oxidation of o-diphenol to o-quinone (diphenolase activity) [29], giving rise to DOPA and Dopaquinone respectively, as shown in Fig. 3.

All three CT-1, CT-2 and CT-3 significantly inhibited the monophenolase reaction of tyrosinase; in particular, it was possible to establish that CT-2 had an IC_{50} = 156 μM. Moreover, peptides CT-1 and CT-3 increased melanin production in mouse B16 melanoma cells, suggesting that they do not inhibit mammalian tyrosinase, while CT-2 inhibited melanin production by >50% at 500 μM. Therefore, they conclude that CT-2 (LQPSHY) is a potent peptide to be used as an agent for melanin-related skin disorder treatment.

On the wave of peptide sequences deriving from rice bran, a study conducted by Kubglomsong et al. makes a comparison between different type of rice bran protein in which it was shown that albumin (RBAlb) is the one with higher anti-tyrosinases activity. Moreover, RBAlb was hydrolyzed and this mixture was analyzed to obtain information on the peptide sequences responsible for TYR inhibition and copper-chelating activity [30]. Thirteen peptides composed of 14–50 residues with molecular weights ranging from 1327 to 4819 Da were identified: DASLTRLDLAGANGTDLAYGISLTVAV (sequence n.1), PRCSASSTTPRSTTSAAWRAPSAPTSSTS (sequence n.2),

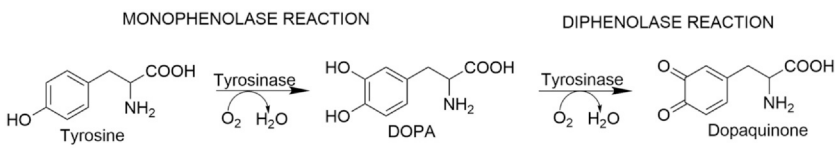


Fig. 3 Tyrosinase monophenolase and diphenolase reaction starting from tyrosine.

QSSKQRRDNGIFFVPQRPYMVLGTLRQQLLYPTW (sequence n.3), DVLEEGNSGDPPLFVSDVLHGAAIEVNEEGTEVAAATVVIMKGRA (sequence n.4), QGWSSSSSEYYGGEGSSSEQGYYGEG (sequence n.5), GWSSSSSEYYGGEGSSSEQGYYGEG (sequence n.6), SSEYYGGEGSSSEQGYYGEG (sequence n.7), VELEEKGEGAAMTE (sequence n.8), AAAVAMAAAVAAGEGGAANYLVFVDPPPSGVVCTAYQLSILAAALGSEEK (sequence n.9), SSHAAARRLRRSSSP (sequence n.10), GEPVAAEERDDGVGVAGE (sequence n.11), SSSEYYGGEGSSSEQGYYGEG (sequence n.12), and LSLIGAVGGIGGSIL (sequence n.13). Remarkably, several peptide sequences (5–7 and 12) had the pattern SSEYYGGEGSSSEQGYYGEG in their peptide sequences, which consists of well-known tyrosinase-inhibitory and metal-chelating amino acids. In particular, serine, glutamic acid, and tyrosine contribute to tyrosinase inhibitory and metal-chelating activities [25,31–37].

3.3.3 Vineyard

A pentapeptide with anti-TYR activity, peptide ECGYF (namely EF-5) was found also in a *Vigna* protein and reported in a study conducted by Shen et al. in which they showed the inhibitory effect of the peptide, but also its radical scavenging effect, and additionally its melanin reducing activity in vitro on A375 melanoma cells. Moreover, the UV-Vis absorption spectra, CD spectra and molecular docking have suggested that EF-5 might induce conformational changes in TYR [38].

3.3.4 Quince

Deng and coworkers reported the activity of peptides derived from Chinese quince (*Chaenomeles speciose*), a plant already in use in Chinese medicine that exhibited excellent anti-inflammatory, antitumor and immunomodulatory properties [39,40]. Hydrolyzed protein fractions were purified, and the amino acid sequence of the purified peptides was identified by MALDI/TOF/TOF mass spectrometry. Their biological properties including antioxidant, copper chelating and tyrosinase inhibitory capacity were studied and the results revealed at least two short sequences with anti-TYR activity: NYRRE (peptide F1-a) and RHAKF (peptide F1-b). The TYR-inhibition IC_{50} of both peptides were calculated and resulted 8.69 mg/mL and 1.15 mg/mL, respectively for F1-a and F1-b. Moreover, as peptide F1-b showed the higher activity in comparison with F1-a, and contain an imidazole ring (His), the authors suggested that this difference in the IC_{50} is due to metal-chelating properties of peptide F1-b [40].

3.3.5 Chia

Peptides deriving from chia seeds of *Salvia hispanica*, and already described for their antioxidant properties, were recently showed to also act as inhibitors of TYR [41,42]. In fact, it was shown by Aguilar-Toalá and Liceaga that fractions hydrolysate from chia seeds containing peptides with molecular mass <3 kDa exhibited inhibitory activity towards different enzymes tested, including TYR, towards which they observed an IC_{50} of 0.66 mg/mL [42].

3.3.6 Walnut

A recent work published by Feng et al. focused on peptides from defatted walnut (*Juglans regia*) meal [43]. This paper represents one among the few examples of well-designed and characterized peptide obtained from natural sources. The authors started from defatted walnut meal protein (DWMP) which was hydrolyzed obtaining defatted walnut meal protein hydrolysates (DWMPHs) and the different fractions obtained were tested to evaluate their possible anti-TYR properties. It was shown that all the four DWMPHs obtained possessed inhibitory activity both for TYR mono- and diphenolase reaction. In particular, it was observed that smaller molecular weight of the ultrafiltration component corresponded to smaller IC_{50} values for the tyrosine monophenolase and diphenolase activity (indicating higher inhibitory properties). DWMPHs-IV displayed the greater TYR inhibitory activity than the other three fractions with IC_{50} values of 3.52 and 2.65 mg/mL for monophenolase and diphenolase, respectively. This fraction was then purified, and the collected fraction were tested again against mushrooms TYR. Among these, fraction F2, was showed to be the most powerful TYR inhibitors, with IC_{50} values of 1.42 and 1.8 mg/mL for monophenolase and diphenolase, respectively. LC-MS/MS analysis on F2 revealed that it was composed of 606 peptide sequences. Considering the large number of peptides to be screened, *in silico* approaches were employed to evaluate the binding ability of TYR to these peptides and to obtain the best inhibitor candidate according to their binding energy based on the affinity scoring function, and peptide FPY exhibited the most stable complex, with the highest binding affinity. Therefore, a synthetic peptide FPY was synthetized and tested, showing excellent TYR inhibitory properties with IC_{50} values of 1.11 and 3.22 mM for monophenolase and diphenolase, respectively.

3.4 Peptides from animal sources

3.4.1 Silk cocoons

Another natural source of TIPs was found in silk cocoons, and in particular in silk sericin hydrolysates recovered from the waste of silk degumming process [32,44]. Despite peptide composition was not described, some key-information were obtained by characterizing the hydrolysates mixture, e.g., that the major amino acid constituent of the hydrolysate is Ser (up to 30%), and the main molecular weight distribution was among 250 and 4000 Da. The tyrosinase inhibiting activity was demonstrated by the decreased conversion of L-DOPA to dopachrome, and the possible mechanism of action proposed was an association with its capacity of chelating elements such as copper and iron by its rich hydroxyl groups. In fact, the correlation between TYR-inhibiting properties and copper-chelating properties is nowadays well known and described [37,45,46].

3.4.2 Fishes

Fish proteins have been also often used as a source of cosmeceutical peptides for the treatment of various skin alterations [47]. One among the most cited studies on this topic is reported by Zhuang et al. and focus on a jellyfish collagen peptide (JPC) rich in Gly, Pro, Ser, Ala, Glu and Asp, with a molecular weight between 400 and 1200 Da. In this study they isolated and purified JCP from jellyfish collagen enzymatic hydrolysate using ion exchange chromatography and gel filtration. The effect on melanogenesis was evaluated by direct inhibition of TYR and melanin content in B16 cells. The observed IC_{50} was 78.2 μg/mL and the content of melanin was decreased to 76.8% at 25 μg/mL and 52.1% at 100 μg/mL compared with the control. JPC was shown to have also copper chelating and antioxidant properties, which is an added value for a melanogenesis regulator, as melanin synthesis is known to be involved in the production of several reactive oxygen species, including $^{\bullet}O2^{-}$, $^{\bullet}OH$ and $NO^{\bullet}$ [46].

JPC case is not unique and there are also other examples of fish collagen-deriving peptides with attributed anti-melanogenic properties, e.g., collagen hydrolysates from *Todarodes pacificus* (squid) skin [48], and collagen peptides from *Chanos chanos* (milkfish) scales [49]. In particular, hydrolysates deriving from squid skin collagen were fractionated and tested to evaluate their hyaluronidase- and TYR-inhibitory activity. The biological assays highlighted that one among the fractions, namely F3, inhibited tyrosinase by 39.65% at 1 mg/mL and was also able to inhibit hyaluronidase. F3 hydrolysate fraction, comprising of 3.4–10 kDa exhibited also strong antioxidant

activities and metal chelating activity due to its high content of Ser, Gly, Val and Pro [48]. Indeed, Chen et al. reported the involvement of peptide extracts from milkfish scales (MSCP) in melanogenesis and their result revealed that 500 and 1000 μg/mL MSCP can repress the tyrosinase activity by 44.8% and 55.1%, respectively, in a dose dependent mode with an IC_{50} of 752.4 μg/mL. Moreover, MSCP inhibits melanin synthesis when used as a treatment in melanoma cells (B16) with a IC_{50} of 887.1 μg/mL, and the authors suggested an involvement in the copper chelating properties, as an explanation for the anti-TYR activity [49].

Unfortunately, as observed for several peptides derived from natural sources, especially for those that originate from protein hydrolysis processes, there is not a precise analytical characterization of the peptides described in these examples, therefore, their sequences are not described.

3.4.3 Sea cucumber

Stichopus japonicus (Sea cucumber) is considered a healthy food with anticancer, immunoregulatory, and anti-aging properties. Considering that it consists of approximately 70% of collagen [50], that is a gelatin source, in a paper published in 2010 by Wang and coworkers, the putative effect of the sea cucumber body wall hydrolysates was evaluated [51]. Gelatin hydrolysates were collected and after HPLC analysis, they were characterized as low molecular-weight gelatin hydrolysates (LMW-GH) as their molecular weight was from 700 up to 1700 Da. The amino acids compositions of these LMW-GH were described, and the most abundant amino acids resulted Gly, Glu, Asp, and Pro (in order). The potential anti-TYR activity and melanin reduction capability of LMW-GH was evaluated in vitro on B16F10 cells at different concentrations (25, 50 and 100 μg/mL). LMW-GH exhibited excellent inhibitory characteristics in both assays. In particular, concerning the inhibition of TYR, LMW-GH activity was 56.9%, 41.4% and 30.8% at 25, 50, and 100 μg/mL, respectively (expressed as the percentage of the absorbance/mg protein in treated cells of the control).

3.4.4 Milk

In 1997 the activity of the so-called whey proteins, milk proteins that remains soluble in whey after casein precipitation, that include α-lactalbumin, β-lactoglobulin (BLG), serum albumin and IgG was firstly described [52]. In this study the authors depicted the activity of the specific milk proteins on the pigmentation of cultured human melanocytes treated for 72 h with each whey protein and added with L-DOPA after cell lysis,

and they showed that β-lactoglobulin decreased TYR activity at a concentration of 1 mg/mL. The mechanism of action proposed is based on the ability of BLG to bind retinol, which is known to inhibit melanin biosynthesis [52,53]. However, Nakajima et al. study considered the whole proteins, instead of specific peptide sequences.

In further studies published some years later, the activity of peptides purified from milk casein and α-lactalbumin was described [54,55]. In particular, peptide YFYPEL was originally isolated from milk casein hydrolysates and several analogues were synthetized in order to study the influence of the deletion of one or more amino acid on the antioxidant activity, founding that the deletion of Y, YF, and YFY causes a loss of activity [54]. Additionally, 42 peptides deriving from α-lactalbumin and BLG hydrolysates with antioxidant properties were described by Hernández-Ledesma and coworkers [55]. Despite in both these cases peptides were only tested for their antioxidant activity and no information on the possible implication in melanogenesis processes were mentioned, they gave rise to further investigation for possible anti-TYR activity. In fact, in a study published in 2020, four types of milk protein-derived peptides deriving from their studies with antioxidant activity (YFYPEL [54], YVEEL, MHIRL and WYSLAMAA [55]) were used as possible leads for anti-TYR peptides, basing on the fact that there could be a correlation between the two activities [56,57]. The authors selected the MHIRL family peptides due to their excellent TYR inhibiting properties, and further studies were conducted to determinate if they could also inhibit melanin production in vitro in different melanocytes (Mel-Ab and B16F10) by interfering with α-MSH binding to MC1R (melanocortin-1 receptor) [58].

In a study conducted by Schurink et al., the activity of several TYR-inhibiting peptides deriving from natural sources, including milk extracts, was described [31]. They started from protein-based octamer peptide libraries made by SPOT synthesis to screen for peptides that show direct interaction with tyrosinase. The authors evaluated the ability of the resulted peptides to bind and inhibit TYR, dividing them into three categories: (I) strong tyrosinase-binding peptides; (II) strong tyrosinase-inhibiting peptides (reported in Table 2); (III) tyrosinase-binding and inhibiting peptides. They observed that the best tyrosinase binding peptides contain an Arg residue and that most tyrosinase-inhibiting peptides contain a Val residue, but also Ala and/or Leu residues (hydrophobic amino acids) seems to be important for the inhibiting activity.

Table 2 List of TYR inhibiting peptides from natural or hybrid sources.

Compound name	Peptide sequence	Natural source (species)	Claimed activity	IC_{50}	References
Compound Ia	Unknown	*Agaricus hortensis* (mushrooms)	Competitive inhibition of TYR in presence of DOPA	Unknown	[15]
Compound Ib	Unknown	*Agaricus hortensis* (mushrooms)	Non-competitive inhibition of TYR in presence of DOPA	Unknown	[15]
Compound 1	Cyclo[PYPV] (reported in Fig. 16)	*Lactobacillus Helveticus* (bacteria)	Inhibition of mushrooms TYR	1.5 mM	[16]
Oscillapeptin G	(reported in Fig. 2)	*Oscillatoria agardhii* (bacteria)	Inhibition of mushrooms TYR	0.1 mM	[17]
Pseudostellarins A	Cyclo[GPYLA]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR	131 μM	[21]
Pseudostellarins B	Cyclo[GIGGGPPF]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR	187 μM	[21]
Pseudostellarins C	Cyclo[GTLPSPFL]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR (I) Antimelanogenesis effect on B16 melanoma cells (II)	63 μM (I) 171 μM (II)	[21]

(continued)

Table 2 List of TYR inhibiting peptides from natural or hybrid sources. (*cont'd*)

Compound name	Peptide sequence	Natural source (species)	Claimed activity	IC_{50}	References
Pseudostellarins D	Cyclo[GGYPLIL]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR (I) Antimelanogenesis effect on B16 melanoma cells (II)	100 μM (I) 49 μM (II)	[21]
Pseudostellarins E	Cyclo[GPPLGPVIF]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR	175 μM	[21]
Pseudostellarins F	Cyclo[GGYLPPLS]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR	50 μM	[21]
Pseudostellarins G	Cyclo[PFSFGPLA]	*Pseudostellaria heterophylla* (plants)	Inhibition of mushrooms TYR (I) Antimelanogenesis effect on B16 melanoma cells (II)	75 μM (I) 102 μM (II)	[21]
P3	RADSRADC	Unknown	Inhibition of mushrooms TYR	123 μM	[25]
P4	YRSRKYSSWY	Unknown	Inhibition of mushrooms TYR (I) Inhibition of mushrooms TYR monophenolase (II)	40 μM (I) 123 μM (II)	[25,27]

TH10	MRSRERSSWY	*Oryza sativa* (plants)	Inhibition of mushrooms TYR (monophenolase)	102 μM	[27]
CT-1	HGGEGGRPY	*Oryza sativa* (plants)	Inhibition of mushrooms TYR	Unknown	[28]
CT-2	LQPSHY	*Oryza sativa* (plants)	Inhibition of mushrooms TYR (I) Inhibition of melanin production on melanoma cells (II)	156 μM (I) 500 μM (II)	[28]
CT-3	HPTSEV	*Oryza sativa* (plants)	Inhibition of mushrooms TYR	Unknown	[28]
RBAlbH fraction 1	Mixture of peptides composed of the recurring sequence SSEYYGGEGSSSE-QGYYGEG	*Oryza sativa* (plants)	Inhibition of mushrooms TYR (I) Copper-chelating activity (II)	1.31 mg/mL (I) 0.62 mg/mL (II)	[30]
Undefined	APLRVYVE	β-Lactoglobulin, whey protein (animals)	On-membrane inhibition of TYR	Unknown	[31]

(continued)

Table 2 List of TYR inhibiting peptides from natural or hybrid sources. (*cont'd*)

Compound name	Peptide sequence	Natural source (species)	Claimed activity	IC_{50}	References
Undefined	ISLLDAQS	β-Lactoglobulin, whey protein (animals)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	VSLLLVGI	α-Lactalbumin, whey protein (animals)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	MMSFVSLL	α-Lactalbumin, whey protein (animals)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	ASVSVSFG	β-Conglycinin, wheat protein (plant)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	MKTFLILV	Gliadin, wheat protein (plants)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	LILVLLAI	Gliadin, wheat protein (plants)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	SVNVHSSL	Ovalbumin, egg white (animals)	On-membrane inhibition of TYR	Unknown	[31]

Undefined	MVLVNAIV	Ovalbumin, egg white (animals)	On-membrane inhibition of TYR	Unknown	[31]
Undefined	IAIMSALA	Ovalbumin, egg white (animals)	On-membrane inhibition of TYR	Unknown	[31]
Silk sericin hydrolysates	Unknown	Wastewater material from cocoons (animals)	Inhibition of mushrooms TYR (I) Ferrous-ion-chelating activity (II)	8.71 mg/mL (I) 0.128 mg/mL (II)	[30,32]
EF-5	ECGYF	*Vigna* (plants)	Inhibition of mushrooms TYR	0.46 mM	[38]
F1-a	NYRRE	*Chaenomeles speciose* (plants)	Inhibition of mushrooms TYR	8.69 mg/mL	[40]
F1-b	RHAKF	*Chaenomeles speciose* (plants)	Inhibition of mushrooms TYR	1.15 mg/mL	[40]
< 3 kDa	Unknown	*Salvia hispanica* (plants)	Inhibition of mushrooms TYR	0.66 mg/mL	[42]
DWMPHs-IV	Unknown	*Juglans regia* (plants)	Inhibition of mushrooms TYR monophenolase (I) Inhibition of mushrooms TYR diphenolase (II)	3.52 mg/mL (I) 2.65 mg/mL (II)	[43]

(continued)

Table 2 List of TYR inhibiting peptides from natural or hybrid sources. (*cont'd*)

Compound name	Peptide sequence	Natural source (species)	Claimed activity	IC_{50}	References
F2	Unknown	*Juglans regia* (plants)	Inhibition of mushrooms TYR monophenolase (I) Inhibition of mushrooms TYR diphenolase (II)	1.42 mg/mL (I) 1.8 mg/mL (II)	[43]
Undefined	FPY	*Juglans regia* (plants)	Inhibition of mushrooms TYR monophenolase (I) Inhibition of mushrooms TYR diphenolase (II)	1.11 mM (I) 3.22 mM (II)	[43]
Silk sericin	Unknown	*Bombyx mori cocoons* (animals)	Inhibition of mushrooms TYR	Unknown	[44]
JPC	Unknown	*Rhopilema esculentum* (animals)	Inhibition of melanin production on melanoma cells (I) Copper-chelating activity (II)	78.2 μg/mL (I) 88.7 μg/mL (II)	[46]
MSCP	Unknown	*Chanos chanos* (animals)	Inhibition of mushrooms TYR (I) Inhibition of TYR and melanin synthesis in melanoma cells (II)	752.4 μg/mL (I) 887.1 μg/mL (II)	[49]
LMW-GH	Unknown	*Stichopus japonicus*	Inhibition of mushrooms TYR	Unknown	[51]

β-Lactoglobulin	Protein Data Bank code: 1BEB	Whey Proteins (animals)	Density of cell blot in cultured melanocytes	Unknown	[52]
Undefined	MHIRL	Whey Proteins (animals)	Inhibition of mushrooms TYR	83 μM	[55,58]
Undefined	HIRL	Whey Proteins (animals)	Inhibition of mushrooms TYR	70 μM	[55,58]
Undefined	MGRY	*Pavlova lutheri* (bacteria)	Reduction of melanin in B16F10 cells Reduction of melanogenesis-related proteins expression	Unknown	[56]
αS-CN hydrolysates	Unknown	*Camelus dromedarius* (animals)	Inhibition of mushrooms TYR	Unknown	[57]
Phosvitin	Unknown	Egg yolk (animals)	Inhibition of mushrooms TYR (I) Inhibition of TYR and melanin synthesis in melanoma cells (II)	≈ 50 μg/mL (II)	[60]
Compound n.3	ILELPFASGDLLML	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]
Compound n.6	GYSLGNWVC-AAK	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]

(continued)

Table 2 List of TYR inhibiting peptides from natural or hybrid sources. (*cont'd*)

Compound name	Peptide sequence	Natural source (species)	Claimed activity	IC_{50}	References
Compound n.10	YFGYTGALRCLV	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]
Compound n.11	HIATNAVLFFGR	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]
Compound n.13	FMMFESQNKDL-LFK	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]
Compound n.18	SGALHCLK	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]
Compound n.21	YFGYTGALR	Egg white proteins (animals)	Predicted monophenolase and diphenolase inhibition	Unknown	[63]
Pt-5	Unknown	Zebrafish (animals)	Inhibition of mushrooms TYR, TRP-1, TRP-2, and MITF expression in melanoma cells	Unknown	[67]
Leucrocin I	NGVQPKY	*Crocodylus siamensis* (animals)	Inhibition of mushrooms TYR	>200 μM	[69]
TILI-1	NGVQPKC	Modified from Leucrocin I	Inhibition of mushrooms TYR	132 μM	[69]
TILI-2	CNGVQPK	Modified from Leucrocin I	Inhibition of mushrooms TYR	113 μM	[69]

As a last example of peptides deriving from milk protein, Addar et al. reported the effect of trypsin and chymotrypsin on αS-casein (αS-CN) collected from *Camelus dromedarius* milk and its hydrolytic fractions that were evaluated for their antioxidant, TYR and urease inhibitory activities. αS-CN and its hydrolysate fractions classified depending on their different molecular weight were tested at 200 μg/mL, and the results showed that low molecular weight fractions obtained from chymotrypsin hydrolysis had better TYR inhibitory effect than the ones at higher molecular weight (>10 kDa) and αS-CN [57]. The highest activity found for chymotrypsin hydrolysates suggests the importance of hydrophobic and aromatic amino acids at the C-terminus position of the peptide for the TYR inhibitory activity, as reported by Schurink et al. [31].

3.4.5 Honey

Tyrosinase inhibiting peptides of approximately 600 Da were also found in honey, however, their sequences were not described and the information regarding their activity were relatively poor and no further investigated [59].

3.4.6 Egg

In 2012 a Korean research group demonstrated the anti-melanogenesis activity of phosvitin [60]. Phosvitin is a phosphoglycoprotein present in egg yolk with specific amino acid composition comprised of 50% serine, 90% of which are phosphorylated [61] that makes this protein a strong metal chelator agent. Additionally, C-terminal and N-terminal of this protein are composed of hydrophobic amino acids that contribute to TYR active site binding [62]. Phosvitin activity was evaluated by the dopachrome method using mushroom tyrosinase, but also in vitro on B16F10 (melanoma cells) by measuring tyrosinase activity and intracellular melanin content. In these experiments, phosvitin resulted capable of inhibiting mushroom tyrosinase, B16F10 tyrosinase expression and decreasing melanin content, even if in a lower manner in comparison with ascorbic acid, that is one of the gold standards. In particular, it was demonstrated that phosvitin not only interferes with the first phase of eumelanogenesis pathway, but also with the second phase by inhibiting MITF expression.

Indeed, in another study the potential anti-tyrosinases activity of egg white proteins fragments obtained by enzymatic digestion was evaluated [63]. The inhibitory effect was evaluated in terms of monophenolase and diphenolase inhibitory activity. From this study emerged that seven sequences deriving from different egg proteins (including e.g., ovalbumin,

ovotransferrin, ovomucoid, ovalbumin-related proteins) could inhibit up to 45.9% monophenolase and up to 48.1% diphenolase, being considered as potential tyrosinase inhibitory peptides. In particular these bioactive peptides were: ILELPFASGDLLML, GYSLGNWVCAAK, YFGYTGALRCLV, HIATNAVLFFGR, FMMFESQNKDLLFK, SGALHCLK and YFGYTGALR [63].

Phosvitin-derived peptides with anti-TYR activity were also found in zebrafishes' eggs. In particular, in light of the antimicrobial immunomodulating properties and antioxidant activity exhibited by phosvitin-derived peptide Pt5 (encompassing the 55C-terminal amino acids of phosvitin) [64–66], Liu et al. conducted a study on the possible involvement of peptide Pt5 in the melanogenesis processes [67]. Peptide Pt5 was shown to inhibit TYR in vitro (dopachrome method) with inhibitory rates of approximately 3.0%, 7.7%, 12.0%, and 16.0% at 5, 10, 50, and 100 μg/mL, respectively. Additionally, Pt5 also inhibits TYR activity and melanin biosynthesis in melanoma cells (B16F10) by reducing TYR, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2), and microphthalmia-associated transcription factor (MITF) levels in cells [67].

3.4.7 Blood

A very recent example of a well characterized peptide deriving from animal source with TYR inhibitory effect, is Leucrocin I (NGVQPKY) originating from *Crocodylus siamensis* (crocodile) white blood cell extract, originally described for its anti-microbial activity [68,69]. In fact, this peptide has a C-terminal tyrosine, a characteristic that can confer tyrosinase inhibitory effect, and this evidence boosted a Thai research group to evaluate its possible implication in the TYR inhibitory processes. Moreover, to understand structure-activity relations, Leucrocin I was rationally modified, and two new peptides were obtained: Tyrosinase Inhibitor Leucrocin I-1 and -2, TILI-1 (NGVQPKC), and TILI-2 (CNGVQPK) respectively. Their efficacy and mechanism were evaluated, surprisingly indicating that the change in the tyrosine of Leucrocin I to cysteine (which led to the TILI-1 sequence) could enhance the inhibitory activity. Additionally, the N-terminal cysteine (TILI-2) showed a higher contribution to the TYR inhibitory activity than the C-terminal one (TILI-1). This effect was depicted both for mushrooms TYR inhibition, and for the reduction in melanin content exhibited when used as treatment in melanoma cells (B16). However, all the tested peptides were relatively weak inhibitors of TYR if compared to other well-known inhibitors, such as kojic acid (IC_{50} = 26 μM) [69].

3.5 Anti-tyrosinase peptides designed from hybrid technologies

Bioactive peptides for different applications can be also designed starting from *in silico* screening of virtual libraries deriving from natural or artificial sources. An example of this application to identify crucial complementary functional groups essential for mushroom TYR inhibition is the study reported by Hsiao et al. [70]. These authors screened compounds reported in a variety of literature references and matched them to find the perfect ligand-based pharmacophore. Among the best 10 molecules found, compound A5 (structure reported in Fig. 4) displayed better results due to the presence of two main region, the first one called Core1, with a tyrosine-like structure and the second one, Core2, similar to a coumarin derivative.

Core1 can act as hydrogen-bond donor, and it can form hydrophobic interactions, while Core2 behave as hydrogen-bond acceptor and can interact through hydrophobic interactions with TYR. Keeping in mind the features of A5, the peptide KFY, with complementary properties, has been identify and tested, exhibiting potent TYR inhibitory activity. Starting from KFY and keeping in mind the constrain of tyrosine in the C-terminal position and the biophysical properties of lysine and phenylalanine, a set of different tripeptides were synthetized. Among them two different peptides CRY and RCY showed considerably high potency in inhibiting tyrosinase activity. The results highlight the necessity of having an arginine residue, both in the N-terminus or in the middle position of the peptide to increase the tyrosine-peptide interaction. CRY particularly showed impressive tyrosinase inhibitory activity, with an IC_{50} value of 6.16 μM, 14-fold lower than kojic acid and 160 times lower than arbutin, a well-known TYR inhibitor. The molecular docking analysis reveal that CRY peptide has features comparable to the model identified from docking studies, forming electrostatic and hydrophobic interactions with glutamic acid and valine residues in the active site of the enzyme. The C-terminal tyrosine instead, is

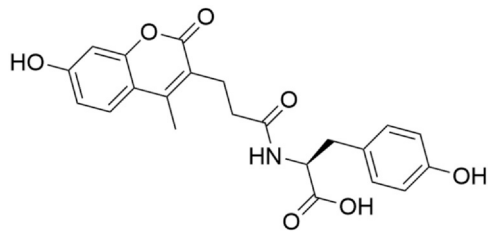


Fig. 4 Structure of A5.

responsible of the establishment of hydrophobic interactions with tryptophan and methionine residues of TYR. Peptide RCY does not display the same interactions of CRY, nor the same fitting inside the enzyme's binding pocket, however its inhibitory potency can be attributed to the presence of cysteine in the C-terminus. Indeed, both tripeptides, possess a cysteine residue close to the copper ions in the active site of mushroom TYR, that thanks to the presence of a thiol group, can act as copper ion chelator.

A second hybrid approach is the use of phage display that represents a potent method for discovering and modifying polypeptides possessing desired functionalities. By merging DNA fragments encoding peptides with genes coding for bacteriophage coat proteins, the resulting fused genes become integrated into phage particles. Subsequently, these particles prominently display the sought-after polypeptides on their exterior surfaces and can be used in screening processes.

Two examples of the application of phage display to the discovery of new peptide TYR inhibitors, are reported by Lee et al. [71], and Nie et al. [72].

In the first example, on the basis of a crystal structure of TYR with a caddie protein in which a short sequence (VSHY) of interaction was shown, the authors exploited the phage display technique to obtain four libraries of tetrapeptides ($CX_1X_2X_3$, $X_3X_2X_1C$, $YX_1X_2X_3$, and $X_3X_2X_1Y$ where C stands for Cys residue and Y stands for non-Cys residue) and screen for new peptide TYR inhibitors. Among these libraries, N-terminal cysteine-containing tetrapeptides showed the most potent tyrosinase-inhibitory abilities, whereas CRVI tetrapeptide exhibited the strongest tyrosinase-inhibitory potency ($IC_{50} = 2.7\ \mu M$), meaning that the N-terminal positioning of the Cys residue is important, but also that the sulfur atom of the thiol group in the cysteine residue is important for copper coordination, as the replacement with Ser negatively affects the potency of the derived peptides [71].

In the second example, indeed, the use of the phage display techniques brought to a novel anti-TYR heptapeptide, whose sequence is IQSHPFF, that was shown to be active both in the mono- and in the diphenolase reactions, with IC_{50} of 1.7 and 4.0 mM, respectively. The authors also reported a kinetic analysis that suggested a competitive and reversible TYR inhibition, possibly occurring through His chelation of copper at the TYR active site, with a inhibition constant (K_i) of 0.765 mM [72].

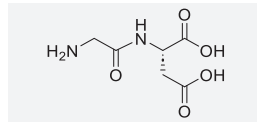
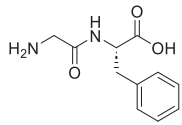
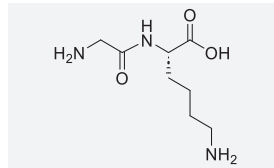
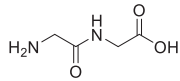
4. Anti-tyrosinase peptides synthetically produced

4.1 Dipeptides and tripeptides

Girelli et al. [73], with the aim of developing non-toxic inhibitors against mushroom TYR, studied the effect on the enzyme of various glycyl-dipeptides using reverse phase HPLC and UV determination of dopachrome formed. The amount of colored product formed is proportional to the enzyme activity and is chromatographically determined both in absence and in presence of the dipeptides. The results obtained evidence that a non-linear reduction of enzyme activity is present for dipeptides GlyAsp, GlyLys, GlyPhe, and GlyGly, as reported in Table 3.

According to the authors, polyphenol oxidase can bind to both the substrate and the inhibitor forming two different complexes, the enzyme-substrate complex and the enzyme inhibitor complex even though also a ternary inactive, non-productive complex formed by the enzyme and both the substrate and the inhibitor. Comparing the inhibition constant of the different dipeptides, it appeared that the dipeptide GlyAsp has the highest

Table 3 Inhibition constant for the dipeptides GlyAsp, GlyPhe, GlyLys and GlyGly express in mM concentration.

Compound:	Structure:	Inhibition constant (mM):
Glycilaspartic Acid (GlyAsp)		0.66
Glycilphenylalanine (GlyPhe)		32.42
Glycillysine (GlyLys)		33.34
Glycilglycine (GlyGly)		33.20

inhibition power, probably due to the interaction of the peptide with the enzyme through the addition al carboxylic group.

Tseng et al. [74] investigate the inhibitory activity of 400 dipeptides against mushroom TYR. The results evidence that cysteine-containing dipeptides abolish the activity of the enzyme, however also dipeptides PD, DY and YK reduce the activity of tyrosinase by 60–70%. The authors also determine the inhibitory potency against polyphenol oxidase of the dipeptides containing cysteine, as reported in Table 4. The IC_{50} values for all the tested compounds ranged from 2.0 to 22.6 μM, with better results for peptides CS, CY, CF, CC, CI, CM and CQ. Peptides with C-terminal cysteine showed lower inhibition efficacy even though some of them such as IC, NC, QC, FC and VC, have comparable IC_{50} values to the N-terminal cysteine containing dipeptides.

To further elucidate how peptides interact with the enzyme, molecular modeling studies were done using CE, CS, CY, CA, and CW and their reverse forms EC, SC, YC, AC, and WC. Molecular modeling studies were performed on both N-terminal and C-terminal cysteine containing

Table 4 IC_{50} values for dipeptides containing N-terminal cysteine or C-terminal cysteine.

	Compound:	Inhibition constant (μM):
	CS	4.5
	CY	3.1
	CF	2.7
N-terminal cysteine	CC	3.2
	CI	4.0
	CM	4.9
	CQ	3.5
	IC	4.5
	NC	5.3
C-terminal cysteine	QC	5.9
	FC	7.9
	VC	8.3

peptides and according to these studies the cysteine residue, independently of its position in the N- or C-terminal side of the peptides, interacts with the binuclear copper ions inside the active site causing the inhibition of the enzyme.

O-dopaquinone scavengers, such as thiol containing compounds are known to react with dopaquinone leading to the formation of thiol-dopa conjugate. Similarly, cysteine containing dipeptides can form dopaquinone conjugates, limiting the formation of dopachrome. For this reason, the dipeptides reported by the authors can hypothetically act in two different ways: (i) interaction with the active sites of TYR and (ii) formation of conjugates with o-dopaquinone with consequent inhibition of dopachrome formation. To confirm this hypothesis Tseng et al. investigated the use of HPLC analysis to determine the formation of the reported peptide-dopa conjugate but no evidence of their presence was found, thus suggesting that their main mechanism of action is the direct inhibition of the enzyme.

Molecular modeling studies were performed also on tyrosine containing peptides, showing that both YK and DY peptides are able to enter deep inside the enzyme binding pocket and form π-π interactions between the phenol ring of tyrosine ad the imidazole ring of the histidine residue of the enzyme. These peptides were also tested in in vitro experiment showing a substrate like inhibitory behavior.

Finally, cytotoxicity assay on cysteine containing peptide YC and CA, with an IC_{50} of 131.6 μM and 9.6 μM respectively, were performed: melanin levels reduction with no significant cytotoxicity was found suggesting a possible use of these peptides in skin whitening products.

Hsiao et al. [70] used molecular docking and pharmacophore modeling to build a pharmacophore model which was subsequently used to screen a vast library of natural compounds with potential activity as mushroom TYR inhibitor as previously reported in the *Anti-tyrosinase peptides designed from hybrid technologies* paragraph.

Luisi et al., as mentioned above**,** studied the effect of sulfur containing amino acids and glutathione, its gamma-oxa-analogue H-Glo(Cys-Gly-OH)-OH, two small synthetic tripeptides Z-tLeu-Asp(OtBu)-Sc and Ac-tLeu-Leu-Asp(OtBu)-Sc (whose structures are reported in Fig. 5), on the activity of TYR [13].

In particular, the two peptides Z-tLeu-Asp(OtBu)-Sc and Ac-tLeu-Leu-Asp(OtBu)-Sc contain a semicarbazone moiety and the natural, non-proteinogenic amino acid tert-leucine, which is characterized by higher

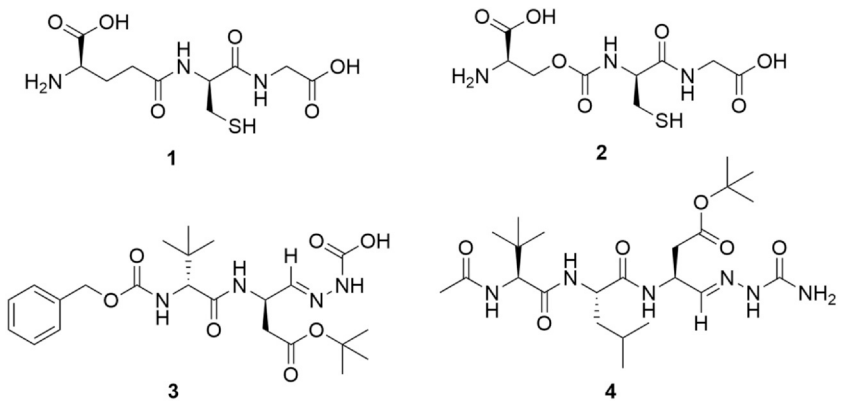


Fig. 5 Structures for glutathione (1), glutathion gamma-oxa-analogue H-Glo(Cys-Gly-OH)-OH (2), peptide Z-tLeu-Asp(OtBu)-Sc (3) and peptide Ac-tLeu-Leu-Asp(OtBu)-Sc (4).

lipophilicity and a bulkier side chain compared to the one of Leucine and Isoleucine. Indeed, this amino acid has been proven to decrease peptide radical formation and increase in vivo stabilization. Results from the dopachrome method are reported in Table 5.

The inhibitory activity of the newly synthetized tripeptides is also accompanied by a good metal reducing power and strong metal chelating activity.

The effect of glutathione on TYR activity has been deeply studied also by Villarama et al. [75]. Glutathione exists in our body in the reduced form GSH which is constantly oxidized and then reduced by the corresponding enzymes. The complex balance among all these species is strongly regulated by internal factors such as hormones and inflammations and external agents like UV lights, chemicals and heat. The mechanism of action of the tripeptide on TYR can be dual: (a) direct inactivation of tyrosinase caused by the binding of the peptide to copper, indeed, TYR is the rate limiting enzyme in the production of melanin; (b) quenching of free radicals and peroxides involved in tyrosinase activation, in particular, GSH can scavenge reactive oxygen species (ROS) generated after UV exposure.

Despite the fact that thiols, and thus GSH, are able to chelate copper ions, present in the active site of TYR, causing the inactivation of the enzyme in a dose dependent manner, TYR is commonly present in an environment rich in cysteine and/or thiol containing compounds without displaying any sign of inhibition. Thus, the reason why glutathione act as TYR inhibitor must be linked to some other phenomena. Indeed, glutathione is involved in TYR transportation, in particular in the inhibition of the transfer of tyrosinase T1 from vesicle to premelanosomes.

Table 5 Tyrosinase inhibitory activity for GSH, its gamma-oxa-analogue and two peptides containing tLeu expressed as milligrams of kojic acid equivalents.

Compound:	Tyrosinase inhibitory activity (mgKAE/g):
GSH	45.60 ± 0.15
H-Glo(Cys-Gly-OH)-OH	216.95 ± 0.17
Z-tLeu-Asp(OtBu)-Sc	163.87 ± 0.90
Ac-tLeu-Leu-Asp(OtBu)-Sc	152.69 ± 1.19

GSH is also involved in the switching process between eumelanin and pheomelanin production. In particular, glutathione interferes with the binding of cysteine to L-dopaquinone causing the induction of cysteinyldopa synthesis and in presence of glutathione-S-transferase it can bind to L-dopaquinone giving glutathionyldopa, a precursor of cystenyldopa causing an increased production of pheomelanin, a yellow-red pigment (as reported in Fig. 6). Thus, glutathione enables the switch from eumelanogenesis to pheaomelanogenesis which results in a lighter skin pigmentation.

Interestingly, as reported by Prota et al., in absence of cysteine or GSH or when there is a decrease in the concentration of these compounds, the hydroxylation of tyrosine followed by dopachrome formation and its transformation into its indole derivative increases, thus increasing eumelanogenesis [76]. The effect of glutathione has also been investigated in a double-blind and placebo-controlled clinical trial of 2014, performed by Watanabe et al. who reported the use of topical lotion containing oxidized glutathione as skin whitening agent [77]. During the 10 weeks of treatment, the skin displayed a progressively lower melanin index, without having marked adverse effects. Although the mechanism of action of GSSG is yet unclear, its efficacy as skin whitening agent could be linked to the presence in the skin of glutathione reductase an enzyme able to rapidly reduce GSSG to GSH.

4.2 Oligopeptides

Mimosine, a non-proteinogenic amino acid reported in Fig. 7, is found in tropical and subtropical plants of genera *Leucaena*. This amino acid has many therapeutic roles, including antitumoral, antiviral and antiproliferative. Therefore, Upadhyay et al. studied as potential TYR inhibitor mimosine-derived tetrapeptides, prepared by solid phase peptide synthesis (SPPS) [78]. The experiments carried out on mushroom tyrosinase highlighted that all the synthetized compounds have better

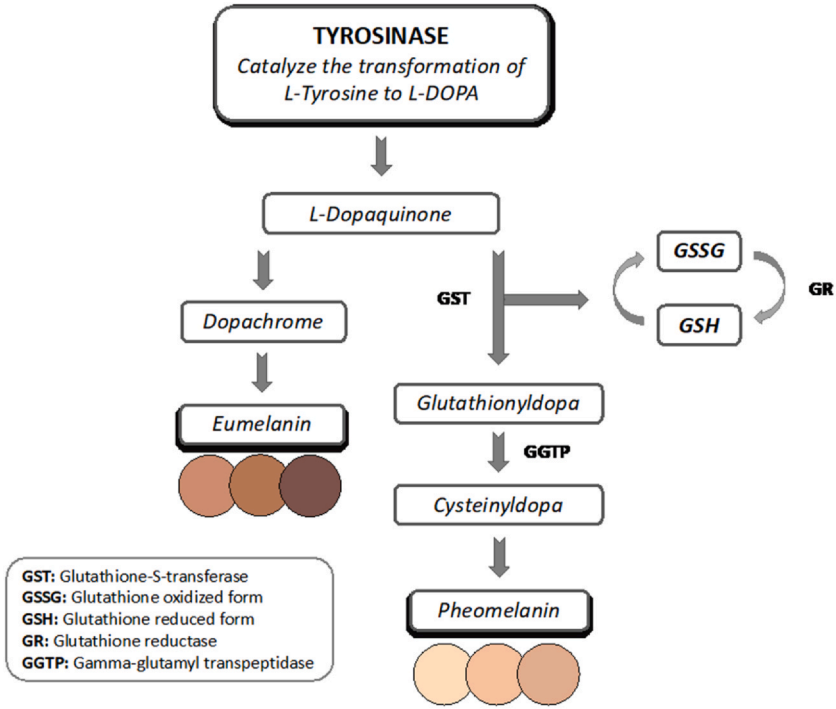


Fig. 6 Scheme of glutathione involvement in melanin synthesis. Highter levels of GHS converted to GSSG led to the synthesis of pheomelanin (red/yellow pigment) in higher percentage than eumelanin (dark-brown/black pigment), resulting in lighter skin tones.

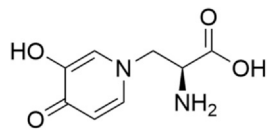


Fig. 7 Structure of mimosine.

inhibitory activity than mimosine itself with an IC_{50} ranging from 5.6 to 36.7 μM. In particular, M-FFY had the best activity amongst the synthetized compounds.

In 2014, Lien and coworkers investigated the effects of acetyl pentapeptides on mushroom tyrosinase as potential inhibitors of melanogenesis [79]. Four different 4n-acetyl-pentapeptides containing Ser, Arg, Phe and Lys were synthetized and tested on B16F10 melanoma cell line. All the compounds were found to be potent TYR inhibitors with IC_{50} below 1 mg/mL, as reported in Table 6.

Table 6 N-Acetyl-pentapeptides inhibitory profile against diphenolase activity of mushroom tyrosinase.

Compound:	IC_{50} (mg/mL):
Ac-P1; Ac-RSRFK	0.75 ± 0.16
Ac-P2; Ac-KSRFR	0.78 ± 0.14
Ac-P3; Ac-KSSFR	0.81 ± 0.02
Ac-P4; Ac-RSRFS	0.29 ± 0.03

Interestingly, the difference in the inhibitory activity of Ac-P1 and Ac-P4 seems to reside in the only different amino acid of the sequence, the serine residue in the C-terminal part of the pentapeptide, suggesting that it plays a critical role on the enzyme activity. Experiments on B16F10 cell line were done using Ac-P4, the most promising compound. The results highlighted that, not only Ac-P4 is not cytotoxic for concentrations ranging from 0.1 to 1.0 mg/mL, but it was also able to reduce the α-MSH induced cellular melanin content in a dose dependent manner, being even more effective than kojic acid. Indeed, IC_{50} for Ac-P4 and kojic acid was 0.09 mM and 1.51 mM, respectively. Taken together, these findings suggest a possible use of Ac-P4 as potential hyperpigmentation reducing agent.

Ubeid et al., after the screening of a proprietary library, identified two promising peptides as tyrosinase inhibitor [25]. The two peptides, P3 and P4 (Seq. P3: RADSRADC as shown in Fig. 8; Seq. P4: YRSRKYSSWY reported in Fig. 9), display enzyme inhibition at very low concentration compared to hydroquinone, with an IC_{50} of 123 μM and 40 μM respectively.

Interestingly, the inhibitory activity of these compounds depends on the substrate and on the presence of cofactors: in presence of L-DOPA as cofactor and L-Tyrosine as substrate, P3 display better inhibitory properties than P4, on the other hand, in presence of only L-tyrosine, P4 gives better results. These findings support the hypothesis of the presence of two different sites on the enzyme, one for the binding of L-DOPA and the other one for the binding of L-tyrosine. Moreover, one of the main advantages of the two peptides is the very low cytotoxicity displayed. While hydroquinone is 100% toxic after 24-hours treatment at 100 μM concentration, P3 does not display cytotoxicity and P4 is only 7% toxic. Furthermore, they both have minimal effects on melanocyte proliferation; thus, they can be potentially used as therapeutic peptides for the treatment of skin

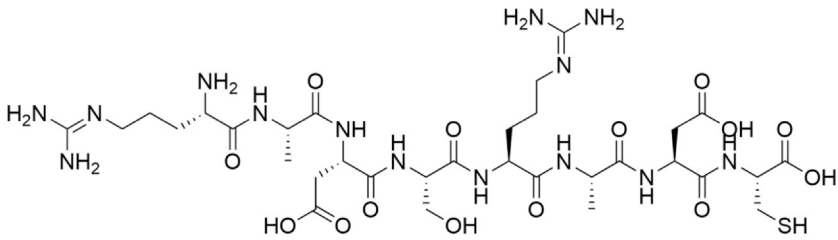


Fig. 8 Structure of peptide P3.

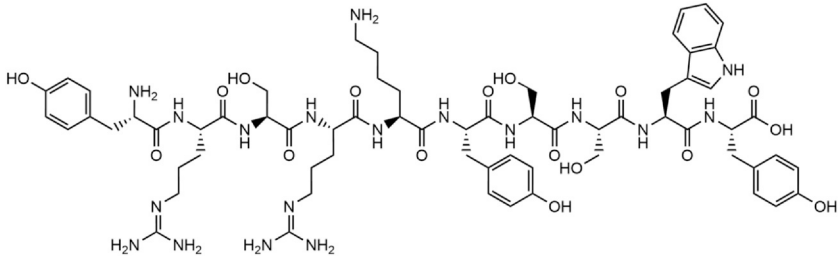


Fig. 9 Structure of peptide P4.

hyperpigmentation. Indeed, P4 is commercialized under the name of Lumixyl ™ a skin-whitening product used to treat melasma [80,81].

In a follow-up study, the same authors described the effect of three octapeptides (Seq. P16: RRWWRRYY, Seq. P17: RRRYWYYR, and Seq. P18: RRYWYWRR, Fig. 10) as tyrosinase inhibitors [82].

The three peptides, which from molecular docking studies, displayed the highest affinity for the TYR catalytic site, have been tested in vitro on human melanocytes, keratinocytes and fibroblast, showing very low cytotoxic effects and revealing a competitive mode of inhibition of the enzyme. The inhibition activity of the peptides was tested both using L-DOPA as substrate and L-tyrosine in presence of L-DOPA as cofactor. As previously reported, these results support the hypothesis of the presence of two catalytic sites as postulated by Hearing et al. [83]. The experiments carried out on primary human melanocytes revealed that the octapeptides are capable of penetrating inside the cells and inhibiting the production of melanin having hypo-pigmenting activity similar to hydroquinone. MTT assays showed minimal toxicity for all the three peptides both after 72 h and 6-days treatments. Interestingly the octapeptides, share the presence of a tryptophan residue that, according to molecular docking simulations, can enter the catalytic site of tyrosinase close to the two copper ions. This

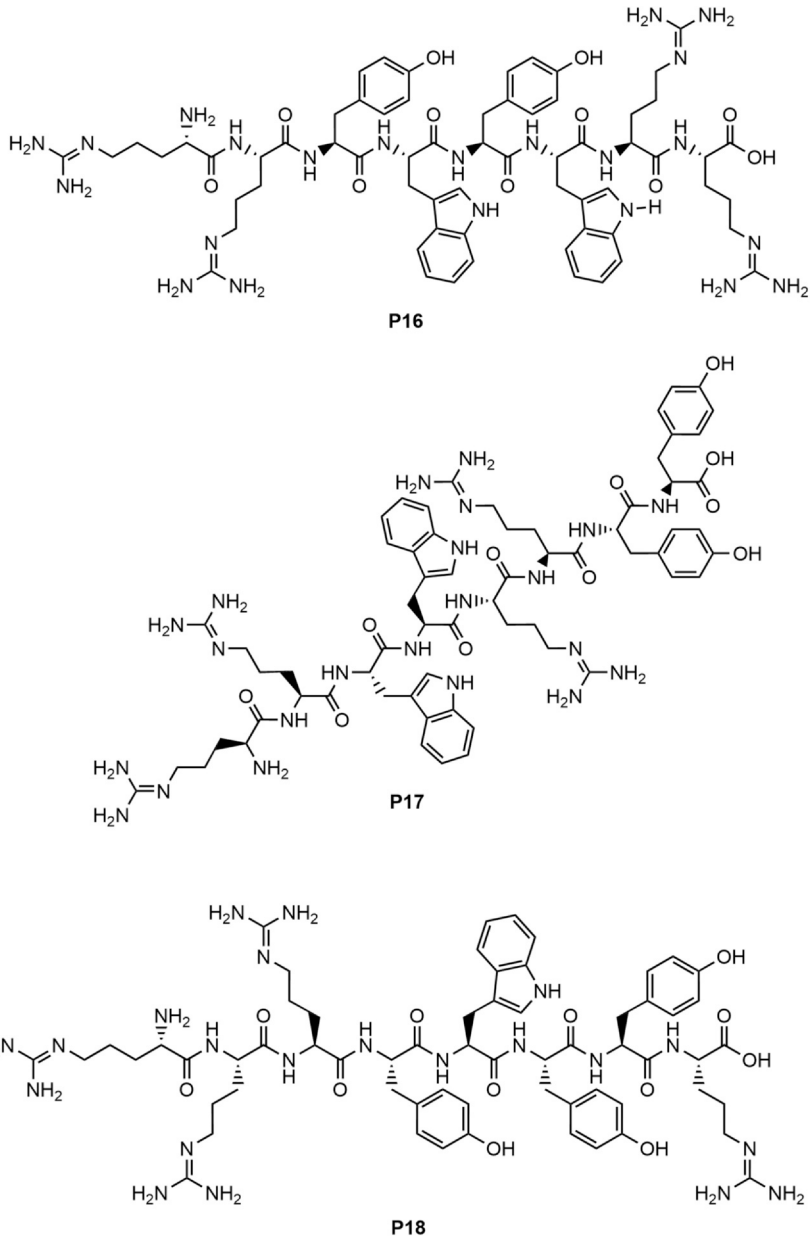


Fig. 10 Structures of peptide P16, P17 and P18.

assumption could be validated by the fact that P17, the only octapeptide with one tryptophan residue has lower inhibitory activity respect to the other two compounds, P16 and P18, which contain two tryptophan residues each. Indeed, the indole ring of tryptophan can form a reversible complex with the copper ion of the catalytic site, which can also interact with the imidazole ring of histidine residues via hydrophobic interactions.

4.3 Peptides conjugated with other chemical moieties

4.3.1 Kojic-acid derivatives

In 2009, Noh and coworkers studied the effect of kojic acid-amino acid conjugates as tyrosinase inhibitors [84]. Kojic acid is employed in many countries as skin whitening agents, however the use in cosmeceutical products has been dramatically reduced due to its low stability during storage and high skin irritability. The new compounds show better activity than kojic acid itself, in particular the ones containing amino acids with aromatic side chains like phenylalanine, tyrosine, tryptophan or histidine. Among them KA-F-NH_2, displays the best tyrosinase inhibitory activity with an IC_{50} of 14.7 μM. Further kinetic studies on the inhibition of L-DOPA oxidation reveal that KA-F-NH_2, can act as a non-competitive inhibitor of mushroom tyrosinase analogously to kojic acid.

The same group also studied the inhibitory activity of tripeptides conjugated with kojic acid [85]. The authors synthetized a library of 22 kojic acid-tripeptides and tested them as inhibitors of TYR comparing the results with the ones previously obtained for kojic acid and kojic acid-tripeptide free acids [86].

Results showed that the inhibitory activity of the conjugated tripeptides were similar to those of the tripeptide-free acids and higher than kojic acid alone. In particular, they investigated the importance of the different residues in the different positions with respect to the kojic acid moiety. Indeed, they find out that peptides having phenylalanine at position KA+1, have the highest inhibitory activity. Substitutions in this position of phenylalanine with hydrophobic amino acids such as tryptophan or tyrosine, create compounds with the best inhibitory activity against TYR, suggesting that this position plays a key role in enzyme inhibition. Fixing the KA+1 and KA+3 positions as phenylalanine and tyrosine the authors studied the effect of the different amino acids in position KA+2 discovering that the amino acid in this position is less relevant for enzyme inhibition. From the combination of all the obtained results the authors selected three different kojic acid-tripeptides free amide with an IC_{50} lower than the

corresponding kojic acid-tripeptides free acid as reported in Table 7. Interestingly, the FWY peptide conjugated with KA both in the free amid or free acid forms, displays the lower IC_{50}.

Two more peptides conjugated with kojic acid with anti-tyrosinase activity where developed by the group of Singh et al. [87]. The two peptides KA-PS and KA-CDPGYIGSR (renamed KA-CR9) were tested on mushroom tyrosinase displaying a dose-dependent inhibitory activity on the enzyme of 82% and 84% respectively. Due to the lower molecular weight of KA-PS conjugate further studies on mouse B16F10 cells and melan-a cells were performed, highlighting that the compound does not display cytotoxicity and retains the same activity of in vitro experiments.

4.3.2 Other moieties

In 2013, Li et al., studied the potential activity as tyrosinase inhibitors of hydroxypyridinone-L-phenylalanine conjugates [88]. Hydroxypyridinone is a kojic acid bioisoster having NH instead of oxygen at position 1 in the pyranone ring. In their work the authors synthetized six different derivatives and tested them as inhibitors for both monophenolase and diphenolase activity of tyrosinase, as displayed in Fig. 11.

The results revealed that amongst all the synthetized compounds 5d and 5e were the only with an IC_{50} lower than the one of kojic acid alone, with values of 19.2 ± 0.8 and $12.6 \pm 0.3\,\mu M$ respectively. Interestingly the inhibitory activity of the compounds increases with increasing hydrophobicity of the compound itself, the only exception being compound 5f where the too high lipophilicity caused a poor water solubility.

Table 7 IC_{50} value for Kojic Acid, Kojic acid free acid conjugated peptides and Kojic acid free amide conjugated peptides.

	Compound:	IC_{50} (μM):
Kojic Acid	KA	94
Free-acid	KA-FWY	1.28
	KA-FHY	4.55
	KA-FRY	5.92
	KA-FWY-NH_2	2.2
Free-amide	KA-FHY-NH_2	2.36
	KA-FRY-NH_2	3.59

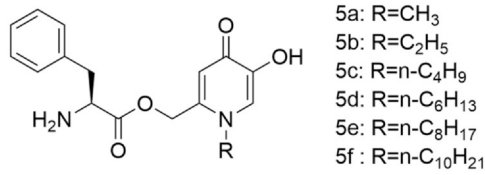


Fig. 11 Structure of hydroxypyridinone-L-phenylalanine conjugates.

Taking into account these findings, the two compounds were also tested on the diphenolase activity of TYR, using L-DOPA as substrate. Even in this case, 5e demonstrated a better inhibitory activity than kojic acid itself, with an IC_{50} of 20 μM. Moreover, from kinetic studies, it was shown that both 5d and 5e molecules were mixed type inhibitors, meaning that they can bind both to the free enzyme and the enzyme-substrate complex. Thus, compound 5e was also tested for its copper chelating ability. 5e can act as bidentate ligand, forming two different complexes with copper ions present in the catalytic site of TYR: CuL^+ and CuL_2. Thanks to spectrophotometric titration, the stability constant, expressed in log scale were calculated for both CuL^+ (log β_1) and CuL_2 (log β_2) exhibiting values of 9.294 ± 0.09 and 15.719 ± 0.11, respectively. Indeed, both log β_1 and log β_2 of 5e were higher than the one of kojic acid (log β_1 = 6.6, log β_2 = 11.7) suggesting a higher affinity of the newly synthetized compound for the enzyme's copper ions.

Given the promising results of Li et al., Zhao and coworkers designed and synthetized hydroxypyridinone- amino acid and hydroxypyridinone-dipeptides and tested them as TYR inhibitors [89]. Among the two different series only compound 6e and 12a, whose structures are reported in Fig. 12, had the strongest monophenolase inhibitory activity with an IC_{50} of 1.95 and 2.79 μM respectively.

Keeping in mind these results the authors also investigated the diphenolase activity of the enzyme using compounds 6e and 12a. In these conditions, compound 6e revealed to be a better inhibitor than compound 12a. however, as mentioned above they can be both regarded as reversible mixed type inhibitors of mushroom tyrosinase. In order to further investigate the inhibitory mechanism of the synthetized derivatives copper chelating reducing and copper chelating ability for compound 6e were determined. From the absorbance measurement at 483 nm (λ for cuprous ions determination), it was possible to confirm that compound 6e had a stronger copper reducing capability than kojic acid itself. Moreover,

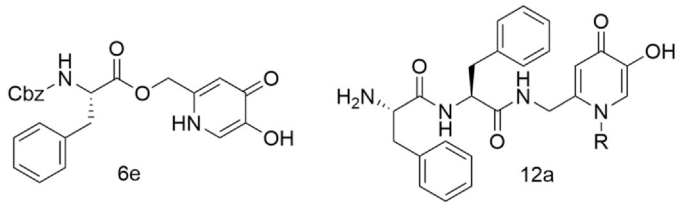


Fig. 12 Structure of hydroxypyridinone-amino acid 6e (Cbz: benzyloxycarbonyl) and hydroxypyridinone-dipeptide 12a.

although the copper chelating ability of compound 6e and kojic acid both increases, increasing the concentration, compound 6e displayed better chelating properties, with values of 91.34 ± 0.28% and 83.34 ± 0.29% at 0.952 mM, at pH 5.0 and 7.4 respectively, while kojic acid at the same values of pH has constants of 82.64 ± 0.25% and 74.35 ± 0.27% at 0.952 mM. Probably compound 6e can entre in the active site of tyrosinase and compete with histidine for the binding of copper ions, causing a loss of enzymatic activity.

Considering that hydroxyphenolic acids and their esters can act as tyrosinase inhibitors and as antioxidant, Noh et al. decided to conjugate protocatechuic acid (PA), a-resocylic acid, gentisic acid (GT) and gallic acid (GA) with aromatic amino acids [90]. The different compounds were tested as inhibitor of TYR using tyrosine as substrate. Amongst them PA-F-NH_2, PA-W-NH_2 and PA-Y-NH_2 display promising results, while the ones conjugated with RA, GT and GA hardly have any inhibitory activity on the enzyme. The IC_{50} values, for both monophenolase and diphenolase activities, of the conjugated compound are listed in Table 8.

All the three compounds can be regarded as reversible competitive-uncompetitive mixed-I type inhibitors. Since PA is a good chelator for copper ions, the mechanism of action of these conjugated molecules can be double. On one hand PA can chelate the copper ions present in the active site of tyrosinase enzyme, on the other hand the aromatic structure of the inhibitor could favor the formation of hydrophobic interactions between the inhibitor itself and the hydrophobic side chains of TYR, thus preventing the entrance of the substrate in the active site.

Recently, peptide fragments derived from β-lactoglobulin, a milk protein, have been reported to be TYR inhibitors [91]. In a work of 2018, Yang and coworkers reported the synthesis of conjugated molecules containing these peptides and caffeic acid (CA), a catechol containing compound with antioxidant activity and which have been proven in a previous

Table 8 IC_{50} value for monophenolase and diphenolase activity of the peptides conjugated with protocatechuic acid.

Compound:	IC_{50} (mM) monophenolase:	IC_{50} (mM) diphenolase:
PA-F-NH_2	0.01 ± 0.001	0.64 ± 0.02
PA-W-NH_2	0.01 ± 0.002	0.56 ± 0.01
PA-Y-NH_2	0.01 ± 0.005	0.66 ± 0.01

study to be able to inhibit α-MSH induced melanin synthesis when in the form of phenethyl ester [92,93]. Using solid phase peptide synthesis, the authors were able to successfully conjugate CA(acetonide)-OH with tetra- and tri-peptides (structure reported in Fig. 13).

The compounds reported in Table 9, were tested against tyrosinase and their inhibitory activity measured using the dopachrome method. To compare the different activities, peptide conjugated with caffeic acids were tested in parallel to the peptides deprived of the caffeic acid moiety.

Results evidence that all the CA-derived peptides were more active against TYR than the unconjugated peptides, suggesting that CA improve the binding of the peptides to the enzyme. Amongst them, CA-MHIR was able to effectively inhibit the enzyme even at a concentration of 50 μM, displaying even better results than Kojic Acid with an IC_{50} of 47.9 μM.

The inhibition mechanism of CA-MHIR (Fig. 14) was investigated revealing a non-competitive type of inhibition. Probably the conjugated peptide can bind outside the active site of the enzyme causing a deformation in the three-dimensional structure of the protein.

Park et al. investigated the effect of caffeic acid and coumaric acid-conjugated peptides in human melanocytes, which in recent studies have been shown to serve as skin-whitening agents inhibiting melanin production more efficiently than KA or arbutin [94]. The group synthetized, using SPPS, peptides containing the -AR- sequence which in previous studies have been proven to be more effective in inhibiting melanogenesis [95]. The peptides were than conjugated to caffeic acid or coumaric acid through a mini-PEG or Gly-Gly-Gly linker to improve the physical properties of the compound and increase its efficacy in inhibiting melanin production, and subsequently tested to measure cell viability, tyrosinase activity and α-MSH-induced melanin content. No signs of cytotoxicity were shown for the different caffeic acid and coumaric acid-conjugated peptides even at 1 mM concentrations. Moreover, the experiment on cells

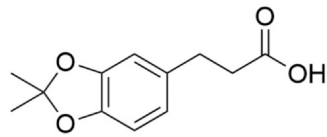


Fig. 13 Structure of caffeic acid acetonide.

Table 9 IC_{50} values for β-lactoglobulin derived peptides with and without caffeic acid moiety.

Compound:	IC_{50} (mM):
CA-MHIR	47.9
CA-HIR	154.8
CA-HIRL	166.2
KA	201.7
HIRL	218.8
MHIR	257.1
HIR	none

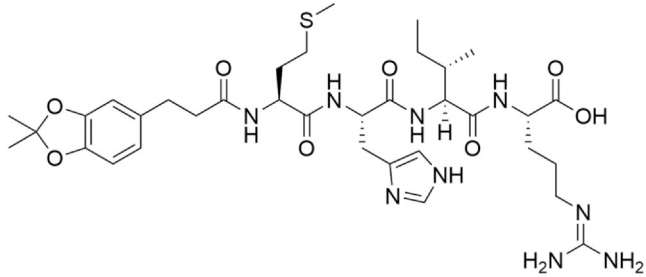


Fig. 14 Structure of CA-MHIR.

treated with α -MSH, to induce melanin production, clearly showed that the conjugated peptides have an inhibitory activity comparable to arbutin, a well-known melanogenesis inhibitor. In particular, four of the synthetized peptides were able to inhibit tyrosinase activity, with coumaric acid-GGG-ARP being stronger than arbutin and thus opening the possibility of use it in cosmetic products to treat or prevent skin pigmentation disorders.

In a study of 2013, Lee at al. synthetized a coumaroyl dipeptide amide, Coumaric acid-LG-NH_2, using SPPS and tested it as skin whitening agent [96]. The peptide, which exhibit very low cytotoxicity in MTT assay, demonstrated to be able to inhibit TYR with an IC_{50} value of 182.4 μM. In experiments on B16F1 melanoma cell lines, the measurement of melanin content revealed that the peptide can successfully reduce α-MSH induced melanin production.

Ascorbic acid, called also vitamin C, is characterized by high instability towards oxygen light and heat. Moreover, it cannot penetrate into cell due to its high hydrophilicity. Thus, especially in the cosmetic industry, there has been the necessity to reduce its rapid degradation and, at the same time, increase its permeability. Choi et al. were able to achieve this, by linking ascorbic acid to pentapeptide KTTKS [97]. The compound was tested in in vitro experiment, and its ability to inhibit TYR was measured. The results highlighted that the stabilized ascorbyl pentapeptide (SAP) had comparable inhibitory activity to ascorbic acid at 500 μM and 1000 μM. Moreover, experiments carried out on B16 murine melanoma cells, showed that SAP had a melanogenesis-inhibitory activity more than 10% higher than arbutin. Indeed, SAP displayed more promising results than ascorbic acid in in vivo tyrosinase inhibition at 1 mM concentration.

Recently, a new study on tripeptides conjugated with thiosemicarbazones have been reported as potential TYR inhibitors [98,99]. Ledwoń et al. in their work investigated the inhibitory effect on diphenolase activity of thiosemicarbazone peptide-conjugated using in vitro experiments on mushroom TYR and on B16F0 cell line [100]. A set of 15 different compounds have been investigated, as reported in Table 10.

Thiosemicarbazones (TSC1, TSC2, and TSC3, Fig. 15) have been designed in order to have the free carboxylic group, responsible of peptide conjugation, distant from the thiourea group involved in the binding to copper ions in the active site of the enzyme.

Tripeptides were selected on the basis of the most active sequences reported in the literature, starting for some key requirements, i.e., the presence of a N-terminal phenylalanine residue, and of aromatic and hydrophobic amino acid (Phe, Tyr, Trp). TYR inhibition assay shows that all TSC and tripeptide-conjugates have a dose dependent inhibitory activity, even if some differences are present such as the lowest ability of TSC2, the bulkiest thiosemicarbazone, to inhibit TYR even at 150 mM concentration. Interestingly, the peptides deprived from the thiosemicarbazone moiety are not active against the enzyme. Indeed, TSC1 and

Table 10 Results of melanin production in B16F0 cell line.

Compound:	IC_{50} of melanin production:
Kojic Acid	121.0
TSC1	101.5
TSC2	19.8
TSC3	126.4
Ac-FFY-OH	245.7
Ac-FYY-OH	174.7
Ac-FWY-OH	140.9
TSC1-FFY-OH	138.4
TSC1-FYY-OH	158.5
TSC1-FWY-OH	150.6
TSC2-FFY-OH	146.1
TSC2-FYY-OH	n/a
TSC2-FWY-OH	**89.9**
TSC3-FFY-OH	162.4
TSC3-FYY-OH	144.9
TSC3-FWY-OH	**67.1**

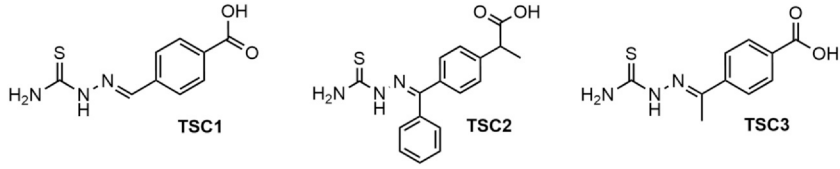


Fig. 15 Structures of TSC1, TSC2, and TSC3.

TSC2 as well as TSC2-FWY-OH and TSC3-FWY-OH are the only compounds displaying stronger potency as melanogenesis inhibitor than kojic acid. On the other hand, melanogenesis inhibition gave different results, highlighting that TSC2 is the most potent melanogenesis inhibitor

among all the tested compounds. This observation, coupled to the previous results, suggest that TSC2 probably interfere with melanogenesis by acting on other key enzymes involved in melanin biosynthesis.

5. Peptidomimetics

The use of peptidomimetic compounds has been widely explored in the past years, since these molecules are expected to feature enhanced stability and bioavailability for in vivo applications, as compared to small peptides. Indeed, although peptides present numerous advantages, they also display some challenges which include their stability and availability, since their half-lives are highly influenced by external conditions such as pH, temperatures and interactions with enzymes. Moreover, their availability also depends from their concentration and distributions/accumulation; e.g., peptides with high molecular weight can present solubility problems at high concentrations, and skin permeability problems [4]. Therefore, to reduce some of the issues connected to the use of peptides, peptidomimetics have been studied and synthetized. In a study of 2007, triazole-containing analogues of a naturally occurring tyrosinase inhibitor have been synthetized and tested against the enzyme [101].

L. helveticus, a gram-positive bacteria, can produce Cyclo[Pro-Tyr-Pro-Val] (Fig. 16, [16]) a small cyclic peptide with TYR inhibitory activity.

However, its synthesis is complex due to the ring closure step. For this reason, taking advantage of the *click chemistry* strategies, analogues containing the triazolyl bridge as amide bond isoster, have been synthetized via Cu(I)-alkyne-azide cycloaddition. Interestingly, the 2022 Nobel Prize in Chemistry recognized the development of *click chemistry* and biorthogonal chemistry to

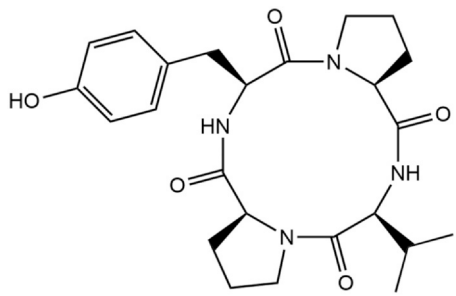


Fig. 16 Structure of cyclo-[Pro-Tyr-Pro-Val].

Table 11 IC_{50} values for the natural compound and the triazolyl-containing peptidomimetics.

Compounds:	IC_{50} (mM):
cyclo-[Pro-Tyr-Pro-Val]	1.5
Triazole analogue 2	0.6
Triazole analogue 3	0.5
Triazole analogue 4	1.6

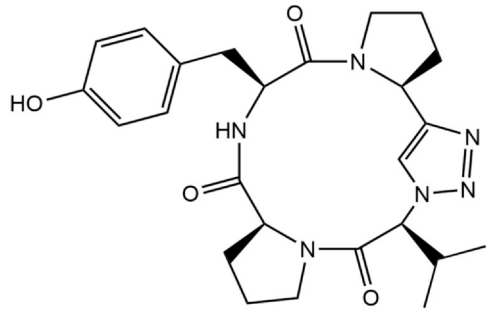


Fig. 17 Structure of the triazole analogue 2.

K. Barry Sharpless, Morten Meldal, and Carolyn R. Bertozzi. The three new peptidomimetics have been tested against mushroom tyrosinase using spectrophotometric assay, displaying the results reported in Table 11.

Surprisingly, not only the analogues retain the inhibitory activity, but triazole analogue 2 (structure reported in Fig. 17) and triazole analogue 3 have better performances compared to the reference compound. Taken together, these results suggest that these triazolyl-containing peptidomimetics, which are readily accessible via click chemistry, could be introduced inside difficult-to-obtain natural compounds, retaining the natural biological activity.

6. Conclusions

This chapter describes the variety of peptides proposed as TIPs, starting from the natural ones, which can be isolated from several sources (i.e., bacteria, mushrooms, plants or animals, as reported in Table 2), taking

into account hybrid approaches in which in silico screening strategies are employed, and ending with totally synthetic peptides.

Naturally derived peptides in some cases are considered a sustainable choice in which by-products can be recycled, however, despite the huge variety of peptides deriving from natural sources, a consideration on their safety emerges spontaneously. In fact, in most of the examples the term "peptide" is used to describe protein hydrolysates, which by definition are mixtures of many unknown peptides, but not as single identity. Nevertheless, in some cases peptide characterization from natural extract has been rigorously carried out, leading to the rational purification of peptide sequences whose activity was deeply investigated [25,27,28,38,40,43,58,69].

Indeed, the advantage of fully synthetic approaches is to obtain products that can be easily purified and fully characterized as single molecules that, upon proper biological assays, may result in safe TIPs.

The number of peptides launched in the pharmaceutical market is getting increased through the years, therefore the demand for optimized methodologies to synthetize peptides consequently increases [102,103]. Undoubtedly, the new frontiers in peptide synthesis are the application of greener reagents and solvents to comply nowadays sustainability perspectives [104–106].

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