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Design and synthesis of aminoester heterodimers containing flavone or chromone moieties as modulators of P-glycoprotein-based multidrug resistance (MDR)

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ABSTRACT

In this study, a new series of heterodimers was synthesized. These derivatives are *N*,*N*-bis(alkanol)amine aryl esters or *N*,*N*-bis(ethoxyethanol)amine aryl esters carrying a methoxylated aryl residue combined with a flavone or chromone moiety. The new compounds were studied to evaluate their P-gp modulating activity on a multidrug-resistant leukemia cell line. Some of the new compounds show a good MDR reversing activity; interestingly this new series of compounds does not comply with the structure-activity relationships (SAR) outlined by previously synthesized analogs carrying different aromatic moieties. In the case of the compounds described in this paper, activity is linked to different features, in particular the characteristics of the spacer, which seem to be critical for the interaction with the pump. This fact indicates that the presence of a flavone or chromone residue influences the SAR of these series of products, and that flexible molecules can find different productive binding modes with the P-gp recognition site. These results support the synthesis of new compounds that might be useful leads for the development of drugs to control P-gp-dependent MDR.

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1. Introduction

Multidrug resistance (MDR) is the major cause of the failure in antineoplastic treatment, and it is mainly due to the overexpression of some transmembrane proteins belonging to the ABC (ATP Binding Cassette) transporters family.[1] This family includes structurally related membrane proteins that are made up of two domains: the nucleotide binding domain (NBD) and the transmembrane spanning domain (TMD).[2] ATP is hydrolysed in the NBD and the resulting energy is used by the TMD to translocate substances through the membrane by conformational changes.[3]

P-gp is the most studied ABC transporter.[4] It is physiologically present in cells of several human tissues where it plays an important role by regulating the permeability of biological membranes, the secretion of physiological lipophilic molecules and the extrusion of xenobiotics,[5] but it is also the first efflux transporter discovered to be involved in drug resistance.[6] In fact, P-gp is overexpressed in many cancer cells as a result of chemotherapy treatment causing an acquired resistance to a variety of anticancer drugs.[7,8] Moreover, in addition to the decrease in the intracellular concentration of chemotherapeutic agents, P-gp might affect resistance also through other mechanism: P-gp overexpressing cells resulted less sensitive to caspase-dependent apoptosis induced by a range of different stimuli. [6]

Inhibition of the functions of P-gp is considered a potential strategy for circumventing MDR. The use of a P-gp modulator in co-administration with the classic antineoplastic drugs could restore drug sensitivity of tumor cells in cancer therapy[9–11]; many compounds have been identified for this purpose, nevertheless few of them have reached clinical trials.[12] The main problem associated with the development of effective P-gp-mediated MDR inhibitors seems due to their influence on the physiological role of P-gp, poor specificity, low affinity for the binding site, and interference with the pharmacokinetics of the associated chemotherapeutic agent.[13]

Despite some doubts related to the future of P-gp inhibitors as a therapeutic approach to overcome MDR, the search for new P-gp-interacting compounds is still of interest, not only in field of cancer resistance.[14] In fact, other potential uses of these agents are emerging, such as that of enhancing drug penetration through biologically protective barriers, such as the blood-brain and blood-cerebrospinal fluid.[15,16] As an example, the overexpression of P-gp has been related to the occurrence of MDR in CNS diseases, such as pharmacoresistant schizophrenia, thus reducing the effectiveness of current thera-

Abbreviations: P-gp, P-glycoprotein; SAR, Structure-activity relationships; DOX, Doxorubicin; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimmide hydrochloride; HOBt, 1-Hydroxybenzotriazole hydrate; DMAP, 4-dimethylaminopyridine

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peutic drugs.[17] Moreover, the important role of ABC transporter proteins like P-gp in stem cells has been evidenced.[18] As a consequence, the increasing interest in the functions and mechanism of action of P-gp indicates the need for new potent and selective molecules to be used as pharmacological tools.

Recently, flavonoids were indicated as a promising family of P-gp modulators. Flavonoids are constituents of fruits and vegetables and constitute the main group of polyphenolic compounds present in human diet. These natural compounds have long been associated with a variety of biochemical and pharmacological properties, including antioxidative, anticarcinogenic, anti-inflammatory and antiviral activities.[19] Their daily consumption is estimated to be as high as 1 g, and it is therefore generally accepted that flavonoids are not toxic. In the last 20 years, flavonoids have attracted attention as putative MDR modulating agents, and their interaction with P-gp was described by different researchers.[19–22] Also some analogs, as functionalized chromones were described as highly active compounds as MDR modulators.[23]

In the last decade, some synthetic flavonoid homodimers and heterodimers as a new class of potent, safe and specific P-gp modulators were reported. Synthetic flavonoid dimers with polyethylene glycol (PEG) as linker displayed, in drug-resistant human breast cancer and leukemia cells, a higher P-gp modulating activity, compared to monomers.[24,25] Introduction of an amine group into the PEG linker improved both the aqueous solubility as well as the modulating activity (Chart 1, structure I).[26] SAR evaluation indicated that flavonoid dimers with non-polar and hydrophobic substituents generally showed a higher activity than that of dimers decorated with polar and hydrophilic residues. Best results were obtained when a benzyl group was introduced on the nitrogen.

In recent years we have studied several families of multidrug resistance modulators including *N*,*N*-bis(alkanol)amine aryl esters characterized by the presence of a basic nitrogen atom linked to two different aromatic ester residues by two identical polymethylenic chains of variable length as spacers (Chart 1, structure II).[27,28] These compounds were designed on the bases of the information that the presence of aromatic moieties and of one or more protonable nitrogen atoms is an important property for the P-gp interaction. In fact, although the atomic structure of human P-gp has not yet been elucidated, current knowledge of the interaction site suggest that it is a large, flexible drug binding domain where different molecules can be accommodated in a plurality of binding modes, establishing π-π, ion-π, hydrogen bonds and hydrophobic interactions.[29]

In our design strategy, a high structural flexibility would allow the molecules to choose the most productive binding mode, within the large P-gp recognition site. Actually, this approach provided good results since most of the synthesized compounds showed to be very potent MDR reversers in a human leukemia cell line.[27,28] Interestingly, a similar approach, labeled as "polyvalency", had been used successfully by Sauna and co-workers, who synthesized several homodimers of the natural MDR inhibitor stipiamide.[30]

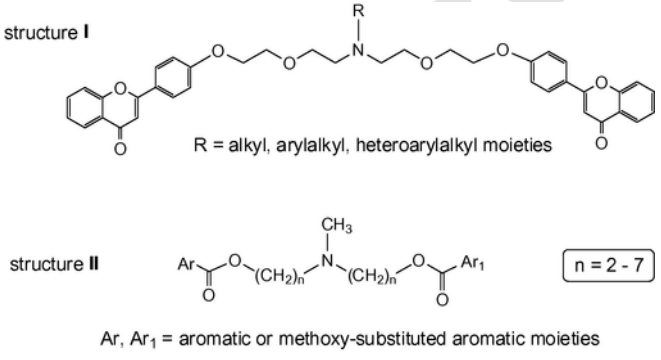

Chart 1. General structures of the lead compounds.

In this study we designed and synthesized a new series of derivatives based on the polyvalency approach. Therefore we synthesized new *N*,*N*-bis(alkanol)amine aryl esters and *N*,*N*-bis(ethoxyethanol)amine aryl esters (**1**−**17**) carrying a methoxylated aryl residue combined with a flavone or chromone moiety. The designed compounds are reported in Chart 2.

Diesters **1**–**10** (Chart 2, structure III) are characterized by the presence of polymethylenic linkers and a *N*-methylated basic portion; the spacers chosen, 3- or 5-methylenes long, were those showing the best results in our previous studies.[27,28] The (*E*)-3-(3,4,5-trimethoxycynnamoyl) aryl moiety was chosen among those that had previously given the best results[27,28] and was always maintained as first ester portion. This moiety was combined with a second ester function carrying different flavonoids: two hydrophilic hydroxychromones (Chart 2, **b** and **d**), two hydrophobic methoxy-substituted chromones (**a** and **c**), and the dihydroxyflavone residue (**e**). Introduction of methoxy groups was designed since these residues often have a positive effect on P-gp modulation. Derivative **11** was synthesized as an example of a poly(ethyleneglycol) analog, which presents two linkers with a length of 5 units. Since *N*-benzyl substitution on non-substituted flavone derivatives gave the best results (Chart 1, structure I) in the series of Chow and Chan,[26] we also synthesized diesters **12**–**17** (Chart 2, structure IV), which are characterized by the presence of a flavone acetic ester and different residues on the nitrogen (methyl, benzyl or hydrogen); in this series both polymethylenic and poly(ethyleneglycol) analogs were obtained, which always show spacers of the same length.

The ability of the synthesized compounds to modulate P-gp was evaluated by the pirarubicin uptake assay on doxorubicin-resistant erythroleukemia K562 cells (K562/DOX cells), and their conformational behavior was investigated by means molecular dynamic simulations.

2. Results and discussion

2.1. Chemistry

The reaction pathways used to synthesize the designed derivatives (**1**−**17**) are reported in Schemes 1–7. All the derivatives were obtained by the esterification of the suitable alcohol monoester with the carboxylic acid carrying the appropriate chromone or flavone residue.

5-Methoxy-4-oxo-4*H*-chromene-2-carboxylic acid **18**, 5-hydroxy-4-oxo-4*H*-chromene-2-carboxylic acid **19**, and 5,7-dihydroxy-4-oxo-4*H*-chromene-2-carboxylic acid **20** were obtained as previously described.[23,31] The dimethoxy analog **23** was prepared as reported in Scheme 1: methylation on position 2 and 4 of 2,4,6-trihydroxyacetophenone gave **21** which was reacted with diethyl oxalate and cyclized under acidic cyclization. Ester **22** was then hydrolyzed obtaining acid **23** after acidification of the obtained solution.

For the synthesis of the dihydroxy flavone acid derivative **26** (Scheme 2), the commercially available flavanone (±) naringenin was alkylated with ethyl bromoacetate to give **24** and oxidized with I_2 in pyridine to give **25**. Alkaline hydrolysis of the ethyl ester and acidification led to the desired acid **26**.

In order to obtain the non-substituted flavone acid derivative **30**, the suitable allyl-protected flavone **27** was synthesized as reported in the literature.[25] As shown in Scheme 3, cleavage of the allyl protecting group by a catalytic amount of tetrakis(triphenylphosphine)palla-

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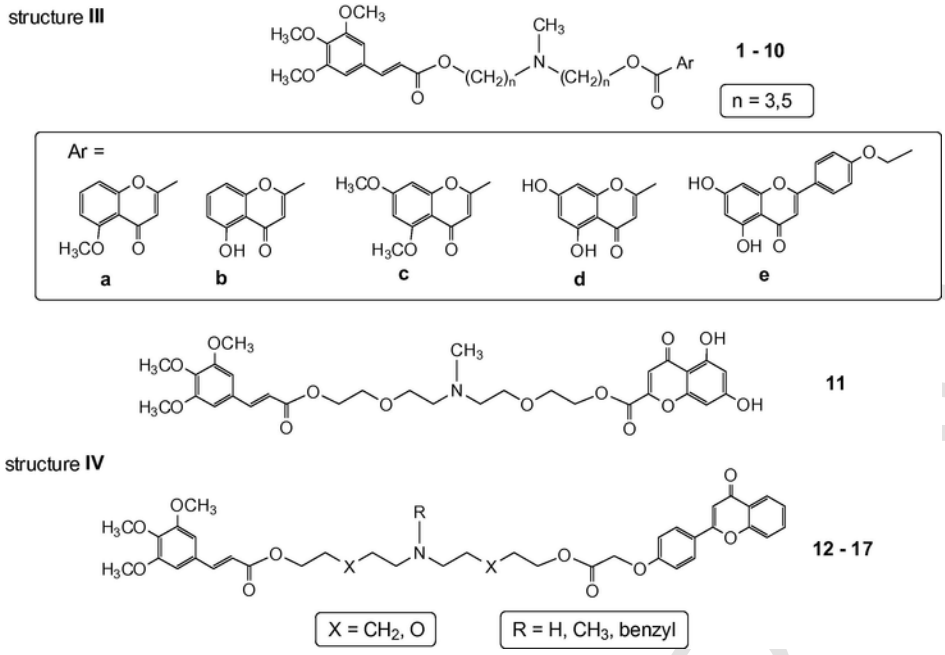
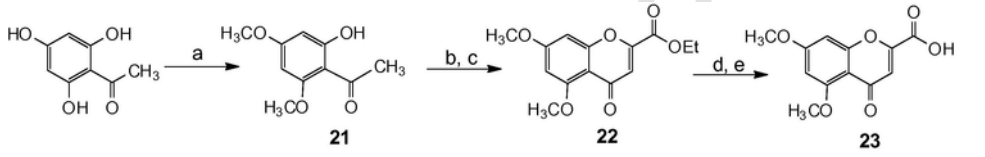
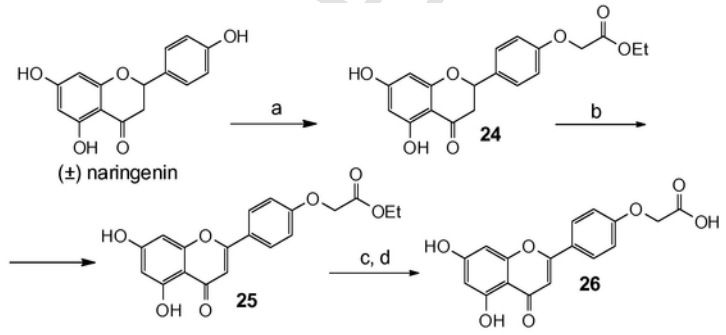


Chart 2. Structures of designed compounds.



Reagents: a) CH_3I, K_2CO_3 acetone; b) $(COOEt)_2$, EtONa, EtOH; c) EtOH, HCl conc.; d) $NaHCO_3$ 10%; e) HCl.

Scheme 1.



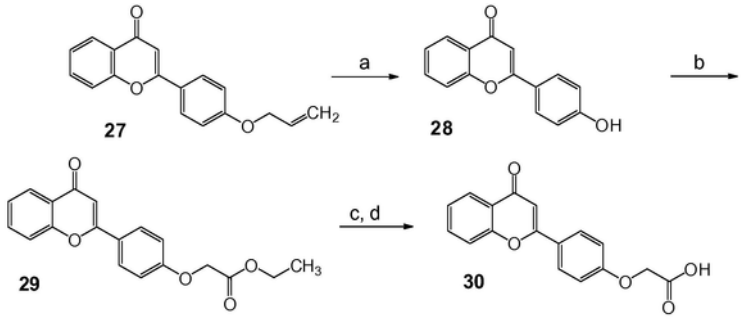
Reagents: a) $BrCH_2COOEt$, K_2CO_3, acetone; b) I_2, pyridine; c) $NaHCO_3$ 10%; d) HCl.

Scheme 2.

dium (0) led to **28**,[25] which was characterized in this study. The deprotected phenolic function was alkylated with ethyl bromoacetate; the obtained ester **29** was transformed into **30** in the usual way.

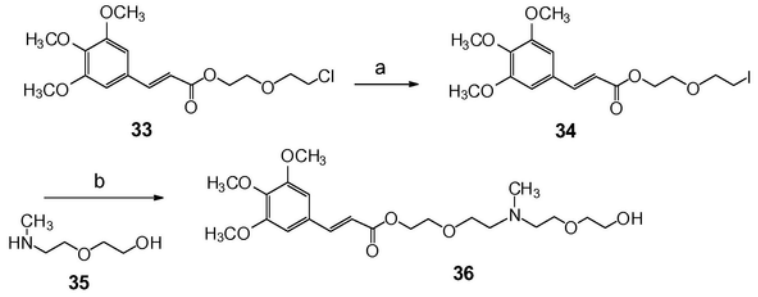
Alcohol **36**, already described by us,[27] was obtained with a more productive procedure (Scheme 4). The chloro derivative **33**[27] was transformed in the corresponding iodo derivative **34** with NaI in acetone, in order to achieve higher yields in the following reaction; **34** was then alkylated with the aminoalcohol **35**[27,33] to give compound **36**.

The synthesis of the benzyl-substituted intermediates is reported in Scheme 5. Reaction of secondary amine **38** with iododerivative **37** and Et_3N in acetonitrile gave alcohol **40**. In the same way **41** was obtained from **39** and **34**. Intermediates **38**[34] and **39**[35] had been already described, but were obtained by us in a different way, by reacting the suitable chloroalcohol and benzylamine in the presence of K_2CO_3 in anhydrous CH_3CN.



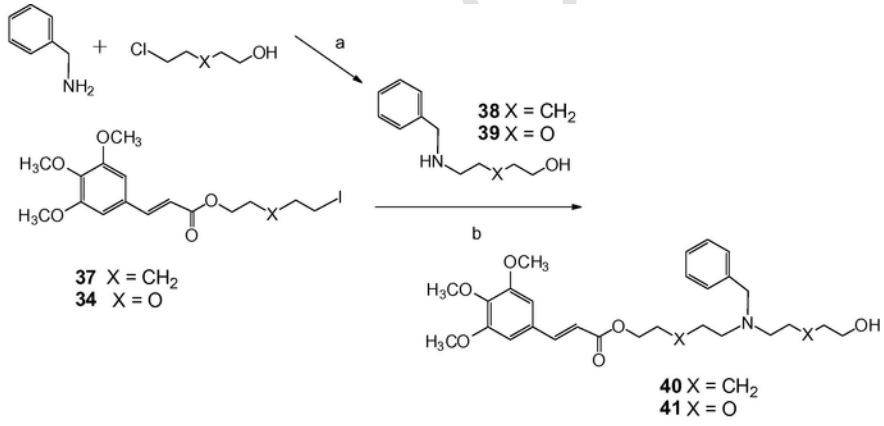
Reagents: a) $Pd(PPh_3)_4$, K_2CO_3, MeOH; b) $BrCH_2COOEt$, K_2CO_3, DMF; c) $NaHCO_3$ 10%; d) HCl. Compound **27** was obtained as reported in ref. 25.

Scheme 3.



Reagents: a) NaI, acetone; b) Et_3N, CH_3CN. Compound **33** was obtained as reported in ref. 27; compound **35** was obtained as reported in ref. 27 and 33.

Scheme 4.

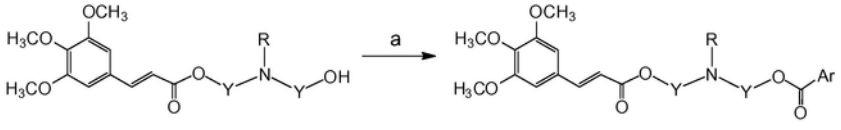


Reagents: a) K_2CO_3, CH_3CN; b) TEA, CH_3CN. Compound **37** was obtained as reported in ref. 32.

Scheme 5.

Final compounds **1**–**13**, **15** and **16** were eventually obtained by reaction of alcohols **31**,[27] **32**,[27] **36**, **40** or **41** with the proper carboxylic acid using the activating agent EDCI in the presence of HOBt in anhydrous CH_2Cl_2 (Scheme 6; for details, see the Experimental Section 4). In particular, starting from the alcohol **31**, final compounds **1**–**5** were obtained; using the alkyl alcohol **32**, compounds **6**–**10** and **12** were synthesized. The ethoxy alcohol **36** led to derivatives **11** and **15**; at last, starting from the *N*-benzyl substituted alcohols **40** and **41**, compounds **13** and **16** were obtained respectively.

In the case of the final compounds **14** and **17**, which carry a non-substituted nitrogen, as first the aminic function was protected, as shown in Scheme 7. Alkyl alcohol **42**[27] and ethoxy alcohol **43**[27] were treated with $(BOC)_2O$ in THF yielding protected derivatives **44** and **45**.[27] These intermediates were reacted with acid **30** and the activating agent EDCI in the presence of DMAP to give **46** and **47** respectively; cleavage of the BOC protecting group with trifluoroacetic acid led to the compounds **14** and **17**.

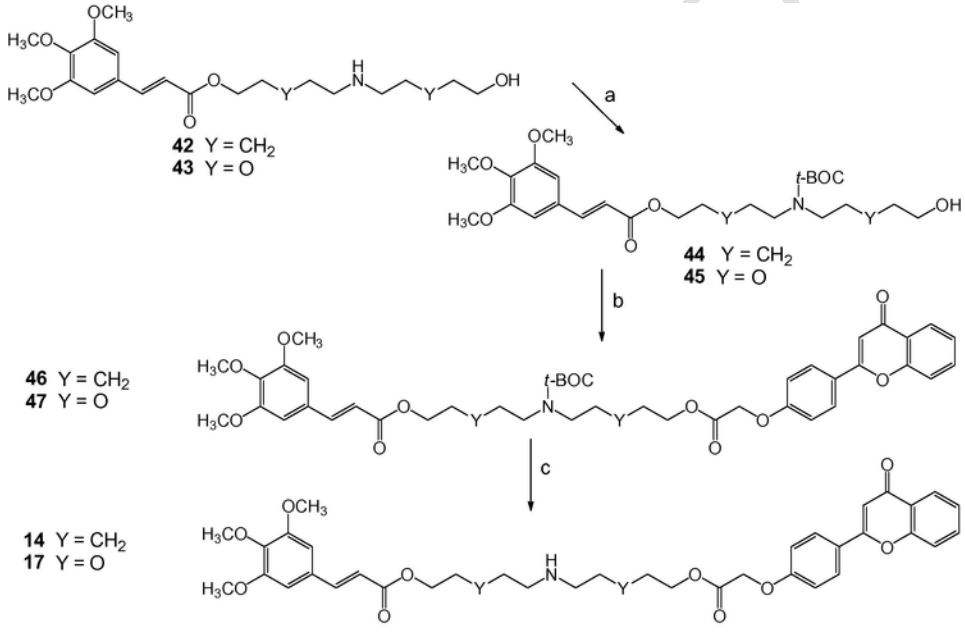


31 Y = $(CH_2)_3$ R = CH_3
32 Y = $(CH_2)_5$ R = CH_3
36 Y = $(CH_2)_2O(CH_2)_2$ R = CH_3
40 Y = $(CH_2)_5$ R = benzyl
41 Y = $(CH_2)_2O(CH_2)_2$ R = benzyl

N	Y	Ar	R
1	$(CH_2)_3$	a	CH_3
2	$(CH_2)_3$	b	CH_3
3	$(CH_2)_3$	c	CH_3
4	$(CH_2)_3$	d	CH_3
5	$(CH_2)_3$	e	CH_3
6	$(CH_2)_5$	a	CH_3
7	$(CH_2)_5$	b	CH_3
8	$(CH_2)_5$	c	CH_3
9	$(CH_2)_5$	d	CH_3
10	$(CH_2)_5$	e	CH_3
11	$(CH_2)_2O(CH_2)_2$	d	CH_3
12	$(CH_2)_5$	f	CH_3
13	$(CH_2)_5$	f	benzyl
15	$(CH_2)_2O(CH_2)_2$	f	CH_3
16	$(CH_2)_2O(CH_2)_2$	f	benzyl

Reagents: a) ArCOOH (**18-20**, **23**, **26**, **30**), EDCI, HOBT. For the meaning of Ar, see Table 1. Compounds **31** and **32** were obtained as reported in ref. 32.

Scheme 6.



42 Y = CH_2
43 Y = O
44 Y = CH_2
45 Y = O
46 Y = CH_2
47 Y = O
14 Y = CH_2
17 Y = O

Reagents: a) $(BOC)_2O$, THF; b) **30**, EDCI, DMAP, CH_2Cl_2, THF; c) CF_3COOH, CH_2Cl_2. Compounds **42**, **43** and **45** were obtained as reported in ref. 27.

Scheme 7.

2.2. Biological studies: modulation of pirarubicin uptake

The ability of the synthesized compounds to modulate P-gp was evaluated on K562/DOX cells. K562 is a human leukemia cell line established from a patient with chronic myelogenous leukemia in blast transformation.[36] K562/DOX doxorubicin resistant cells overexpress only the membrane glycoprotein P-gp.[37–39] Before evaluating the biological activity of the derivatives, their intrinsic toxicity was assessed on both K562 and K562/DOX cell lines using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. The compounds tested at 1.0 and 3.0 μM concentrations have no intrinsic toxicity both to the parental and resistant cell line. Data and experimental details are reported in the Supplementary material.

The uptake of THP-adriamycin (pirarubicin) was measured by means of a continuous spectrofluorometric signal of anthracycline at 590 nm (λ_{ex} = 480 nm) after cell incubation, following the protocols reported in previous papers.[40,41] The P-gp modulating activity of the studied compounds on the pirarubicin uptake test is expressed by: *i*) $[I]_{0.5}$, which measures the potency of the modulator and represents the concentration that causes a half-maximal increase (α = 0.5) in the nuclear concentration of pirarubicin, and *ii*) α_{max}, which represents the efficacy of the modulator and is the maximum increase in the nuclear concentration of pirarubicin in resistant cells that can be ob-

tained with a given compound. The value of α varies between 0 (in the absence of the inhibitor) and 1 (when the amount of pirarubicin in resistant cells is the same as in sensitive cells).

The results obtained are reported in Table 1 together with those of verapamil, the gold standard of P-gp inhibition, used as reference compound.

As regards the polymethylenic derivatives **1**–**10**, it clearly emerges that, regardless of the flavone or chromone residue which is combined with the (*E*)-3-(3,4,5-trimethoxycynnamoyl)moiety, the length of the linker is essential for the activity. In fact all the compounds carrying two 3-carbon alkyl chains are almost inactive, showing an efficacy between 4% and 30%. Therefore for compounds **1**–**5**, $[I]_{0.5}$ values could not be evaluated.

More interesting results were instead obtained with the analogs carrying two 5-carbon alkyl chains. Derivatives **6**–**10** are able to completely reverse P-gp-dependent pirarubicin extrusion (α_{max} close to 1) with potency values ($[I]_{0.5}$) in the micromolar range. In particular, the hydroxychromone compound **7** and the dihydroxyflavone derivative **10** show the highest reversing activity in the series, with potencies higher than that of verapamil ($[I]_{0.5}$ value of 0.35 μM and 0.96 μM with respect to 1.6 μM). In this series, insertion of an ethoxylated chain of the same number of atoms (see compound **11** *vs* compound **9**) completely abolishes the activity (α_{max} value: not measurable for **11** and 0.99 for **9**).

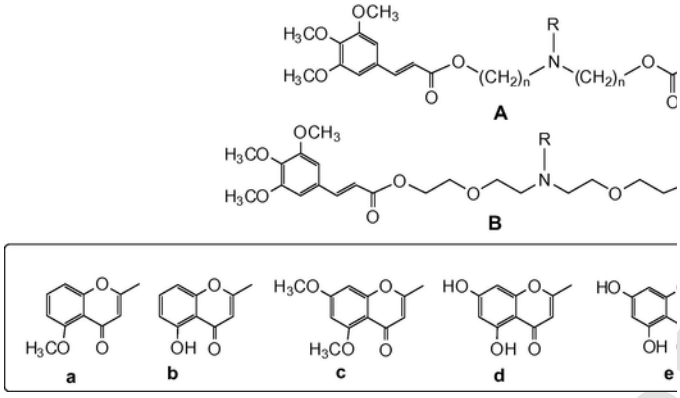
A similar behavior is maintained in the series of compounds **12**–**17**, carrying the flavone residue **f** and different residues on the nitrogen. In this case, regardless from the group present on the basic nitrogen, the polymethylenic derivatives are always more active than their ethoxyethylic analogs (compare **12** *vs* **15**, **13** *vs* **16**, **14** *vs* **17**). As regard the influence of the *N*-residue, in the polymethylenic set of compounds, the methyl group confers the best activity (**12**, $[I]_{0.5}$ = 0.34 μM), followed by the *N*-benzyl group (**13**, $[I]_{0.5}$ = 0.43 μM), while the activity is less pronounced in the case of the secondary amine (**14**, $[I]_{0.5}$ = 1.32 μM). In the ethoxylated series, only the *N*-methyl substituted derivative **15** shows a remarkable efficacy (α_{max} = 0.86), with a potency of 1.23 μM.

These data are somehow unexpected. In fact, in the series of *N*-methyl derivatives previously synthesized in our laboratory,[27,28,32] compounds with two 3-carbon alkyl chains showed in most cases a good MDR modulating activity, even if compounds bearing two 5-carbon alkyl chains were often more potent. As regard the efficacies, the different series of compounds showed mixed results, depending from the inserted aryl residue. Surprisingly, in the series synthesized in this study the presence of the shorter linker (compounds **1**–**5**) completely or nearly completely abolishes the activity, regardless of the different size of the aryl group Ar_1 (Table 1). On the contrary, activity is maintained in the presence of the longer chain (compounds **6**–**10**, **12**–**14**), and as expected the MDR modulation activity of this set of derivatives depends on the nature of the aromatic ester. Evaluation of the effect of the flavonoid residue is not straightforward: insertion of the monohydroxy chromone **b** leads to the most interesting compound in this series, but also the dihydroxy flavone **e** has a positive influence on the activity. The presence of the lipophilic non-substituted flavone **f** confers a good activity to the molecule, confirming the results previously reported.[26]

If we extend the analysis to compounds **11** and **15**–**17**, the results indicate that P-gp modulation is mainly related on the nature of the chain, rather than on its length. The ethoxyethylic derivatives indeed are always less potent with respect to the polymethylenic ones, even if the two spacers contain the same number of atoms. These data contrast with the results of Chow and Chan,[26], since the very active flavonoid dimers described always carry two ethoxyethylic chains. It must however be kept in mind that the pharmacological assays and the cell lines employed in the case of these flavonoid compounds are different from those used in our studies. If we consider the effect of the substituent on the basic nitrogen, the presence of the methyl group seems to confer the best characteristics to the molecules, while the benzyl residue, which is reported to have a positive effect in the flavonoid heterodimers,[26] in our series has not such a good influence, in particular when combined with an ethoxylated spacer. In the case of the secondary amines **14** and **17** the results are further less interesting.

The fact that this new series of compounds does not comply with the SAR outlined by our previous derivatives opens a new scenario. For our "hybrid" heterodimers the activity is linked to different features, in particular the characteristics of the spacer, which seem to be critical for the interaction with the pump. In fact, compounds bearing two 5-carbon alkyl chains always show P-gp modulating activity. Likely enough, the presence of a flavone or chromone residue modifies the molecular characteristics in terms of both lipophilic/hy-

Table 1
P-gp modulating activity of compounds **1**–**17**. Verapamil is reported as comparison.



N	Structure	n	R	Ar_1	$[i]_{0.5}$ μM[a]	α_{max}[b]
1	A	3	CH_3	a	–	0.12 ± 0.03
2	A	3	CH_3	b	–	0.06 ± 0.02
3	A	3	CH_3	c	–	0.04 ± 0.008
4	A	3	CH_3	d	–	0.20 ± 0.03
5	A	3	CH_3	e	–	0.30 ± 0.02
6	A	5	CH_3	a	3.50 ± 0.5	0.98 ± 0.02
7	A	5	CH_3	b	0.35 ± 0.05	0.94 ± 0.03
8	A	5	CH_3	c	1.32 ± 0.57	0.99 ± 0.01
9	A	5	CH_3	d	2.43 ± 0.48	0.99 ± 0.01
10	A	5	CH_3	e	0.96 ± 0.20	0.99 ± 0.01
11	B		CH_3	d	–	n.a[c]
12	A	5	CH_3	f	0.34 ± 0.07	0.99 ± 0.01
13	A	5	benzyl	f	0.43 ± 0.12	0.99 ± 0.01
14	A	5	H	f	1.32 ± 0.22	0.69 ± 0.17
15	B		CH_3	f	1.23 ± 0.17	0.86 ± 0.13
16	B		benzyl	f	–	0.27 ± 0.01
17	B		H	f	–	0.26 ± 0.01
verapamil					1.6 ± 0.3	0.70 ± 0.07

[a] Concentration of the inhibitor that causes a 50% increase in nuclear concentration of pirarubicin (α = 0.5).

[b] Efficacy of MDR-modulator and maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells. Results are expressed as the mean ± SE of three independent experiments done at least three times.

[c] Not active.

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drophilic balance and ability to establish H-bonds. This evidence seems to suggest, for the compounds described in this work, a particular way of interaction with the large P-gp recognition site. Furthermore, the flexibility of the designed molecules allow each compound to bind in its own way, finding the best productive binding mode. This fact is a further proof of the validity of the polyvalent binding approach.

2.3. Molecular modeling studies

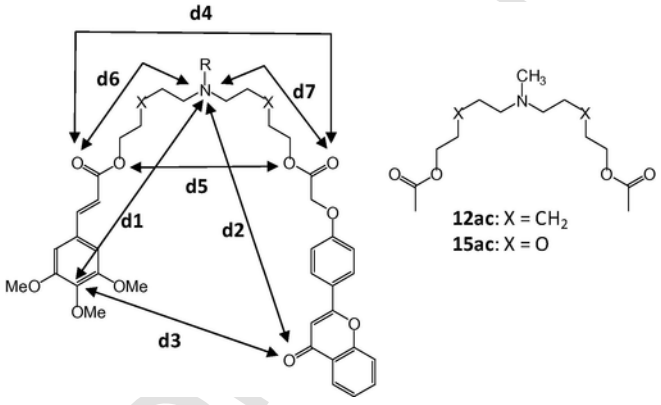
In order to investigate the different conformational behavior of ethoxyethylic and polymethylenic derivatives, the conformational effect of the O/CH_2 substitution in compounds **12**–**17** was evaluated by means of molecular dynamic simulations. This methodology was chosen owing to the high conformational flexibility of the compounds, which makes a conformational search difficult either by means of systematic dihedral rotation or simulated annealing.

The compounds, in the neutral and protonated forms, underwent a 10 ns dynamic simulation in the vacuum. All molecules adopt bended conformations showing the (*E*)-3-(3,4,5)-trimethoxycynnamyl and flavone moieties close in space. A set of seven distances has been selected to monitor changes in the conformational behavior possibly due to the O/CH_2 substitution; for compounds **12**–**17** in the neutral form the mean values of d1-d7 (Table 2) recorded in the last 3 ns of the dynamic simulation, do not show a clear trend, suggesting a similar dynamic behavior for the two sets of compounds **12**–**14** and **15**–**17**. On the contrary, a difference was found for the protonated forms. In fact, an intramolecular hydrogen bond has been observed between the NH^+ group and the flavone C═O for compounds **12**–**14**, while for **15**–**17** the H-bond is formed between the NH^+ group and the C═O of the acetate moiety; accordingly, mean values below 3 Å have been found for d2 (compounds **12**–**14**) and d7 (compounds **15**–**17**), and the H-bond interactions were found stable within the time window of observation (see Fig. S2 in the Supplementary material). In Fig. 1 two conformations of **12** (right) and **15** (left), highlighting the different H-bond. In addition, a second H-bond is formed for both the *N*-unsubstituted derivatives **14** and **17** involving the C═O of the trimethoxycinnamyl moiety (d6 < 3 Å, Table 2).

To avoid a possible interference of the intramolecular π-stacking, calculations were repeated for **12** and **15** in which both aromatic appendages have been deleted, transforming the ester moieties into simpler acetates. Visual inspection of the dynamic simulation showed that these molecules adopt both bended and extended conformations, with the former being more frequent. Again, in the protonated molecules some distances were shorter than in the neutral forms due to intramolecular H-bonds, which however were less stable than in the parent molecules (data not shown). No clear difference between O- and CH_2-containing compounds has been noticed.

Therefore, the performed dynamic simulations suggest a different preference for the carbonyl groups involved in H-bond with the pro-

Table 2
Distances (Å) between selected atoms throughout a 3 ns dynamic simulation.[a]



N	X	d1	d2	d3	d4	d5	d6	d7
12	CH_2	9.8±0.7	8.9±2.1	4.9±1.2	7.8±1.0	7.1±1.5	6.5±0.5	7.6±0.8
13	CH_2	9.2±0.6	8.0±0.5	6.0±0.7	10.0±0.5	6.6±0.4	5.6±0.5	7.4±0.2
14	CH_2	10.0±1.1	8.8±2.9	5.6±2.3	9.1±1.7	7.3±1.4	6.8±0.6	6.7±0.9
15	O	8.4±08	8.6±1.5	5.1±0.7	9.1±1.7	7.7±1.2	6.0±0.6	6.6±0.8
16	O	9.0±0.8	9.5±2.2	5.3±1.2	8.7±1.9	7.1±1.5	6.2±0.6	5.7±0.8
17	O	10.3±1.6	9.3±2.8	7.8±2.9	7.1±1.2	4.9±1.0	6.2±0.7	5.9±0.9
$12H^+$	CH_2	8.1±0.6	2.8±0.1	5.9±0.4	12.0±1.0	10.9±0.4	6.0±0.8	7.6±0.7
$13H^+$	CH_2	8.0±0.7	2.7±0.1	6.0±0.7	12.3±0.9	10.8±0.4	5.3±1.2	8.2±0.7
$14H^+$	CH_2	8.5±0.5	2.7±0.1	7.8±2.9	8.2±1.3	9.4±1.3	2.7±0.1	7.9±0.5
$15H^+$	O	9.1±1.1	10.0±1.8	5.4±2.1	5.6±1.8	6.5±0.9	5.8±0.4	3.0±0.3
$16H^+$	O	9.2±0.8	8.6±1.7	5.7±1.3	4.8±0.8	6.6±0.9	5.1±0.7	3.0±0.2
$17H^+$	O	8.5±0.6	9.1±1.9	8.9±3.3	4.0±0.5	6.7±0.7	2.9±0.2	2.8±0.1
12ac	CH_2	–	–	–	6.4±1.8	5.6±1.2	7.0±0.7	7.4±0.8
15ac	O	–	–	–	7.7±2.1	6.2±1.9	6.3±0.8	6.1±0.1
$12acH^+$	CH_2	–	–	–	5.1±1.1	6.5±1.6	3.4±0.7	4.5±1.8
$15acH^+$	O	–	–	–	5.3±0.9	6.1±1.1	3.8±0.5	4.2±1.4

[a] Values are the mean ± SD recorded during the last 3 ns of a 10 ns simulation performed in vacuum. H^+ means that the molecule has been calculated in the protonated form.

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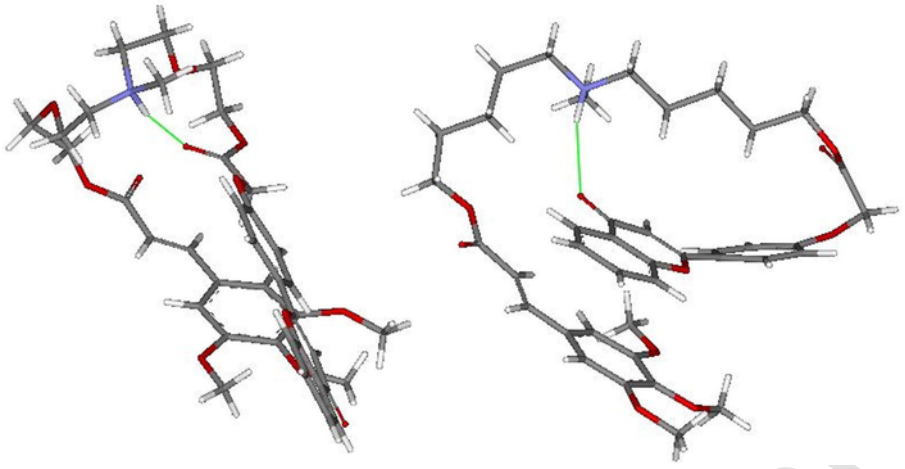

Fig. 1. Conformations of **12** (right) and **15** (left), sampled from the dynamic simulation, showing the different H-bond (green).

tonated nitrogen atom between the polymethylenic compounds **12**–**14** and the corresponding ethoxyethylic derivatives **15**–**17**. Further studies are needed to understand if this difference is the reason for the different activity of the two sets of compounds.

3. Conclusions

In this study, a new series of heterodimers was synthesized. These derivatives are *N*,*N*-bis(alkanol)amine aryl esters or *N*,*N*-bis(ethoxyethanol)amine aryl esters, containing a flavone or chromone moiety. Some of the new compounds show a good P-gp modulating activity on the pirarubicin uptake test; interestingly this new series of compounds does not comply with the SAR outlined by our previous derivatives. In the case of the compounds described in this paper, different structure-activity relationships can be drawn, confirming that the presence of a flavone or chromone residue influences the SAR of these series of products, and that flexible molecules can find different productive binding modes with the P-gp recognition site. Molecular dynamic simulations suggest that the O/CH_2 substitution elicits some effects on the conformation adopted by the molecules in the protonated forms, where a difference was found. Some of these compounds show an interesting P-gp modulating activity, which can hopefully be increased by inserting different residues; these results support the synthesis of new compounds that might be useful leads for the development of drugs to control P-gp-dependent MDR.

4. Experimental

4.1. Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer Spectrum RX I FT-IR spectrophotometer in Nujol mull for solids and neat for liquids. 1D and 2D (COSY and HSQC) NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR). Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Yields are given after purification, unless otherwise stated.

ESI-MS spectra were obtained using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by Electrospray Source (ESI) operating in both positive and negative ions.

The data were acquired in scan mode between the range 150–800 *m*/*z*. In the instrumental conditions used, the most abundant ion species expected for the analytes will be $[M+H]^+$ or $[M-H]^-$.

Compounds **1**–**17** were obtained in a purity ≥95%. Their combustion analyses are indicated by symbols, and the analytical results are within ±0.4% of the theoretical values. Compounds were named following IUPAC rules as applied by Reaxys database. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen. Free bases **1**–**17** were transformed into the hydrochloride by treatment with a solution of acetyl chloride (1.1 eq) in anhydrous CH_3OH, or into the oxalate by treatment with 1.05 eq of oxalic acid in ethyl acetate. The salts were crystallized from abs. ethanol/petroleum ether.

*4.1.1. 1-(2-Hydroxy-4,6-dimethoxyphenyl)ethan-1-one **21***

2,4,6-Trihydroxyacetophenone (2 g, 10.7 mmol), methyl iodide (2.70 mL, 42.9 mmol) and K_2CO_3 (2.96 g, 21.4 mmol) were dissolved in 50 mL of acetone. The mixture was heated to reflux for 24 h in the dark. The reaction mixture was filtered under vacuum, and the filtrate was evaporated under reduced pressure. The crude product was purified by flash chromatography using cyclohexane/ethyl acetate 95:5 as eluting system; 1.13 g of title compound **21** were obtained. Yield: 36%. ^{1}H NMR ($CDCl_3$) δ: 6.05 (d, J=2.4 Hz, 1H, CH arom.); 5.92 (d, J=2.4 Hz, 1H, CH arom.); 3.85 (s, 3H, OCH_3); 3.81 (s, 3H, OCH_3); 2.60 (s, 3H, CH_3) ppm.

*4.1.2. Ethyl 5,7-dimethoxy-4-oxo-4H-chromene-2-carboxylate **22***

A solution of **21** (884 mg, 4.51 mmol) in ethyl oxalate (2.4 mL, 18.04 mmol) was added to a freshly prepared solution of EtONa in abs. EtOH (obtained by addition of 623 mg (27.1 mmol) of sodium to 10 mL of abs. EtOH). The mixture was heated to reflux for 10 h, then it was cooled to room temperature and water was added. The solvent was evaporated, and the obtained brown residue was treated with HCl 2N and extracted with ethyl acetate. The organic layer was washed with brine, dried on Na_2SO_4 and evaporated under vacuum. The crude residue was dissolved in 15 mL of EtOH 96% and heated to reflux for 15 min; conc. HCl (0.6 mL) was added, and the obtained solution was maintained to reflux for 1 h. The reaction mixture was cooled to room temperature, and the solvent was evaporated. The crude brown product was purified by flash chromatography, using cy-

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clohexane/ethyl acetate 50:50 as eluting system, yielding 800 mg (71%) of a light yellow solid (mp 151–153 °C). ^{1}H NMR ($CDCl_3$) δ: 6.98 (s, 1H, CH-CO); 6.60 (d, 1H, J=2.4 Hz, CH arom.); 6.38 (d, J=2.4 Hz, 1H, CH arom.); 4.43 (q, J=7.2 Hz, 2H, CH_2CH_3); 3.94 (s, 3H, OCH_3); 3.89 (s, 3H, OCH_3); 1.41 (t, J=7.2 Hz, 3H, CH_2CH_3) ppm.

*4.1.3. 5,7-Dimethoxy-4-oxo-4H-chromene-2-carboxylic acid **23***

To 200 mg (0.57 mmol) of **22**, 20 mL of a solution of 10% $NaHCO_3$ were added. The mixture was heated at 80 °C for 3 h. After cooling, the solution was acidified with conc. HCl and extracted with ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4 and evaporated under vacuum, yielding 170 mg (95%) of a pale yellow solid (mp 241–243 °C). ^{1}H NMR (CD_3OD) δ: 6.86 (s, 1H, CH-CO); 6.74 (d, J=2.4 Hz, 1H, CH arom.); 6.56 (d, J=2.4 Hz, 1H, CH arom.); 3.94 (s, 3H, OCH_3); 3.91 (s, 3H, OCH_3) ppm.

*4.1.4. Ethyl 2-[4-(5,7-dihydroxy-4-oxo-3,4-dihydro-2H-1-benzopyran-2-yl)phenoxy]acetate **24***

To a solution of 200 mg of (±)naringenin (0.73 mmol) in 7 ml of acetone, K_2CO_3 (101.5 mg, 0.73 mmol) was added. The mixture was stirred at room temperature for 15 min, then ethyl bromoacetate (0.08 ml, 0.73 mmol) dissolved in 3 mL acetone was added dropwise. The reaction mixture was stirred 24 h at room temperature, filtered under vacuum, and the filtrate was evaporated under reduced pressure. The crude product was purified by flash chromatography using CH_2Cl_2/MeOH 99:1 as eluting system. 158 mg of **24** were obtained as a white solid (mp 185–186 °C), yield 60%. MS m/z (%): 359.1 (100) $[M+H]^+$; ^{1}H NMR (CD_3OD) δ: 7.32 (d, J=8.4 Hz, 2H, CH arom.); 6.81 (d, J=8.4 Hz, 2H, CH arom.); 6.06–6.03 (m, 2H, CH arom.); 5.41–5.37 (m, 1H, CHO); 4.74 (s, 2H, OCH_2CO); 4.25 (q, J=7.2 Hz, 2H, CH_2CH_3); 3.20–3.13 (m, 1H, CH*H*CO); 2.77–2.72 (m, 1H, C*H*HCO); 1.28 (t, J=7.2 Hz, 3H, CH_2CH_3) ppm.

4.1.5. Ethyl 2-[4-(5,7-dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy]acetate 25

To a solution of 150 mg (0.42 mmol) of **24** in 8 mL of anhydrous pyridine, 106.34 mg (0.42 mmol) of I_2 were added. The mixture was kept at 90 °C for 6 h then it was cooled to room temperature and poured on ice. The solution was extracted twice with ethyl acetate, then the organic layer was washed three times with a saturated solution of $Na_2S_2O_3$, once with a saturated solution of NaCl, and dried on Na_2SO_4. Evaporation of the solvent and distillation of the pyridine yielded 149 mg of **25** as yellow solid, mp 185–187 °C. The compound was not further purified. Yield: 100%. ESI-MS: $[M-H]^-$ species at m/z 355. ^{1}H NMR (CD_3OD) δ: 7.88 (d, J=9.0 Hz, 2H, CH arom.); 6.93 (d, J=9.0 Hz, 2H, CH arom.); 6.67 (d, J=2.4 Hz, 1H, CH arom); 6.65 (s, 1H, CHCO); 6.37 (d, J=2.4 Hz, CH arom); 4.82 (s, 2H, OCH_2CO); 4.28 (q, J=7.2 Hz, 2H, CH_2CH_3); 1.29 (t, J=7.2 Hz, 3H, CH_2CH_3) ppm.

*4.1.6. 2-[4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy]acetic acid **26***

With the same procedure described for **23**, and starting from 149 mg (0.42 mmol) of **25** and 15 ml of a solution of 10% $NaHCO_3$, 130 mg of **26** as yellow solid were obtained, that did not need further purification (mp 159–161 °C). Yield: 94%. ^{1}H NMR (CD_3OD) δ: 7.90 (d, J=8.8 Hz, 2H, CH arom.); 6.95 (d, J=8.8 Hz, 2H, CH arom.); 6.68 (d, J=2.4 Hz, 1H, CH arom.); 6.67 (s, 1H, CHCO); 6.39 (d, J=2.4 Hz, 1H, CH arom.); 4.81 (s, 2H, OCH_2CO) ppm.

*4.1.7. 2-(4-Hydroxyphenyl)-4H-chromen-4-one **28***

A catalytic amount of $Pd(PPh_3)_4$ (280 mg, 0.24 mmol) was added to a solution of **27**[25] (2.26 g, 8.1 mmol) and K_2CO_3 (4.49 g, 32.5 mmol) in 40 mL of anhydrous MeOH at reflux. The reaction mixture was stirred at reflux for 4 h; then it was filtered to remove K_2CO_3. The obtained brown solution was diluted with water, acidified to pH 3 using 2 N HCl in ice and extracted twice with diethyl ether. The organic layer was dried on Na_2SO_4, evaporated and purified by a filtration on silica gel with CH_2Cl_2/MeOH 9:1. Yield: 1.90 g (98.2%). ^{1}H NMR (CD_3OD) δ: 8.12 (d, J=8.0 Hz, 1H, CH arom.); 7.91 (d, J=8.4 Hz, 2H, CH arom,); 7.78 (t, J=8.0 Hz, 1H, CH arom.); 7.69 (d, J=8.0 Hz, 1H, CH arom.); 7.47 (t, J=8.0 Hz, 1H, CH arom.); 6.94 (d, J=8.4 Hz, 2H, CH arom.); 6.79 (s, 1H, CHCO) ppm.

*4.1.8. Ethyl 2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetate **29***

K_2CO_3 (1.10 g, 8.0 mmol) and ethyl bromoacetate (1.06 mL, 9.6 mmol) were added to a solution of 1.90 g (8.0 mmol) of **28** in 50 mL of DMF. The mixture was stirred at room temperature for 24 h, then it was filtered under vacuum by adding a little amount of MeOH. The gold-yellow solution was evaporated, yielding 2.52 g (97.4%) of title compound as light-yellow solid (mp 131–132 °C). ^{1}H NMR ($CDCl_3$) δ: 8.14 (d, J=7.6 Hz, 1H, CH arom.); 7.81 (d, J=8.4 Hz, 2H, CH arom.); 7.59 (t, J=7.6 Hz, 1H, CH arom.); 7.47 (d, J=8.0 Hz, 1H, CH arom.); 7.34 (t, J=8.0 Hz, 1H, CH arom.);6.96 (d, J=8.4 Hz, 2H, CH arom.); 6.67 (s, 1H, CHCO); 4.63 (s, 2H, OCH_2CO); 4.22 (q, J=7.2 Hz, 2H, CH_2CH_3); 1.25 (t, J=7.2 Hz, 3H, CH_2CH_3) ppm.

*4.1.9. 2-[4-(4-Oxo-4H-chromen-2-yl)phenoxy]acetic acid **30***

To 2.52 g (7.8 mmol) of **29** 100 mL of a solution of 10% $NaHCO_3$ were added. The mixture was stirred at 100 °C for 5 h and then 18 h at room temperature. After cooling, the solution was acidified with HCl 2 N. A yellow solid was obtained which was collected by under vacuum/suction filtration. Yield 2.13 g (92.6%) (mp 219–221 °C). ^{1}H NMR (CD_3OD) δ: 8.13 (d, J=8.4 Hz, 1H, CH arom.); 8.03 (d, J=8.8 Hz, 2H, CH arom.); 7.80 (t, J=8.4 Hz, 1H, CH arom.); 7.73 (d, J=7.6 Hz, 1H, CH arom.); 7.50 (t, J=7.6 Hz, 1H, CH arom.); 7.12 (d, J=8.8 Hz, 2H, CH arom.); 6.85 (s, 1H, CHCO); 4.78 (s, 2H, OCH_2CO) ppm.

*4.1.10. 2-(2-Iodoethoxy)ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **34***

NaI (367 mg, 2.45 mmol) was added to a solution of 211 mg (0.61 mmol) of **33**[27] in 10 mL of acetone. The reaction was stirred at reflux in the dark for 20 h. The mixture was then cooled to room temperature, the solvent was evaporated, and the residue was treated with water and extracted three times with CH_2Cl_2. The organic layer was dried on Na_2SO_4 and evaporated to give a light-yellow oil (231 mg, 87%) that was no further purified. ^{1}H NMR ($CDCl_3$) δ: 7.59 (d, J=16.0 Hz, 1H, *CH*═CH); 6.71 (s, 2H, CH arom.); 6.35 (d, J=16.0 Hz, 1H, CH═*CH*); 4.33 (t, J=4.8 Hz, 2H, CH_2O); 3.83 (s, 9H, OCH_3); 3.76–3.73 (m, 4H, CH_2OCH_2); 3.23 (t, J=6.8 Hz, 2H, CH_2I) ppm.

*4.1.11. 2-(2-{[2-(2-Hydroxyethoxy)ethyl](methyl)amino}ethoxy)ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **36***[27]

227 mg (0.52 mmol) of **34** were reacted with 70 mg (0.59 mmol) of **35**[27,33] and 0.2 mL of anhydrous Et_3N in 4 mL of anhydrous acetonitrile. The reaction mixture was stirred at 60 °C for 18 h. Then CH_2Cl_2 was added, and the solution was washed twice with basic wa-

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ter, dried on Na_2SO_4 and evaporated to give the crude product that was purified by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 95:5:0.5 as eluting system. 100 mg (50.0%) of title compound were obtained. ^{1}H NMR ($CDCl_3$) δ: 7.61 (d, J=16.0 Hz, 1H, CH═*CH*); 6.75 (s, 2H, CH arom.); 6.38 (d, J=16.0 Hz, 1H, *CH*═CH); 4.35 (t, J=4.8 Hz, 2H, CH_2OCO); 3.87 (s, 9H, OCH_3); 3.72 (t, J=4.8 Hz, 2H, CH_2OH); 3.68–3.57 (m, 8H, CH_2OCH_2); 2.69 (t, J=5.6 Hz, 2H, CH_2N); 2.65 (t, J=5.2 Hz, 2H, CH_2N); 2.33 (s, 3H, NCH_3) ppm.

4.1.12. 5-(Benzylamino)pentan-1-ol ***38***[34]

To a solution of benzylamine (0.66 mL, 6.03 mmol) and 5-chloropentan-1-ol (0.42 mL, 4.02 mmol) in 5 mL of anhydrous acetonitrile, 555 mg of K_2CO_3 (4.02 mmol) were added. The mixture was stirred at 80 °C for 48 h and concentrated in vacuo. The residue was dissolved with CH_2Cl_2, and the organic layer was washed twice with a solution of 10% NaOH, dried on Na_2SO_4 and evaporated to give the crude product that was purified by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 95:5:0.5 as eluting system. 160 mg (20.6%) of title compound as a yellow oil were obtained. ^{1}H NMR ($CDCl_3$) δ: 7.33–7.26 (m, 5H, CH arom.); 3.78 (s, 2H, NCH_2Ph); 3.61 (t, J=6.4 Hz, 2H, CH_2OH); 2.65 (t, J=6.8 Hz, 2H, NCH_2); 1.88 (s, 1H, NH); 1.61–1.50 (m, 4H, CH_2); 1.47–1.42 (m, 2H, CH_2) ppm.

4.1.13. 5-[Benzyl-(5-hydroxypentyl)amino]pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***40***

Compounds **38** (160 mg, 0.83 mmol), **37**[32] (360 mg, 0.83 mmol) and anhydrous triethylamine (0.36 ml, 2.72 mmol) were dissolved in 15 mL of anhydrous acetonitrile. The mixture was stirred at reflux for 27 h, then it was evaporated. The obtained residue was dissolved in CH_2Cl_2, and the organic layer was washed three times with a solution of 10% NaOH, dried on Na_2SO_4 and evaporated to give the crude product. After purification by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 95:5:0.5 as eluting system, 124 mg (30.0%) of **40** as pale-yellow oil were obtained. ^{1}H NMR ($CDCl_3$) δ: 7.59 (d, J=16.0 Hz, 1H, *CH*═CH); 7.31–7.22 (m, 5H, CH arom.); 6.75 (s, 2H, CH arom.); 6.34 (d, J=16.0 Hz, 1H, CH═*CH*); 4.18 (t, J=6.4 Hz, 2H, CH_2OCO); 3.89 (s, 6H, OCH_3); 3.88 (s, 3H, OCH_3); 3.61–3.56 (m, 4H, NCH_2Ph and CH_2OH); 2.45–2.41 (m, 4H, CH_2N); 1.70–1.64 (m, 4H, CH_2); 1.55–1.47 (m, 4H, CH_2); 1.43–1.33 (m, 4H, CH_2) ppm.

4.1.14. 2-[2-(Benzylamino)ethoxy]ethan-1-ol ***39***[35]

To a solution of benzylamine (0.88 mL, 8.04 mmol) and 2-(2-chloroethoxy)ethanol (0.84 mL, 8.04 mmol) in 5 mL of anhydrous acetonitrile, 1.11 g of K_2CO_3 (8.04 mmol) were added. The mixture was stirred at reflux for 48 h and concentrated in vacuo. The residue was dissolved with CH_2Cl_2, and the organic layer was washed three times with a solution of 10% NaOH, dried on Na_2SO_4 and evaporated to give the crude product that was purified by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 93:7:0.3 as eluting system. 323 mg (20.6%) of **39** as a yellow oil were obtained. ^{1}H NMR ($CDCl_3$) δ: 7.34–7.25 (m, 5H, CH arom.); 3.83 (s, 2H, NCH_2Ph); 3.69 (t, J=4.2 Hz, 2H, O*CH*$_2$$CH_2OH$); 3.63 (t, J=5.2 Hz, 2H, O*CH*$_2$$CH_2N$); 3.55 (t, J=4.2 Hz, 2H, CH_2OH); 2.83 (t, J=5.2 Hz, 2H, N*CH*$_2$$CH_2O$) ppm.

4.1.15. 2-(2-{Benzyl[2-(2-hydroxyethoxy)ethyl]amino}ethoxy)ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***41***

Iododerivative **34** (492 mg, 1.13 mmol), aminoalcohol **39** (220 mg, 1.13 mmol) and anhydrous triethylamine (0.45 mL, 3.40 mmol) were dissolved in 10 mL of anhydrous acetonitrile. The mixture was stirred at 82 °C for 24 h, then it was evaporated. The obtained residue was dissolved in CH_2Cl_2, and the organic layer was washed three times with a solution of 10% NaOH, dried on Na_2SO_4 and evaporated to give the crude product. After purification by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 95:5:0.5 as eluting system, 225 mg (39.6%) of **41** as yellow oil were obtained. ^{1}H NMR ($CDCl_3$) δ: 7.60 (d, J=16.0 Hz, 1H, *CH*═CH); 7.33–7.21 (m, 5H, CH arom.); 6.73 (s, 2H, CH arom.); 6.36 (d, J=16.0 Hz, 1H, CH═*CH*); 4.33 (t, J=4.4 Hz, 2H, CH_2OCO); 3.89 (s, 6H, OCH_3); 3.86 (s, 3H, OCH_3); 3.71–3.51 (m, 12H, OCH_2 and NCH_2Ph and CH_2OH); 2.79–2.75 (m, 4H, CH_2N) ppm.

4.1.16. General procedure for the synthesis of diester compounds ***1****–****13****,* ***15*** *and* ***16***

To a solution of the suitable acid (**18**,[23] **19**,[23] **20**,[31] **23**, **26** or **30**) (1 eq.) in anhydrous THF, EDCI (1.2 eq.), HOBt (1.2 eq.) and a solution of the proper alcohol (**31**,[32] **32**,[32] **36**, **40**, **41**) (1 eq.) in anhydrous CH_3CN or CH_2Cl_2 were added. The mixture was stirred at room temperature for 48 h, then it was evaporated. The obtained residue was dissolved in CH_2Cl_2, and the organic layer was washed twice with water and once with a saturated solution of $NaHCO_3$, dried on Na_2SO_4 and evaporated to give the desired ester. As reported hereinafter, when necessary the oily product was purified by flash chromatography using the appropriate eluting system. All the compounds were transformed into the corresponding hydrochloride or oxalate. The salts were crystallized from abs. ethanol/petroleum ether and were obtained as white solids.

4.1.16.1. (3-({3-[(E)-5-Methoxy-4-oxo-4H-chromene-2-carbonyloxy]propyl}(methyl)amino)propyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***1***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Yield 93%. ^{1}H NMR ($CDCl_3$) δ: 7.58 (t, J=8.4 Hz, 1H, CH arom.); 7.56 (d, J=16.0 Hz, 1H, *CH*═CH); 7.12 (d, J=8.4 Hz, 1H, CH arom.); 6.98 (s, 1H, CHCO); 6.81 (d, J=8.4 Hz, 1H, CH arom.); 6.72 (s, 2H, CH arom.); 6.32 (d, J=16.0 Hz, 1H, CH═*CH*); 4.45 (t, J=6.4 Hz, 2H, CH_2O); 4.26 (t, J=6.4 Hz, 2H, CH_2O); 3.96 (s, 3H, OCH_3); 3.88 (s, 6H, OCH_3); 3.87 (s, 3H, OCH_3); 2.55–2.50 (m, 4H, NCH_2); 2.27 (s, 3H, NCH_3); 1.99–1.89 (m, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 178.01 (C); 166.94 (C); 160.00 (C); 159.01 (C); 157.98 (C); 153.38 (C); 149.55 (C); 144.71 (*CH*═CH); 134.72 (CH); 129.87 (C); 117.25 (CH); 116.42 (CH═*CH*); 110.53 (CH); 106.84 (CH); 103.21 (CH); 65.02 (CH_2O); 62.74 (CH_2O); 60.96 (OCH_3); 56.48 (OCH_3); 56.15 (OCH_3); 54.22 (NCH_2); 53.79 (NCH_2); 41.89 (NCH_3); 26.63 (CH_2); 26.35 (CH_2) ppm.

Oxalate: mp 87–89 °C. Anal: $C_{32}H_{37}NO_{14}$ (C, H, N).

4.1.16.2. 3-({3-[(E)-5-Hydroxy-4-oxo-4H-chromene-2-carbonyloxy]propyl}(methyl) amino)propyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***2***

Free base: yield 93%. ^{1}H NMR ($CDCl_3$) δ: (t, J=8.4 Hz, 1H, CH arom.); 7.55 (d, J=16.0 Hz, 1H, *CH*═CH); 7.02 (s, 1H, CHCO); 7.01 (d, J=8.4 Hz, 1H, CH arom.); 6.82 (d, J=8.4 Hz, 1H, CH arom.); 6.72 (s, 2H, CH arom.); 6.31 (d, J=16.0 Hz, 1H, CH═*CH*); 4.48 (t, J=6.4 Hz, 2H, CH_2O); 4.26 (t, J=6.4 Hz, 2H, CH_2O); 3.87 (s, 9H, OCH_3); 2.53–2.47 (m, 4H, NCH_2); 2.25 (s, 3H, NCH_3); 2.00–1.84 (m, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 183.72 (C); 166.92 (C); 160.68 (C); 159.89 (C); 153.42 (C); 153.00 (C); 149.56 (C); 144.73 (*CH*═CH); 136.48 (CH); 129.83 (C); 117.22 (CH═*CH*); 113.46 (CH); 112.04 (CH); 107.71 (CH); 105.26 (CH); 65.39 (CH_2O); 62.77 (CH_2O); 60.95 (OCH_3); 56.16 (OCH_3); 54.22 (NCH_2); 53.75 (NCH_2); 41.97 (NCH_3); 26.73 (CH_2); 26.42 (CH_2)

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ppm.

Oxalate: mp 106–107 °C. Anal: $C_{31}H_{35}NO_{14}$ (C, H, N).

*4.1.16.3. 3-({3-[(E)-5,7-Dimethoxy-4-oxo-4H-chromene-2-carbonyloxy]propyl}(methyl)amino)propyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **3***

Free base: chromatographic eluent: CH_2Cl_2/MeOH 95:5. Yield 54%. ^{1}H NMR ($CDCl_3$) δ: 7.50 (d, J=16.0 Hz, 1H, *CH*═CH); 6.87 (s, 1H, CHCO); 6.68 (s, 2H, CH arom.); 6.50 (d, J=2.0 Hz, 1H, CH arom.); 6.28 (d, J=2.0 Hz, 1H, CH arom.); 6.26 (d, J=16.0 Hz, 1H, CH═*CH*); 4.39 (t, J=6.4 Hz, 2H, CH_2O); 4.20 (t, J=6.4 Hz, 2H, CH_2O); 3.85 (s, 3H, OCH_3); 3.83 (s, 6H, OCH_3); 3.82 (s, 6H, OCH_3); 2.49–2.42 (m, 4H, NCH_2); 2.21 (s, 3H, NCH_3); 1.91–1.79 (m, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 176.94 (C); 166.88 (C); 164.75 (C); 160.82 (C); 160.53 (C); 159.65 (C); 153.33 (C); 149.73 (C); 144.63 (CH═CH); 140.01 (C); 129.83 (C); 117.21 (CH═CH); 116.60 (CH); 109.97 (C); 105.20 (CH); 96.69 (CH); 93.07 (CH); 64.91 (CH_2O); 62.71 (CH_2O); 60.88 (OCH_3); 56.34 (OCH_3); 56.11 (OCH_3); 55.85 (OCH_3); 54.18 (NCH_2); 53.73 (NCH_2); 41.86 (NCH_3); 26.62 (CH_2); 26.33 (CH_2) ppm.

Oxalate: mp 120–121 °C. Anal: $C_{33}H_{39}NO_{15}$ (C, H, N).

*4.1.16.4. 3-({3-[(E)-5,7-Dihydroxy-4-oxo-4H-chromene-2-carbonyloxy]propyl}(methyl)amino)propyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **4***

Free base: chromatographic eluent: CH_2Cl_2/MeOH 95:5. Yield 71%. ^{1}H NMR ($CDCl_3$) δ: 7.52 (d, J=16.0 Hz, 1H, *CH*═CH); 6.75 (s, 1H, CHCO); 6.70 (s, 2H, CH arom.); 6.31 (d, J=16.0 Hz, 1H, CH═*CH*); 6.30 (d, J=2.0 Hz, 1H, CH arom.); 6.20 (d, J=2.0 Hz, 1H, CH arom.); 4.39 (t, J=6.4 Hz, 2H, CH_2O); 4.25 (t, J=6.4 Hz, 2H, CH_2O); 3.86 (s, 6H, OCH_3); 3.85 (s, 3H, OCH_3); 2.67–2.61 (m, 4H, NCH_2); 2.35 (s, 3H, NCH_3); 2.02–1.93 (m, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 181.70 (C); 167.21 (C); 165.41 (C); 162.26 (C); 159.87 (C); 157.58 (C); 153.38 (C); 151.67 (C); 145.17 (*CH*═CH); 140.09 (C); 129.74 (C); 116.85 (CH); 113.34 (CH═*CH*); 105.78 (C); 105.25 (CH); 100.35 (CH); 95.03 (CH); 64.57 (CH_2O); 62.58 (CH_2O); 60.97 (OCH_3); 56.15 (OCH_3); 54.11 (NCH_2); 53.49 (NCH_2); 41.24 (NCH_3); 25.96 (CH_2); 25.78 (CH_2) ppm.

Oxalate: mp 175–177 °C. Anal: $C_{31}H_{35}NO_{15}$ (C, H, N).

*4.1.16.5. 3-{[3-({2-[4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy] acetyl}oxy)propyl](methyl)amino}propyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **5***

Free base: chromatographic eluent: CH_2Cl_2/MeOH 95:5. Yield 12%. ^{1}H NMR ($CDCl_3$) δ: 7.68 (d, J=8.8 Hz, 2H, CH arom.); 7.58 (d, J=16.0 Hz, 1H, *CH*═CH); 6.91 (d, J=8.8 Hz, 2H, CH arom.); 6.77 (s, 2H, CH arom.); 6.56–6.52 (m, 2H, CH arom. and CHCO); 6.36 (d, J=16.0 Hz, 1H, CH═*CH*); 6.33–6.28 (m, 1H, CH arom.); 4.64 (s, 2H, OCH_2CO); 4.29 (t, J=6.4 Hz, 2H, CH_2O); 4.23 (t, J=6.4 Hz, 2H, CH_2O); 3.86 (s, 9H, OCH_3); 2.54–2.47 (m, 4H, NCH_2); 2.27 (s, 3H, NCH_3); 1.89–1.87 (m, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 182.34 (C); 168.14 (C); 167.14 (C); 164.45 (C); 163.22 (C); 162.17 (C); 160.56 (C); 157.42 (C); 153.38 (C); 145.03 (*CH*═CH); 139.99 (C); 129.81 (C); 128.30 (CH); 122.24 (C); 117.05 (CH═*CH*); 116.30 (CH); 105.91 (C); 105.16 (CH); 103.67 (CH); 98.26 (CH); 93.31 (CH); 65.14 (OCH_2CO); 63.85 (CH_2O); 62.84 (CH_2O); 61.01 (OCH_3); 56.13 (OCH_3); 54.11 (NCH_2); 53.80 (NCH_2); 41.71 (NCH_3); 26.18 (CH_2); 26.08 (CH_2) ppm. **Oxalate**: mp 112–113 °C. Anal: $C_{38}H_{41}NO_{16}$ (C, H, N).

*4.1.16.6. 5-({5-[(E)-5-Methoxy-4-oxo-4H-chromene-2-carbonyloxy]pentyl}(methyl)amino)pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **6***

Free base: chromatographic eluent: CH_2Cl_2/diethyl ether/petroleum ether/abs.EtOH/NH_4OH 120:120:300:60:3.3. Yield 81%. ^{1}H NMR ($CDCl_3$) δ: 7.60 (t, J=8.4 Hz, 1H, CH arom.); 7.58 (d, J=16.0 Hz, 1H, *CH*═CH); 7.15 (d, J=8.4 Hz, 1H, CH arom.); 6.98 (s, 1H, CHCO); 6.83 (d, J=8.4 Hz, 1H, CH arom.); 6.74 (s, 2H, CH arom.); 6.33 (d, J=16.0 Hz, 1H, CH═*CH*); 4.36 (t, J=6.8 Hz, 2H, CH_2O); 4.19 (t, J=6.8 Hz, 2H, CH_2O); 3.98 (s, 3H, OCH_3); 3.88 (s, 6H, OCH_3); 3.87 (s, 3H, OCH_3); 2.37–2.33 (m, 4H, NCH_2); 2.22 (s, 3H, NCH_3); 1.80–1.43 (m, 12H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 178.05 (C); 167.01 (C); 160.02 (C); 159.04 (C); 157.92 (C); 153.44 (C); 149.57 (C); 144.59 (CH); 134.72 (*CH*═CH); 129.95 (C); 117.47 (CH═*CH*); 116.45 (CH); 110.61 (CH); 106.89 (CH); 105.27 (CH); 66.77 (CH_2O); 64.56 (CH_2O); 60.96 (OCH_3); 57.67 (NCH_2); 57.52 (NCH_2); 56.52 (OCH_3); 56.18 (OCH_3); 42.17 (NCH_3); 28.72 (CH_2); 28.42 (CH_2); 26.83 (CH_2); 23.99 (CH_2); 23.84 (CH_2) ppm.

Oxalate: mp 88–89 °C. Anal: $C_{36}H_{45}NO_{14}$ (C, H, N).

*4.1.16.7. 5-({5-[(E)-5-Hydroxy-4-oxo-4H-chromene-2-carbonyloxy]pentyl}(methyl)amino)pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **7***

Free base: chromatographic eluent: CH_2Cl_2/MeOH 95:5. Yield 73%. ^{1}H NMR ($CDCl_3$) δ: 7.59–7.53 (m, 2H, CH arom. and *CH*═CH); 7.04 (d, J=8.0 Hz, 1H, CH arom.); 7.03 (s, 1H, CH arom.); 6.83 (d, J=8.0 Hz, 1H, CH arom.); 6.74 (s, 2H, CH arom.); 6.33 (d, J=16.0 Hz, 1H, CH═*CH*); 4.39 (t, J=6.4 Hz, 2H, CH_2O); 4.19 (t, J=6.4 Hz, 2H, CH_2O); 3.88 (s, 6H, OCH_3); 3.87 (s, 3H, OCH_3); 2.39–2.36 (m, 4H, NCH_2); 2.23 (s, 3H, NCH_3); 1.84–1.68 (m, 6H, CH_2); 1.61–1.42 (m, 6H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 167.01 (C); 160.70 (C); 153.44 (C); 153.00 (C); 149.56 (C); 144.63 (CH═CH); 136.52 (CH); 129.93 (C); 117.43 (CH═CH); 113.51 (CH); 112.08 (CH); 107.77 (CH); 105.27 (CH); 67.09 (CH_2O); 64.53 (CH_2O); 60.97 (OCH_3); 57.64 (NCH_2); 57.48 (NCH_2); 56.18 (OCH_3); 42.12 (NCH_3); 29.70 (CH_2); 28.71 (CH_2); 28.38 (CH_2); 26.84 (CH_2); 23.97 (CH_2); 23.80 (CH_2) ppm.

Oxalate: mp 106–107 °C. Anal: $C_{35}H_{43}NO_{14}$ (C, H, N).

*4.1.16.8. 5-({5-[(E)-5,7-Dimethoxy-4-oxo-4H-chromene-2-carbonyloxy]pentyl}(methyl)amino)pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **8***

Free base: chromatographic eluent: CH_2Cl_2/MeOH 95:5. Yield 68%. ^{1}H NMR ($CDCl_3$) δ: 7.57 (d, J=16.0 Hz, 1H, *CH*═CH); 6.92 (s, 1H, CH arom.); 6.73 (s, 2H, CH arom.); 6.59 (d, J=2.0 Hz, 1H, CHCO); 6.37 (d, J=2.0 Hz, 1H, CH arom.); 6.32 (d, J=16.0 Hz, 1H, CH═*CH*); 4.34 (t, J=6.4 Hz, 2H, CH_2O); 4.18 (t, J=6.4 Hz, 2H, CH_2O); 3.92 (s, 3H, OCH_3); 3.87 (s, 6H, OCH_3); 3.85 (s, 6H, OCH_3); 2.49–2.45 (m, 4H, NCH_2); 2.33 (s, 3H, NCH_3); 1.82–1.46 (m, 12H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 181.60 (C); 167.46 (C); 167.03 (C); 162.20 (C); 160.07 (C); 157.91 (C); 153.40 (C); 151.78 (C); 144.75 (*CH*═CH); 140.10 (C); 129.83 (C); 117.21 (CH═*CH*); 113.32 (CH); 105.42 (C); 105.22 (CH); 100.90 (CH); 95.45 (CH); 66.41 (CH_2O); 64.27 (CH_2O); 60.90 (OCH_3); 57.12 (NCH_2); 56.85 (NCH_2); 56.30 (OCH_3); 56.10 (OCH_3); 55.72 (OCH_3); 41.12 (NCH_3); 28.49 (CH_2); 28.09 (CH_2); 25.42 (CH_2); 25.31 (CH_2); 23.76 (CH_2); 23.68 (CH_2) ppm.

Oxalate: mp 110–111 °C. Anal: $C_{37}H_{47}NO_{15}$ (C, H, N).

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4.1.16.9. 5-({5-[(E)-5,7-Dihydroxy-4-oxo-4H-chromene-2-carbonyloxy]pentyl}(methyl)amino)pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***9***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 90:10:0.5. Yield 71%. ^{1}H NMR ($CDCl_3$) δ: 7.55 (d, J=16.0 Hz, 1H, *CH*═CH); 6.81 (s, 1H, CHCO); 6.72 (s, 2H, CH arom.); 6.33 (d, J=2.0 Hz, 1H, CH arom.); 6.30 (d, J=16.0 Hz, 1H, CH═*CH*); 6.19 (d, J=2.0 Hz, 1H, CH arom.); 4.29 (t, J=6.4 Hz, 2H, CH_2O); 4.16 (t, J=6.4 Hz, 2H, CH_2O); 3.86 (s, 6H, OCH_3); 3.85 (s, 3H, OCH_3); 2.62–2.58 (m, 4H, NCH_2); 2.42 (s, 3H, NCH_3); 1.77–1.63 (m, 8H, CH_2); 1.44–1.40 (m, 4H, $2CH_2$) ppm. ^{13}C NMR ($CDCl_3$) δ: 181.59 (C); 167.48 (C); 167.05 (C); 162.18 (C); 160.06 (C); 157.90 (C); 153.41 (C); 151.78 (C); 144.78 (*CH*═CH); 140.11 (C); 129.87 (C); 117.25 (CH═*CH*); 113.37 (CH); 105.40 (C); 105.28 (CH); 100.94 (CH); 95.48 (CH); 66.46 (CH_2O); 64.22 (CH_2O); 60.94 (OCH_3); 57.00 (NCH_2); 56.86 (NCH_2); 56.17 (OCH_3); 41.12 (NCH_3); 28.49 (CH_2); 28.09 (CH_2); 25.42 (CH_2); 25.31 (CH_2); 23.76 (CH_2); 23.68 (CH_2) ppm.

Oxalate: mp 160–167 °C. Anal: $C_{35}H_{43}NO_{15}$ (C, H, N).

4.1.16.10. 5-{[5-({2-[4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy] acetyl}oxy)pentyl](methyl)amino}pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***10***

Free base: chromatographic eluent: CH_2Cl_2/MeOH 95:5. Yield 18%. ^{1}H NMR (CD_3OD) δ: 7.85 (d, J=8.4 Hz, 2H, CH arom.); 7.57 (d, J=16.0 Hz, 1H, *CH*═CH); 6.92 (d, J=8.4 Hz, 2H, CH arom.); 6.88 (s, 2H, CH arom.); 6.64 (d, J=2.0 Hz, 1H, CH arom.); 6.62 (s, 1H, CHCO); 6.43 (d, J=16.0 Hz, 1H, CH═*CH*); 6.34 (d, J=2.0 Hz, 1H, CH arom.); 4.87 (s, 2H, OCH_2CO); 4.26 (t, J=6.4 Hz, 2H, CH_2O); 4.21 (t, J=6.4 Hz, 2H, CH_2O); 3.84 (s, 6H, OCH_3); 3.79 (s, 3H, OCH_3); 2.99–2.91 (m, 4H, NCH_2); 2.72 (s, 3H, NCH_3); 1.78–1.68 (m, 12H, CH_2) ppm.

Oxalate: mp 165–166 °C. Anal: $C_{42}H_{49}NO_{16}$ (C, H, N).

4.1.16.11. 2-{2-[(2-{2-[(E)-5,7-Dihydroxy-4-oxo-4H-chromene-2-carbonyloxy] ethoxy}ethyl)(methyl)amino]ethoxy}ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***11***

Free base: chromatographic eluent: CH_2Cl_2/petroleum ether/abs.EtOH/NH_4OH 340:60:65:8. Yield 79%. ^{1}H NMR ($CDCl_3$) δ: 7.53 (d, J=16.0 Hz, 1H, *CH*═CH); 6.71 (s, 1H, CHCO); 6.69 (s, 2H, CH arom.); 6.29 (d, J=16.0 Hz, 1H, CH═*CH*); 6.28 (d, J=2.0 Hz, 1H, CH arom.); 6.20 (d, J=2.0 Hz, 1H, CH arom.); 4.38 (t, J=6.4 Hz, 2H, CH_2O); 4.28 (t, J=6.4 Hz, 2H, CH_2O); 3.85 (s, 9H, OCH_3); 3.78–3.70 (m, 2H, OCH_2); 3.69–3.61 (m, 6H, OCH_2); 2.83–2.75 (m, 4H, NCH_2); 2.42 (s, 3H, NCH_3) ppm. ^{13}C NMR ($CDCl_3$) δ: 181.54 (C); 166.89 (C); 166.06 (C); 162.06 (C); 159.78 (C); 157.52 (C); 153.37 (C); 151.27 (C); 145.17 (*CH*═CH); 140.07 (C); 129.77 (C); 116.86 (CH═*CH*); 113.48 (CH); 105.50 (C); 105.29 (CH); 100.63 (CH); 95.18 (CH); 69.07 (CH_2O); 68.91 (CH_2O); 68.45 (CH_2O); 68.32 (CH_2O); 68.22 (CH_2O); 68.06 (CH_2O); 65.76 (CH_2O); 63.64 (CH_2O); 56.94 (NCH_2); 56.78 (NCH_2); 56.20 (OCH_3); 56.06 (OCH_3); 42.50 (NCH_3) ppm.

Oxalate: mp 53–56 °C. Anal: $C_{33}H_{39}NO_{17}$ (C, H, N).

4.1.16.12. 5-{Methyl-[5-({2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy) pentyl]amino}pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***12***

Free base: chromatographic eluent: CH_2Cl_2/diethyl ether/petroleum ether/abs.EtOH/NH_4OH 120:120:300:60:3.3. Yield 78.5%. ^{1}H NMR ($CDCl_3$) δ: 8.17 (d, J=8.0 Hz, 1H, CH arom.), 7.84 (d, J=9.2 Hz, 2H, CH arom.); 7.64 (t, J=8.0 Hz, 1H, CH arom.); 7.55 (d, J=16.0 Hz, 1H, *CH*═CH); 7.49 (d, J=8.0 Hz, 1H, CH arom.); 7.36 (t, J=8.0 Hz, 1H, CH arom.); 6.98 (d, J=9.2 Hz, 2H, CH arom.); 6.71 (s, 2H, CH arom.); 6.69 (s, 1H, CHCO); 6.31 (d, J=16.0 Hz, 1H, CH═*CH*); 4.67 (s, 2H, OCH_2CO); 4.20–4.16 (m, 4H, CH_2OCO); 3.84 (s, 9H, OCH_3); 2.31–2.26 (m, 4H, CH_2N); 2.17 (s, 3H, NCH_3); 1.70–1.63 (m, 4H, CH_2); 1.52–1.25 (m, 8H, CH_2); ppm. ^{13}C NMR ($CDCl_3$) δ: 178.23 (C); 168.33 (C); 166.97 (C); 163.01 (C); 160.48 (C); 156.13 (C); 153.40 (C); 144.58 (*CH*═CH); 133.62 (CH); 129.90 (C); 128.02 (CH); 125.62 (CH); 125.12 (CH); 125.04 (C); 123.89 (C); 117.95 (CH); 117.42 (CH═*CH*); 115.06 (CH); 106.43 (CH); 105.22 (CH); 65.52 (CH_2O); 65.21 (CH_2O); 64.50 (CH_2O); 60.92 (OCH_3); 57.62 (CH_2N); 57.53 (CH_2N); 56.13 (OCH_3); 56.10 (OCH_3); 42.13 (NCH_3); 28.68 (CH_2); 28.45 (CH_2); 26.87 (CH_2); 26.82 (CH_2); 23.93 (CH_2); 23.75 (CH_2) ppm.

Hydrochloride: mp 105–107 °C. Anal: $C_{40}H_{48}ClNO_{10}$ (C, H, N).

4.1.16.13. 5-{Benzyl[5-({2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy)pentyl] amino}pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***13***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Yield 50.8%. ^{1}H NMR ($CDCl_3$) δ: 8.20 (d, J=7.6 Hz, 1H, CH arom.); 7.87 (d, J=8.8 Hz, 2H, CH arom.); 7.66 (t, J=7.6 Hz, 1H, CH arom.); 7.58 (d, J=16.0 Hz, 1H, *CH*═CH); 7.52 (d, J=7.6 Hz, 1H, CH arom.); 7.39 (t, J=7.6 Hz, 1H, CH arom.); 7.31–7.20 (m, 5H, CH arom.); 7.00 (d, J=8.8 Hz, 2H, CH arom.); 6.74 (s, 1H, CHCO); 6.72 (s, 2H, CH arom.); 6.33 (d, J=16.0 Hz, 1H, CH═*CH*); 4.68 (s, 2H, OCH_2CO); 4.20–4.15 (m, 4H, CH_2OCO); 3.87 (s, 6H, OCH_3); 3.85 (s, 3H, OCH_3); 3.53 (s, 2H, NCH_2Ph); 2.42–2.38 (m, 4H, CH_2N); 1.67–1.60 (m, 4H, CH_2); 1.53–1.45 (m, 4H, CH_2); 1.41–1.29 (m, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 178.30 (C); 168.34 (C); 167.01 (C); 163.06 (C); 160.51 (C); 156.18 (C); 153.44 (C); 144.61 (*CH*═CH); 140.13 (C); 133.64 (CH); 129.92 (C); 128.79 (CH); 128.16 (CH); 128.06 (CH); 125.68 (CH); 125.15 (CH); 123.94 (C); 117.97 (CH); 117.44 (CH═*CH*); 115.09 (CH); 106.50 (CH); 105.26 (CH); 65.59 (CH_2O); 65.24 (CH_2O); 64.57 (CH_2O); 60.96 (OCH_3); 58.61 (NCH_2Ph); 56.16 (OCH_3); 53.62 (CH_2N); 53.52 (CH_2N); 28.63 (CH_2); 28.38 (CH_2); 26.66 ($2CH_2$); 23.77 (CH_2); 23.57 (CH_2) ppm.

Hydrochloride: mp 58–60 °C. Anal: $C_{46}H_{52}ClNO_{10}$ (C, H, N).

4.1.16.14. 2-{2-[Methyl-({2-[2-({2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetyl}oxyethoxy]ethyl})amino]ethoxy}ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***15***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 93:7:0.3 Yield 6.2%. ^{1}H NMR ($CDCl_3$) δ: 8.21 (d, J=7.6 Hz, 1H, CH arom.); 7.88 (d, J=8.8 Hz, 2H, CH arom.); 7.68 (t, J=7.6 Hz, 1H, CH arom.); 7.62–7.53 (m, 2H, CH arom. and *CH*═CH); 7.41 (t, J=7.6 Hz, 1H, CH arom.); 7.03 (d, J=8.8 Hz, 2H, CH arom.); 6.75 (s, 2H, CH arom.); 6.74 (s, 1H, CHCO); 6.37 (d, J=16.0 Hz, 1H, CH═*CH*); 4.76 (s, 2H, OCH_2CO); 4.39–4.34 (m, 4H, $2CH_2OCO$); 3.88 (s, 9H, OCH_3); 3.74–3.49 (m, 8H, CH_2O); 2.72–2.64 (m, 4H, CH_2N); 2.36 (s, 3H, NCH_3) ppm. ESI-MS: [M−H]+ species at *m*/*z* 706.

Hydrochloride: low melting solid. Anal: $C_{38}H_{44}ClNO_{12}$ (C, H, N).

4.1.16.15. 2-{2-[Benzyl({2-[2-({2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy)ethoxy]ethyl})amino]ethoxy}ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate ***16***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Yield 73.0%. ^{1}H NMR ($CDCl_3$) δ: 8.20 (d, J=8.0 Hz, 1H, CH arom.); 7.87 (d, J=8.8 Hz, 2H, CH arom.); 7.66 (t, J=8.0 Hz, 1H, CH arom.); 7.59 (d, J=16.0 Hz, 1H, *CH*═CH); 7.53 (d, J=8.0 Hz, 1H, CH arom.); 7.39 (t, J=8.0 Hz, 1H, CH arom.);

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7.35–7.20 (m, 5H, CH arom.); 7.00 (d, J=8.8 Hz, 2H, CH arom.); 6.73 (s, 1H, CHCO); 6.72 (s, 2H, CH arom.); 6.36 (d, J=16.0 Hz, 1H, CH═*CH*); 4.69 (s, 2H, OCH_2CO); 4.35–4.31 (m, 4H, CH_2OCO); 3.87 (s, 6H, OCH_3); 3.85 (s, 3H, OCH_3); 3.71–3.64 (m, 4H, OCH_2); 3.62 (s, 2H, NCH_2Ph); 3.60–3.55 (m, 4H, OCH_2); 2.79–2.73 (m, 4H, CH_2N) ppm. ^{13}C NMR ($CDCl_3$) δ: 178.33 (C); 168.24 (C); 166.85 (C); 163.06 (C); 160.43 (C); 156.19 (C); 153.43 (C); 145.09 (*CH*═CH); 133.64 (CH); 129.81 (C); 128.24 (CH); 128.07 (CH); 125.69 (CH); 125.15 (CH); 123.94 (C); 117.97 (CH); 117.04 (CH═*CH*); 115.11 (CH); 106.53 (CH); 105.31 (CH); 69.00 (OCH_2); 68.54 (OCH_2); 65.11 (OCH_2); 64.44 (OCH_2); 63.59 (OCH_2); 60.96 (OCH_3); 59.74 (NCH_2Ph); 56.16 (OCH_3); 53.83 (CH_2N) ppm.

Hydrochloride: low melting solid. Anal: $C_{44}H_{48}ClNO_{12}$ (C, H, N).

*4.1.17. 5-{[(tert-Butoxy)carbonyl](5-hydroxypentyl)amino}pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **44***

To a solution of **42**[32] (278 mg, 0.68 mmol) in 10 mL of THF stirred at 0 °C, a solution of 185 mg of di-*tert*-butyl dicarbonate (0.85 mmol) in 5 mL of THF and triethylamine (0.19 mL, 1.36 mmol) were added, and the mixture was stirred 30 min in ice-bath and 18 h at room temperature. The solution was concentrated in vacuo and the oily residue was dissolved in CH_2Cl_2; the organic solution was washed twice with water, dried on Na_2SO_4, and evaporated yielding 275 mg (yield: 79.5%) of title compound as a yellow oil that was not further purified. ^1H NMR ($CDCl_3$) δ: 7.58 (d, J=15.8 Hz, 1H, *CH*═CH); 6.75 (s, 2H, CH arom.); 6.33 (d, J=15.8 Hz, 1H, CH═*CH*); 4.19 (t, J=6.4 Hz, 2H, CH_2OCO); 3.89 (s, 6H, OCH_3); 3.87 (s, 3H, OCH_3); 3.64–3.61 (m, 2H, CH_2OH); 3.20–3.14 (m, 4H, CH_2N); 1.74–1.69 (m, 4H, CH_2); 1.45 (s, 9H, *t*-butyl); 1.74–1.32 (m, 8H, CH_2) ppm.

*4.1.18. 5-{[(tert-Butoxy)carbonyl][5-({2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy)pentyl]amino}pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **46***

To 275 mg (0.54 mmol) of alcohol **44** dissolved in 25 mL of anhydrous CH_2Cl_2, a solution of 240 mg (0.81 mmol) of acid **30** in 25 mL of anhydrous THF, 53 mg (0.43 mmol) of DMAP and 186 mg (0.97 mmol) of EDCI were added. The reaction mixture was stirred at room temperature for 70 h, then it was evaporated. The obtained residue was dissolved in CH_2Cl_2, and the organic layer was washed twice with water and one time with a saturated solution of $NaHCO_3$, dried on Na_2SO_4 and evaporated to give the crude ester. After purification by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 95:5:0.5 as eluting system, 189 mg (44.5% yield) of title compound as colorless oil were obtained. ^1H NMR ($CDCl_3$) δ: 8.21 (d, J=8.0 Hz, 1H, CH arom.); 7.88 (d, J=9.2 Hz, 2H, CH arom.); 7.67 (t, J=8.0 Hz, 1H, CH arom.); 7.56–7.49 (m, 2H, CH arom. and *CH*═CH); 7.41 (t, J=8.0 Hz, 1H, CH arom.); 7.02 (d, J=9.2 Hz, 2H, CH arom.); 6.74 (s, 2H, CH arom.); 6.73 (s, 1H, CHCO); 6.32 (d, J=16.0 Hz, 1H, CH═*CH*); 4.69 (s, 2H, OCH_2CO); 4.23–4.18 (m, 4H, CH_2OCO); 3.87 (s, 6H, OCH_3); 3.86 (s, 3H, OCH_3); 3.18–3.13 (m, 4H, CH_2N); 1.78–1.67 (m, 4H, CH_2); 1.56–1.49 (m, 4H, CH_2); 1.44 (s, 9H, *t*-butyl); 1.40–1.29 (m, 4H, CH_2) ppm.

*4.1.19. 2-(2-{[(tert-Butoxy)carbonyl]({2-[2-({2-[4-(4-oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy)ethoxy]ethyl})amino}ethoxy)ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **47***

To 99 mg (0.19 mmol) of alcohol **45**[27] dissolved in 15 mL of anhydrous CH_2Cl_2, a solution of 86 mg (0.10 mmol) of acid **30** in 15 mL of anhydrous THF, 19 mg (0.29 mmol) of DMAP and 66 mg (0.35 mmol) of EDCI were added. The reaction mixture was stirred at room temperature for 70 h, then it was evaporated. The obtained residue was dissolved in CH_2Cl_2, and the organic layer was washed twice with water and one time with a saturated solution of $NaHCO_3$, dried on Na_2SO_4 and evaporated to give the crude ester. After purification by flash chromatography, using CH_2Cl_2/MeOH/NH_4OH 95:5:0.5 as eluting system, 72 mg (47.2% yield) of title compound as yellow oil were obtained. ^1H NMR ($CDCl_3$) δ: 8.20 (d, J=7.6 Hz; 1H, CH arom.); 7.87 (d, J=8.8 Hz, 2H, CH arom.); 7.66 (t, J=7.6 Hz, 1H, CH arom.); 7.59 (d, J=15.8 Hz, 1H, *CH*═CH); 7.53 (d, J=8.0 Hz, 1H, CH arom.); 7.40 (t, J=8.0 Hz, 1H, CH arom.); 7.02 (d, J=8.8 Hz, 2H, CH arom.); 6.75 (s, 2H, CH arom.); 6.73 (s, 1H, CHCO); 6.36 (d, J=15.8 Hz, 1H, CH═*CH*); 4.73 (s, 2H, OCH_2CO); 4.37–4.32 (m, 4H, CH_2OCO); 3.87 (s, 3H, OCH_3); 3.86 (s, 6H, OCH_3); 3.72–3.53 (m, 8H, OCH_2); 3.50–3.42 (m, 4H, CH_2N); 1.44 (s, 9H, *t*-butyl) ppm.

*4.1.20. General procedure for the synthesis of diester compounds **14** and **17***

To a solution of the suitable BOC-protected ester (**46** or **47**) (0.1 mmol) in anhydrous CH_2Cl_2, 26 eq of trifluoroacetic acid were added. The mixture was stirred 45 min at room temperature, then it was evaporated. The obtained residue was dissolved in CH_2Cl_2, and the organic layer was washed three times with a saturated solution of $NaHCO_3$, dried on Na_2SO_4 and evaporated to give the crude ester, which was purified by flash chromatography using the appropriate eluting system, as reported hereinafter. The compounds were transformed into the corresponding hydrochloride as white solid. The salts were crystallized from abs. ethanol/petroleum ether.

*4.1.20.1. 5-{[5-({2-[4-(4-Oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy)pentyl]amino}pentyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **14***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 97:3:0.3. Yield 88.7%. ^1H NMR ($CDCl_3$) δ: 8.20 (d, J=8.0 Hz, 1H, CH arom.); 7.88 (d, J=8.8 Hz, 2H, CH arom.); 7.67 (t, J=8.0 Hz, 1H, CH arom.); 7.57 (d, J=15.6 Hz, 1H, *CH*═CH); 7.53 (d, J=8.0 Hz, 1H, CH arom.); 7.39 (t, J=8.0 Hz, 1H, CH arom.); 7.02 (d, J=8.8 Hz, 2H, CH arom.); 6.74 (s, 3H, CHCO and CH arom.); 6.33 (d, J=15.6 Hz, 1H, CH═*CH*); 4.70 (s, 2H, OCH_2CO); 4.23–4.16 (m, 4H, CH_2OCO); 3.87 (s, 9H, OCH_3); 2.64–2.59 (m, 4H, CH_2N); 1.71–1.33 (m, 12H, CH_2) ppm. ^{13}C NMR ($CDCl_3$) δ: 178.33 (C); 168.36 (C); 167.00 (C); 163.09 (C); 160.51 (C); 156.19 (C); 153.44 (C); 144.67 (*CH*═CH); 133.66 (CH); 129.91 (C); 128.08 (CH); 125.68 (CH); 125.17 (CH); 123.92 (C); 117.97 (CH); 117.39 (CH═*CH*); 115.10 (CH); 106.50 (CH); 105.28 (CH); 65.44 (OCH_2); 65.25 (OCH_2); 64.42 (OCH_2); 60.95 (OCH_3); 56.17 (OCH_3); 49.64 (CH_2N); 49.56 (CH_2N); 29.31 (CH_2); 29.21 (CH_2); 28.65 (CH_2); 28.41 (CH_2); 23.79 (CH_2); 23.61 (CH_2) ppm.

Hydrochloride: 110–112 °C. Anal: $C_{39}H_{46}ClNO_{10}$ (C, H, N).

*4.1.20.2. 2-[2-({2-[2-({2-[4-(4-Oxo-4H-chromen-2-yl)phenoxy]acetyl}oxy)ethoxy]ethyl}amino)ethoxy]ethyl (2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **17***

Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Yield 87.6%. ^1H NMR ($CDCl_3$) δ: 8.20 (d, J=8.0 Hz, 1H, CH arom. 7.87 (d, J=8.8 Hz, 2H, CH arom.); 7.66 (t, J=8.0 Hz, 1H, CH arom.); 7.59 (d, J=16.0 Hz, 1H, *CH*═CH); 7.53 (d, J=8.0 Hz, 1H, CH arom.); 7.40 (t, J=8.0 Hz, 1H, CH arom.); 7.02 (d, J=8.8 Hz, 2H, CH arom.); 6.73 (s, 3H, CHCO and CH arom.); 6.37 (d, J=16.0 Hz, 1H, CH═*CH*); 4.73 (s, 2H, OCH_2CO); 4.37–4.33 (m, 4H, CH_2OCO); 3.85 (s, 9H, OCH_3); 3.74–3.57 (m, 8H, OCH_2); 2.85–2.80 (m, 4H, CH_2N) ppm. ^{13}C NMR ($CDCl_3$) δ: 178.33 (C); 168.27 (C); 166.86 (C); 163.06 (C); 160.44 (C); 156.17 (C); 153.42

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(C); 145.12 (*CH*═CH); 133.64 (CH); 129.80 (C); 128.08 (CH); 125.67 (CH); 125.15 (CH); 123.91 (C); 117.97 (CH); 117.01 (CH═*CH*); 115.12 (CH); 106.49 (CH); 105.30 (CH); 70.57 (OCH_2); 69.11 (OCH_2); 68.66 (OCH_2); 65.12 (OCH_2); 64.37 (OCH_2); 63.55 (OCH_2); 60.95 (OCH_3); 56.15 (OCH_3); 49.19 (CH_2N); 49.15 (CH_2N) ppm.

Hydrochloride: 122–123 °C. Anal: $C_{37}H_{42}ClNO_{12}$ (C, H, N).

4.2. Biology

4.2.1. Cell lines and cultures

The K562 is an undifferentiated erythroleukemia cell line originally derived from a patient with chronic myelogenous leukemia.[36] The K562 leukemia cells and the P-gp over-expressing K562/DOX cells were obtained from Prof. J.P. Marie (Hopital Hotel-Dieu, Paris, France). The cells were cultured following a previously reported protocol.[42]

4.2.2. Drugs and chemicals

Purified verapamil and pirarubicin were purchased by Sigma-Aldrich (Milan – Italy). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11{,}500\ M^{-1}\ cm^{-1}$. Stock solutions were prepared just before use. Buffer solutions were HEPES buffer containing 5 mM HEPES, 132 mM NaCl, 3.5 mM $CaCl_2$, 5 mM glucose, at pH 7.3.

The uptake of pirarubicin in cells was followed by monitoring the decrease in the fluorescence signal at 590 nm ($\lambda_{ex} = 480$ nm) according to the previously described method.[43,44]

4.3. Molecular dynamics simulations

A 10 ns MD simulation was performed for all molecules using GROMACS v5.1 program[45]. The DS ViewerPro 6.0 program[46] was used to build the initial conformations of molecules. The partial atomic charge of the structures, were calculated with CHIMERA[47] using AM1-BCC method and the topology was created with ACPYPE[48] based on the routine Antechamber[49].

The OPLS-AA/L all-atom force field[50] parameters were applied to all the structures.

The simulation was performed in vacuum. To remove bad contacts, energy minimization was performed using the steepest descent algorithm until convergence is achieved or for 50,000 maximum steps. In the consecutive steps, equilibrations of the system were conducted in two phases: (1) canonical NVT ensemble, a 100 ps position-restrained of molecules at 300 K was carried out using a Temperature coupling thermostat (velocity rescaling with a stochastic term) to ensure the proper stabilization of the temperature[51]; (2) isothermalisobaric NPT ensemble, a 100 ps position-restrained of molecules at 300 K and 1 bar was carried out without using barostat pressure coupling to stabilize the system. These were then followed by a 10 ns MD run without position restraints at 300 K. the Lincs algorithm[52] was used for bond constraints to maintain rigid bond lengths.

The initial velocity was randomly assigned taken from Maxwell–Boltzman distribution at 300 K and computed with a time step of 2 fs, and the coordinates were recorded every 0.1 ns.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.11.016.

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