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Original article

Enhancement of drug affinity for cell membranes by conjugation with lipoamino acids. I. Synthesis and biological evaluation of lipophilic conjugates of tranylcypromine

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Abstract

Conjugation with lipoamino acids (LAAs) increases the lipophilicity of drug molecules. Because of their amphipatic nature, they also provide the conjugated drugs a '*membrane-like character*', capable to facilitate their interaction with and penetration through cell membranes and biological barriers. To study such a feature, our aim is to collect experimental and computational data using a novel series of lipophilic conjugates between a model drug (tranylcypromine (TCP)) and LAA residues containing a short, a medium or a long alkyl side chain (C-4 to C-16), to provide a wide range of lipophilicity. For comparison, a corresponding set of amides of TCP with alkanoic or fatty acids was prepared and characterized. Their in vitro monoamine oxidase inhibitory activity also tested.

Keywords: Lipoamino acids; Tranylcypromine; MAO inhibition; Lipophilicity; Prodrugs

1. Introduction

Enhancing the lipophilicity of drugs is a widely pursued strategy to overcome their difficulty in entering cells and/or crossing biological barriers, including the blood–brain barrier (BBB) [1–3]. Conjugation of drug molecules to lipoamino acids (LAAs) has shown to increase biological uptake and intracellular concentration [4–7].

LAAs are α-amino acids containing an alkyl side chain; the length and structure of the side chain, and the number of LAA residues can ultimately modulate the lipophilicity, stability and solubility of the resulting drug conjugate [4,6]. LAAs combine the physico-chemical properties of both lipids and amino acids; because of their amphipatic structure, their linkage to drugs, apart from enhancing their lipophilicity, can facilitate their interaction with cell membranes and penetration across absorption and biological barriers [4]. Depending on the lability of the drug-LAA linkage, drug conjugation to LAAs can result in either bioreversible prodrugs or stable derivatives, displaying their own biological activity [4].

We have synthesized two series of lipophilic derivatives of the monoamino oxidase inhibitor (MAOI) tranylcypromine (*trans*-(+)-2-phenylcyclopropanamine (TCP)) (Fig. 1). TCP is an irreversible MAO inhibitor that causes a long lasting, non-competitive inhibition of the enzyme complex [8]. Although more recent drugs are available in therapy, that act with different mechanisms and lower side effects, the use of classical MAOIs is still useful in particular cases, e.g. in

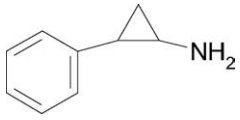

Fig. 1. Structure of tranylcypromine (TCP).

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patients not responding to tricyclic antidepressant drugs [9]. However, in this study TCP was only chosen as a model drug.

TCP was linked *via* an amide bond to either LAA residues or linear alkanoic (fatty) acids (FA), both containing a short, a medium or a long alkyl side chain (from 4 to 16 carbon atom chain). While TCP-FA are characterized by an increased lipophilicity, the conjugation of LAAs to TCP is expected to modulate the amphiphilicity of the final compounds [10].

The final goal of the present research, whose second part is forthcoming, is to assess the lipophilic character of TCP-LAA and TCP-FA derivatives using computational approaches and experimental (calorimetry) experiments. By the latter the interaction of these compounds with biomembrane models, consisting of phospholipid liposomes, will be analyzed.

In this first note, the synthesis and characterization of TCP lipophilic derivatives were reported. A preliminary in vitro biological assay was also carried out to correlate the MAO inhibitory activity of the prepared derivatives with their lipophilicity (calculated Log*P*).

2. Results and discussion

2.1. Synthesis and characterization of TCP-LAA and TCP-FA conjugates

TCP conjugates with LAAs and FAs were obtained in high purity and yields using a reported method [5]. TCP hydrochloride was reacted with Boc-protected 2-aminocarboxylic acids (LAAs) or alkyl carboxylic acis (FA) using a hydrosoluble carbodiimide (EDAC HCl) to make easier the further work-out of the reaction mixture, and HOBt to prevent isomerization reactions. The Boc-protecting group was easily removed using a classical treatment with diluted TFA.

The final compounds were obtained with good yields and purity following separation by column chromatography. The structure of conjugates **1**–**7** was confirmed by mass spectrometry, ^{1}H-NMR and FT-IR. In the latter (data not shown), the condensation of TCP with the LAA or FA moiety was confirmed by the appearance of weak signals due to the amide carbonyl, at 1690–1630 cm^{-1}, and the contemporary disappearance of the strong peak (around 3425 cm^{-1}) of the aromatic amine group in TCP.

A preliminary evaluation of the stability of conjugates **1a**–**7a** toward the hydrolytic cleavage indicated that all the compounds are stable at 37 °C and pH 7.4 up to 6 h, i.e. well over the conditions used in the biological tests. This ensured us that the enzyme inhibition activity registered can be attributed to the intact conjugates and not to the free TCP release upon their hydrolysis.

2.2. Biological evaluation

The inhibitory activity of TCP-LAA conjugates **1a**–**7a** against rat liver mitochondria MAO was evaluated by an in vitro assay, compared with the parent drug (Fig. 2). The assay consisted in measuring spectrophotometrically the amount of 4-OH-quinoline formed from kynuramine in the presence of MAO. The inihibition of the activity of the latter by TCP or derivatives **1**–**7** was linearly correlated to the reduction in 4-OH-quinoline formation.

All the tested conjugates still possess an inhibitory activity against MAO enzymes, however reduced with respect to TCP. To reach a 100% inhibition of the enzyme, we used a high drug concentration (100 μM); such a concentration was taken as reference for the other compounds, although beyond a therapeutic value.

Except the lower homologue **1a**, whose activity was negligible even at the highest dose, the other terms of the series showed a dose-related activity profile. At the concentration of 100 μM the order of activity was associated with the lipophilicity of conjugates, i.e. the length of side alkyl chain. The compound with the longest chain (C16, compound **7a**) was however less active, most probably because of its low solubility in the (aqueous) test medium.

On the other hand, the reduction of the inhibitory concentration observed with conjugates **1a**–**7a** in respect to the parent drug (pI_{50} raised from 0.8 for TCP to 30–50 μM for the more active conjugates), as well as the shape of the inhibition profiles (Fig. 2), suggest that these conjugates do not act with a mechanism-based enzyme inhibition, like TCP itself, but probably because of a competitive inhibition to MAO.

An optimal value of lipophilicity/solubility then exists at which the conjugates are able to interact with and inhibit stronger these mitochondrial enzymes, to reach whom the drug must penetrate the cell membrane. These findings are not of course a novelty in the field of lipophilic prodrugs; however,

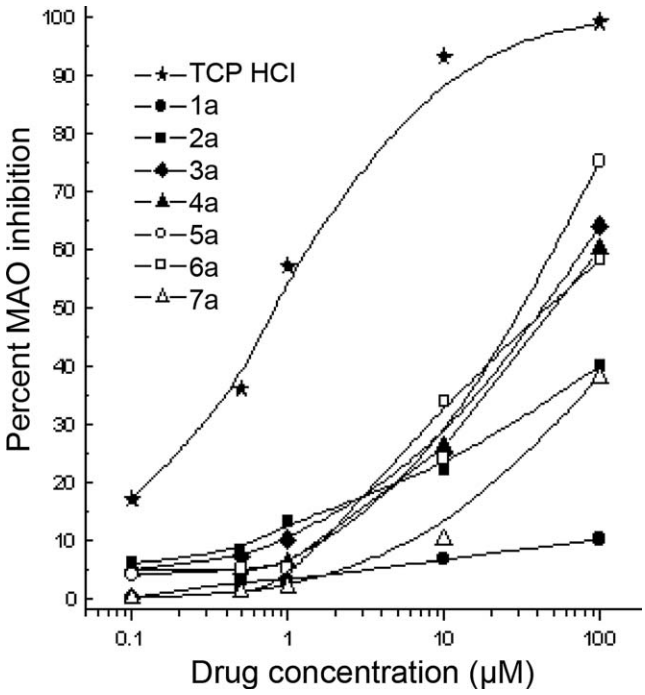


Fig. 2. Semi-logarithmic plot of the inhibitory activity of TCP and TCP-LAA conjugates against rat liver mitochondrial MAOs.

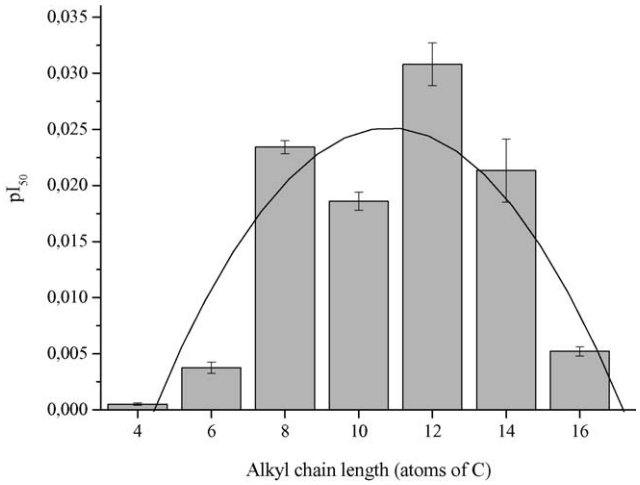


Fig. 3. Relationship between the lipophilicity and MAO inhibitory activity of compounds **1a**–**7a** (at the concentration of 100 μM).

it is noteworthy that the parabolic relationship obtained by plotting the MAO inhibitory activity (expressed as pI_{50}) vs. the length of alkyl side chain of the LAA moiety (Fig. 3), is completely superimposable with the experimental DSC data relative to the thermotropic effects exerted by these conjugates on DMPC liposomes (R. Pignatello, unpublished data). Also in those DSC experiments, in facts, the intermediate terms of the series (from the octyl to the dodecyl conjugates **3a**–**5a**) were those ones able to interact more deeply and at a similar extent with the phospholipid bilayers taken as biomembrane model, and perturb their ordered packing, whereas the lower and higher homologues showed a reduced enzyme inhibitory activity and a poorer interaction with liposomes.

It is conceivable that the inhibitory activity observed for these compounds is somewhat related to the presence of a free amino group in the LAA residue; similarly to parent TCP, his group is in fact involved in the interaction with the MAO enzyme complex. As a confirmation, we tested two amides of TCP with alkanoic acids (compounds **2b** and **5b**, Table 2). These compounds, which lack a free amino group, showed only a very low inhibitory activity against MAO (10% and 12% of inhibition, respectively, at the highest tested concentration of 100 μM) (Fig. 2).

In conclusion, in this paper the conjugation of TCP, a classical MAO inhibitor, with short, medium, and long alkyl side chain LAA residues has been carried out to provide a set of molecular tools useful to evaluate the effect of increasing lipophilicity/amphiphilicity on their interaction with biological membrane models. These latter results, that will be published in a following paper, will be also correlated with the biological data here reported.

3. Materials and methods

3.1. Materials

TCP hydrochloride hemihydrate (Sigma), shorter alkyl chain containing LAAs (L-2-aminobutyric acid, DL-norleucine, and L-2-aminooctanoic acid), the carboxylic acids and all other reactants were Fluka or Aldrich products (Sigma–Aldrich Chimica S.r.l., Milan, Italy). Long-chain containing LAAs were prepared as described elsewhere [11].

3.2. Chemistry

3.2.1. General

IR spectra were recorded in nujol using a Perkin–Elmer 1600 spectrophotometer. ^{1}H-NMR spectra were recorded in $CDCl_3$ with a Varian instrument operating at 250 MHz; chemical shifts were reported in ppm, using TMS as the internal standard. Mass analysis was performed with a triple quadrupole spectrometer (IPE sciex API 3000) operating in SIM mode with a positive ions electrospray. TLC was used to check the completion of the reactions and compound purity, and was performed on G60 silica gel aluminum sheets (Merck $F_{254+366}$), using a dichloromethane–methanol 95:5, v/v eluent mixture. Spots were evidenced either by using UV light, treatment with ninhydrin or acid–base reactant (bromocresol purple).

3.2.2. Synthesis of BOC-LAA

The BOC protection of the amino terminal of the LAAs, were carried out using a published method [11].

3.2.3. General synthesis of TCP-LAA amide conjugates

BOC-LAA (0.5 mmol) was dissolved in dry dichloromethane (5 ml) and 1-hydroxybenzotriazole hydrate (HOBt; 0.5 mmoles; 76.5 mg) and triethylamine (0.625 mmoles; 87 μl) were added to the solution. The 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (0.75 mmoles; 106.5 mg) was added to the mixture and stirred for 2 h at –5 °C. A solution of TCP (0.25 mmoles; 42.5 mg) in dry dichloromethane (5 ml) was then added and the mixture was stirred at room temperature for 24 h.

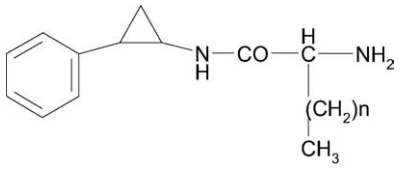
The solvent was removed in vacuum and the residue was dissolved in dichloromethane (30 ml) and washed in following steps with 20-ml aliquots of brine, 5% sodium hydrogen carbonate solution, brine, 5% acetic acid solution, and finally water. The organic phase was dried with anhydrous sodium sulfate and evaporated in vacuo. The residue was purified by column chromatography on silica gel (dichloromethane/methanol, 95:5, v/v).

The BOC-protecting group was removed by treating the conjugates with a 30% (v/v) solution of TFA in dichloromethane for 4 h at room temperature. Volatiles were removed under high vacuum and the residue was purified by flash column chromatography, with the above eluent mixture.

All the reaction products were yellowish oily or waxy substances and were stored in the refrigerator and away from direct light. Their physico-chemical data are reported in Table 1.

*3.2.3.1. 2-Amino-*N*-(2-phenylcyclopropyl)butanamide (**1a**).* Yield: 78%; analysis for $C_{13}H_{18}N_2O$ (218.29): % found (calc.) C 72.00 (71.53); H 8.39 (8.31); N 13.12 (12.83); IR (nujol,

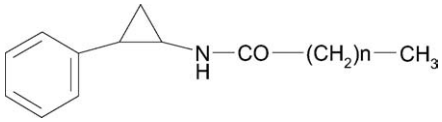
Table 1
Structure and physico-chemical properties of TCP-LAA conjugates **1a**–**7a**



Compound	*n*	CLog*P*[a]			CLogD$^{7.4}$
		A	B	C	B
1a	1	1.46	1.31	1.52	0.51
2a	3	2.52	2.33	2.45	1.73
3a	5	3.58	3.35	3.38	2.39
4a	7	4.46	4.37	4.30	3.52
5a	9	5.71	5.39	5.23	4.50
6a	11	7.10	7.22	6.16	6.23
7a	13	7.83	7.43	7.09	6.51
TCP[b]	–	*1.21*	*1.37*	*1.59*	*–0.89*

[a] Predictions were made using the following softwares: (A) ACD Log*P* 5.15; (B) Pallas 3.0; (C) Osiris Property Explorer.
[b] For TCP an experimental Log*P* value (n-octanol/water) of 1.58 has been reported [15].

Table 2
Structure and physico-chemical properties of TCP-FA amides **1b**–**7b**



Compound	n	CLog*P*[a]		
		A	B	C
1b	2	2.38	2.28	3.07
2b	4	3.44	3.30	4.00
3b	6	4.51	4.34	4.93
4b	8	5.57	5.36	5.86
5b	10	6.63	6.36	6.78
6b	12	7.70	7.37	7.71
7b	14	8.76	8.39	8.64

[a] Predictions were made using the following softwares: (A) ACD Log*P* 5.15; (B) Pallas 3.0; (C) Osiris Property Explorer.

cm^{-1}): 3405, 1678, 1206, 1138, 841, 802; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.36–7.18 (5H, m, aromatics), 5.99 (s, 1H, NH), 4.17 (m, 1H, α-CH), 2.56 (1H, m, CH), 2.32 (1H, m, CH), 1.9–1.6 (m, 2H, CH_2), 0.84 (t, 3H, ω-CH_3), 0.75 (m, 2H, ciclo-CH_2).

*3.2.3.2. N^l-(2-phenylcyclopropyl)norleucinamide (**2a**).* Yield: 82%; analysis for $C_{15}H_{22}N_2O$ (246.35): % found (calc.) C 73.33 (73.13); H 8.87 (9.00); N 11.12 (11.37); IR (nujol, cm^{-1}): 3405, 1678, 1206, 1138, 841, 802; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.38–7.18 (5H, m, aromatics), 5.99 (s, 1H, NH), 4.15 (m, 1H, α-CH), 2.56 (1H, m, CH), 2.31 (1H, m, CH), 1.9–1.65 (m, 6H, CH_2), 0.88 (t, 3H, ω-CH_3), 0.75 (m, 2H, ciclo-CH_2).

*3.2.3.3. 2-Amino-*N*-(2-phenylcyclopropyl)octanamide (**3a**).* Yield: 65%; analysis for $C_{17}H_{26}N_2O$ (274.40): % found (calc.) C 74.34 (74.41); H 9.88 (9.55); N 10.76 (10.21); IR (nujol, cm^{-1}): 3450 (br), 1670, 1206; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.5–7.2 (5H, m, aromatics), 5.99 (s, 1H, NH), 4.17 (m, 1H, α-CH), 2.56 (1H, m, CH), 2.31 (1H, m, CH), 2.2–1.6 (m, 10H, CH_2), 0.89 (t, 3H, ω-CH_3), 0.75 (m, 2H, ciclo-CH_2).

*3.2.3.4. 2-Amino-*N*-(2-phenylcyclopropyl)decanamide (**4a**).* Yield: 85%; analysis for $C_{19}H_{30}N_2O$ (302.45): % found (calc.) C 75.46 (75.45); H 9.85 (10.00); N 9.11 (9.26); IR (nujol, cm^{-1}): 1671, 1210, 840, 800; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.6–7.3 (5H, m, aromatics), 5.97 (s, 1H, NH), 4.21 (m, 1H, α-CH), 2.55 (1H, m, CH), 2.32 (1H, m, CH), 1.95–1.47 (m, 14H, CH_2), 0.85 (t, 3H, ω-CH_3), 0.76–0.71 (m, 2H, ciclo-CH_2).

*3.2.3.5. 2-Amino-*N*-(2-phenylcyclopropyl)dodecanamide (**5a**).* Yield: 80%; analysis for $C_{21}H_{34}N_2O{\cdot}H_2O$(348.53): % found (calc.) C 72.98 (72.37); H 10.31 (10.41); N 7.96 (8.04); IR (nujol, cm^{-1}): 3390, 1671, 1212, 840, 796; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.35–7.1 (5H, m, aromatics), 6.00 (s, 1H, NH), 4.18 (m, 1H, α-CH), 2.56 (1H, m, CH), 2.32 (1H, m, CH), 1.9–1.6 (m, 18H, CH_2), 0.88 (t, 3H, ω-CH_3), 0.73 (m, 2H, ciclo-CH_2).

*3.2.3.6. 2-Amino-*N*-(2-phenylcyclopropyl)tetradecanamide (**6a**).* Yield: 90%; analysis for $C_{23}H_{38}N_2O$ (358.56): % found (calc.) C 76.83 (77.04); H 10.83 (10.68); N 8.12 (7.81); IR (nujol, cm^{-1}): 3261, 1669, 1200, 1144, 840, 802; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.4–7.1 (5H, m, aromatics), 6.00 (s, 1H, NH), 4.19 (m, 1H, α-CH), 2.62 (1H, m, CH), 2.30 (1H, m, CH), 1.95–1.6 (m, 22H, CH_2), 0.84 (t, 3H, ω-CH_3), 0.74–0.72 (m, 2H, ciclo-CH_2).

*3.2.3.7. 2-Amino-*N*-(2-phenylcyclopropyl)hexadecanamide (**7a**).* Yield: 67%; analysis for $C_{25}H_{42}N_2O$ (386.61): % found (calc.) C 77.99 (77.67); H 10.87 (10.95); N 7.37 (7.25); IR (nujol, cm^{-1}): 3300, 1669, 1210, 841, 801; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.46–7.30 (5H, m, aromatics), 5.97 (s, 1H, NH), 4.20 (br m, 1H, α-CH), 2.55 (1H, m, CH), 2.30 (1H, m, CH), 2.1–1.64 (m, 26H, CH_2), 0.89 (t, 3H, ω-CH_3), 0.75 (m, 2H, ciclo-CH_2).

*3.2.4. TCP-FA amides **1b**–**7b***

The chosen carboxylic acid (0.15 mmoles) was reacted with equimolar TCP under the same conditions above described for TCP-LAA conjugates. The physico-chemical characteristics are summarized in Table 2.

*3.2.4.1. N-(2-phenylcyclopropyl)butanamide (**1b**).* Yield: 78%; analysis for $C_{13}H_{17}NO$ (203.28): % found (calc.) C 76.54 (76.81); H 8.47 (8.43); N 6.32 (6.89); IR (nujol, cm^{-1}): 1653; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.35–7.28 (5H, m, aromatics), 5.72 (s, 1H, NH), 3.05 (1H, br m, CH), 2.35 (1H, m, CH), 2.08 (t, 2H, CH_2), 1.27 (m, 2H, CH_2), 0.89 (t, 3H, ω-CH_3), 0.76–0.72 (m, 2H, ciclo-CH_2).

3.2.4.2. N-*(2-phenylcyclopropyl)hexanamide (**2b**).* Yield: 78%; analysis for $C_{15}H_{21}NO$ (231.333): % found (calc.) C 78.21 (77.88); H 9.67 (9.15); N 6.12 (6.05); IR (nujol, cm^{-1}): 1650, 1568; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.32–7.2 (5H, m, aromatics), 5.69 (s, 1H, NH), 3.15 (1H, br m, CH), 2.35 (1H, m, CH), 2.11 (t, 2H, CH_2), 1.63 (m, 4H, CH_2), 1.18 (m, 2H, CH_2), 0.89 (t, 3H, ω-CH_3), 0.76–0.72 (m, 2H, ciclo-CH_2).

3.2.4.3. N-*(2-Phenylcyclopropyl)octanamide (**3b**).* Yield: 79%; analysis for $C_{17}H_{25}NO$ (259.387): % found (calc.) C 78.93 (78.72); H 9.17 (9.71); N 5.13 (5.40); IR (nujol, cm^{-1}): 1646, 1564; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.4–7.2 (5H, m, aromatics), 5.89 (s, 1H, NH), 3.1 (1H, br m, CH), 2.33 (1H, m, CH), 2.12 (t, 2H, CH_2), 1.60 (m, 8H, CH_2), 1.26 (m, 2H, CH_2), 0.84 (t, 3H, ω-CH_3), 0.75 (m, 2H, ciclo-CH_2).

3.2.4.4. N-*(2-Phenylcyclopropyl)decanamide (**4b**).* Yield: 88%; analysis for $C_{19}H_{29}NO$ (287.44): % found (calc.) C 79.02 (79.39); H 9.89 (10.17); N 5.12 (4.87); IR (nujol, cm^{-1}): 1637; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.39–7.27 (5H, m, aromatics), 5.75 (s, 1H, NH), 3.02 (1H, br m, CH), 2.34 (1H, m, CH), 2.08 (t, 2H, CH_2), 1.63–1.58 (m, 12H, CH_2), 1.25–1.22 (m, 2H, CH_2), 0.89 (t, 3H, ω-CH_3), 0.73(m, 2H, ciclo-CH_2).

3.2.4.5. N-*(2-Phenylcyclopropyl)dodecanamide (**5b**).* Yield: 90%; analysis for $C_{21}H_{33}NO$ (315.49): % found (calc.) C 80.33 (79.95); H 10.12 (10.54); N 4.11 (4.44); IR (nujol, cm^{-1}): 1630, 1544; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.32–7.21 (5H, m, aromatics), 5.7 (s, 1H, NH), 3.25 (1H, br m, CH), 2.35 (1H, m, CH), 2.08 (t, 2H, CH_2), 1.65–1.62 (m, 16H, CH_2), 1.28 (m, 2H, CH_2), 0.85 (t, 3H, ω-CH_3), 0.76–0.70 (m, 2H, ciclo-CH_2).

3.2.4.6. N-*(2-Phenylcyclopropyl)tetradecanamide (**6b**).* Yield: 70%; analysis for $C_{23}H_{37}NO$ (343.55): % found (calc.) C 81.01 (80.41); H 10.87 (10.86); N 4.00 (4.08); IR (nujol, cm^{-1}): 1632, 1544; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.42–7.33 (5H, m, aromatics), 5.49 (s, 1H, NH), 3.02 (1H, br m, CH), 2.35 (1H, m, CH), 2.18 (t, 2H, CH_2), 1.62–1.48 (m, 20H, CH_2), 1.28–1.22 (m, 2H, CH_2), 0.88 (t, 3H, ω-CH_3), 0.76–0.70 (m, 2H, ciclo-CH_2).

3.2.4.7. N-*(2-Phenylcyclopropyl)hexadecanamide (**7b**).* Yield: 74%; analysis for $C_{25}H_{41}NO$ (371.6): % found (calc.) C 80.33 (80.80); H 11.47 (11.12); N 4.11 (3.77); IR (nujol, cm^{-1}): 1638, 1554; ^{1}H-NMR ($CDCl_3$; δ, ppm vs. TMS): 7.32–7.22 (5H, m, aromatics), 5.73 (s, 1H, NH), 3.11 (1H, br m, CH), 2.35 (1H, m, CH), 2.09 (t, 2H, CH_2), 1.68 (m, 24H, CH_2), 1.28 (m, 2H, CH_2), 0.90 (t, 3H, ω-CH_3), 0.82–0.72 (m, 2H, ciclo-CH_2).

3.3. Calculation of the physico-chemical properties

The Log*P* values of the synthesized conjugates were calculated using three different softwares (to validate each other and compensate for the approximation of the databases): ACD Log*P* 5.15 software (Advanced Chemistry Development Inc., Toronto, Canada); Pallas 3.0 (CompuDrug International, Inc., San Francisco, USA); and OSIRIS Property Explorer (www.actelion.com).

The distribution coefficient at pH 7.4 (apparent partition coefficient, $LogD_{7.4}$) was calculated using the Pallas 3.0 software. All the calculated parameters are gathered in Tables 1 and 2.

3.4. Stability tests

The hydrolytic stability of the amide bond in conjugates **1a**–**7a** was assessed by incubating the compounds at 37 ± 2 °C in phosphate buffered saline (PBS, pH 7.4) for 8 h. At predetermined time intervals 0.5-ml aliquots of the solution were withdrawn, evaporated to dryness under a nitrogen flow, re-dissolved in methanol and analyzed by HPLC.

A Varian LC Star 9010 System was used, equipped with a Symmetry C18 column (Water Corp., Milford, MA, USA), a Varian 9050 UV–Vis detector set at 264 nm, and a Varian 4400 Integrator. An isocratic flow of 1.0 ml min^{-1} made up of 50 mM KH_2PO_4 (pH 4.5)/acetonitrile/water (20:20:60) was used, as a slight modification of a reported method [12].

3.5. MAO inhibitory activity of TCP-LAA conjugates

3.5.1. Preparation of mitochondrial suspensions

MAO (EC 1.4.3.4) enzymatic activity was evaluated using rat liver mitochondria [13], in the absence or presence of either different concentrations of TCP or the tested conjugates.

Rat livers were homogenized in 5 mM HEPES buffer solution at pH 7.4, containing 0.1-mM EGTA, 70-mM saccharose and 220-mM mannitol. The homogenate was centrifuged at 800 × *g* for 10 min; the supernatant was centrifuged again at 10,000 × *g* for 10 min. The resulting pellet was suspended in the buffer solution and centrifuged at 800 × *g* for 10 min. The mitochondria fraction was finally obtained by centrifugation of the supernatant at 10,000 × *g* for 10 min.

The mitochondria pellet was suspended in an HEPES-EGTA buffer solution (pH 7.4) and then fractionated into aliquots and stored at –80 °C. Protein concentration was dosed according to Lowry et al. [14].

3.5.2. Inhibition of MAO activity

Each sample, containing 4 μg of mitochondria proteins, was incubated for 5 min at 37 °C in the presence or without different concentrations of TCP or compounds **1**–**7** (0.1, 0.5, 1, 10, or 100 μM), and dissolved in DMSO. After adding 30 μl of kynuramine (3.07 mM), samples were incubated for 15 min, afterwards the reaction was stopped by adding 0.4 M perchloric acid and the samples were centrifuged at 12,000 × *g* for 2 min. Aliquots of 1 ml of the supernatant were then added to 2 ml of 1 M NaOH and the fluorescence due to the formed 4-OH-quinoline was measured at $\lambda_{ex} = 315$ nm and $\lambda_{ex} = 380$ nm. The MAO enzymatic activity was calculated

as nmoles of 4-OH-quinoline per mg of mitochondria proteins, using as reference a calibration curve performed with known concentrations of 4-hydroxyquinoline (0.25–3 μM).

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