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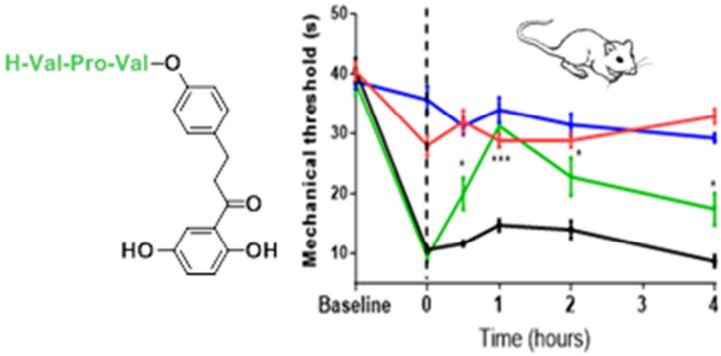

Amino acid and peptide prodrugs of diphenylpropanones positive allosteric modulators of α7 nicotinic receptors with analgesic activity

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Key words: Diphenylpropanones, Prodrugs, Diketopiperazines, DDP-IV, α7 nicotinic receptors, Allosteric modulation

Research Highlights

- Amino acid/peptide prodrugs of 1,3-diphenylpropanone α7 nAChRPAMs are described
- Val-Val and Val-Pro-Val are suitable promoieties
- Val-Pro-Val derivative exhibited long-lasting analgesic effect than the parent drug

ABSTRACT: α7 Nicotinic acetylcholine receptors (nAChRs) are ion channels implicated in a number of CNS pathological processes, including pain and psychiatric, cognitive and inflammatory diseases. Comparing with orthosteric agonism, positive allosteric modulation of these channels constitutes an interesting approach to achieve selectivity versus other nicotinic receptors. We have recently described new chalcones and 1,3-diphenylpropanones as positive allosteric modulators (PAMs) of α7 nAChRs, which proved to have good analgesic activities but poor pharmacokinetic properties. Here we report the preparation of amino acid and peptide derivatives as prodrugs of these modulators with the aim of improving their *in vivo* biological activity. While the valine derivative showed very short half life in aqueous solutions to be considered a prodrug, Val-Val and Val-Pro-Val are suitable precursors of the parent 1,3-diphenylpropanones, via chemical and enzymatic transformation, respectively. Compounds **19** (Val-Val) and **21** (Val-Pro-Val), prodrugs of the 2',5',4-trihydroxy-1,3-diphenylpropan-1-one **3**, showed significant antinociceptive activity in *in vivo* assays. The best compound, **21**, displayed a better profile in the analgesia test than its parent compound **3**, exhibiting about the same potency but long-lasting effects.

INTRODUCTION

Nicotinic acetyl choline receptors (nAChRs) are ligand-gated ion channels widely expressed in the nervous system. Among them, α7 receptors (α7 nAChRs) are implicated in a number of CNS pathological processes, from psychiatric [1-3], and cognitive disorders [4, 5] to inflammatory processes [6, 7], being associated to different sorts of pain [8-10], such as inflammatory [8, 11, 12], nociceptive [13, 14], and neuropathic pain [15-17]. While most of the described modulators of these channels are agonists, competitive with acetyl choline (Ach), the endogenous ligand, in recent years, efforts have been focused on the search for positive allosteric modulators (PAMs), agents that enhanced the positive response evoked by ACh, interacting with the receptor at allosteric sites. One of the advantages of PAMs is the higher receptor subtype selectivity comparing with true agonists, given that they bind to sites other than the well-conserved orthosteric binding domain. This, together with the fact that they show no effect in the absence of the natural neurotransmitter, made PAMs an interesting alternative to competitive agonists [18, 19]. There are not many compounds reported as PAMs of nAChRs, but several of them have been progressed achieving clinical phases [20-22].

We have recently described some new chemotypes that proved to be potent PAMs of the α7 nAChRs with structures of chalcone [23] and 1,3-diphenylpropanone [24]. These compounds were identified by screening a heterogeneous collection of small natural molecules, which led to the identification of a polyhydroxy substituted chalcone, Isoliquiritigenin, as a selective PAM of α7 nAChRs. This finding was the origin of a research project that permitted the identification of the polyhydroxy derivative **1** (Figure 1), as a potent and selective PAM of α7 nAChR [23]. To further explore the potential of

the series, and taking into account the high chemical reactivity that the α,β-unsaturated carbonyl system confers to the chalcone structure, hydrogenation of the double bond was performed. This led to the identification of new 1,3-diphenylpropan-1-ones, **2** and **3** (Figure 1), which also behaved as PAMs of the α7 nicotinic receptor with lower but still significant enhancing activity (see Table 1) [24].

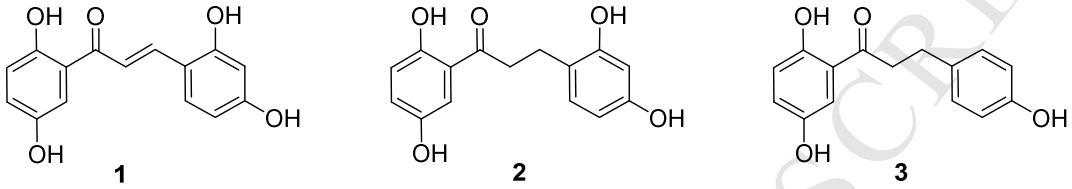


Figure 1. Structure of parent compounds.

The low aqueous solubility and the short times of action found in the *in vivo* experiments performed with these compounds [23, 24] prompted us to take a brief *in vitro* study of their pharmacokinetic (PK) properties. This study revealed that compounds **1-3** had in fact a poor PK profile. From the three compounds the best results were found for compound **3**, that showed a bit better aqueous solubility and permeability than compounds **1** and **2**. In particular, the values found for compound **3** are on the range of 160 µM regarding solubility in PBS and 33.5 x 10^{-6} cm/s permeability in the Caco-2 assay. However, the low values obtained for the half-live time (18-42 min), together with the high intrinsic clearance found in general for the three studied compounds, **1-3**, with values on the range of 165-387 µL/min/mg [24], was the fact that really encouraged us to prepare some derivatives that could behave as carrier-prodrugs, especially regarding their *in vivo* biological behavior, liberating the parent drug after the *in vivo* administration in the alive organism.

In the last decades a lot of work around the development and use of new prodrug strategies have ended in the improvement of the PK properties of many drugs [25, 26]. Among them, we have considered three different approaches based on the preparation of amino acid or peptide derivatives since they are easily excreted and non-toxic [27].

On one hand, we planned to prepare amino acid derivatives, in particular Valine-derived compounds that could act in a similar way as the well-known commercialized valacyclovir, the valine derivative of the antiherpes agent acyclovir. This approach is based on the lability of the ester valine bond, which permits the *in vivo* liberation of the active substance [28].

On the other hand, we also contemplated the preparation of dipeptide and tripeptide derivatives following two different strategies found in the literature. The first approach described by the group of P. Gomes, is based on the preparation of dipeptide derivatives of the active substance, which will be recovered after the intramolecular cyclization of the dipeptide fragment, giving rise to the corresponding diketopyperazine (DKP) [29, 30]. The second one, described by S. Velazquez and co-workers, is an enzyme-based prodrug approach centered on the recognition of specific peptide moieties by dipeptidyl peptidase IV (DPP IV/CD26) [31]. This enzyme is able to cleave oligopeptide moieties having a Pro residue located in the penultimate position of the N-terminus.

Following the indicated prodrug strategies, in this paper we describe the preparation of amino acid (Val) and peptide (Val-Val and Val-Pro-Val) derivatives of the selected compounds **1-3**, by ester formation with the OH group in position 4. In the case of Val derivatives, the new formed ester bond should be sensitive to chemical hydrolysis or to the action of esterases, which will liberate the parent compounds into the organism. For Val-Val derivatives, the ability of the dipeptide to form a DKP should control drug release. Compounds having the tripeptide moiety Val-Pro-Val will suffer first the action

of CD26, which will liberate Val-Pro dipeptide and the Val-compound, followed by the chemical or esterase-mediated hydrolysis of the valine residue, which will lead to the corresponding parent drugs [32]. Moreover, amino acid and peptide prodrugs presumably would be able to improve drug permeability, because they could be recognized by specific transporting proteins [33].

RESULTS AND DISCUSSION

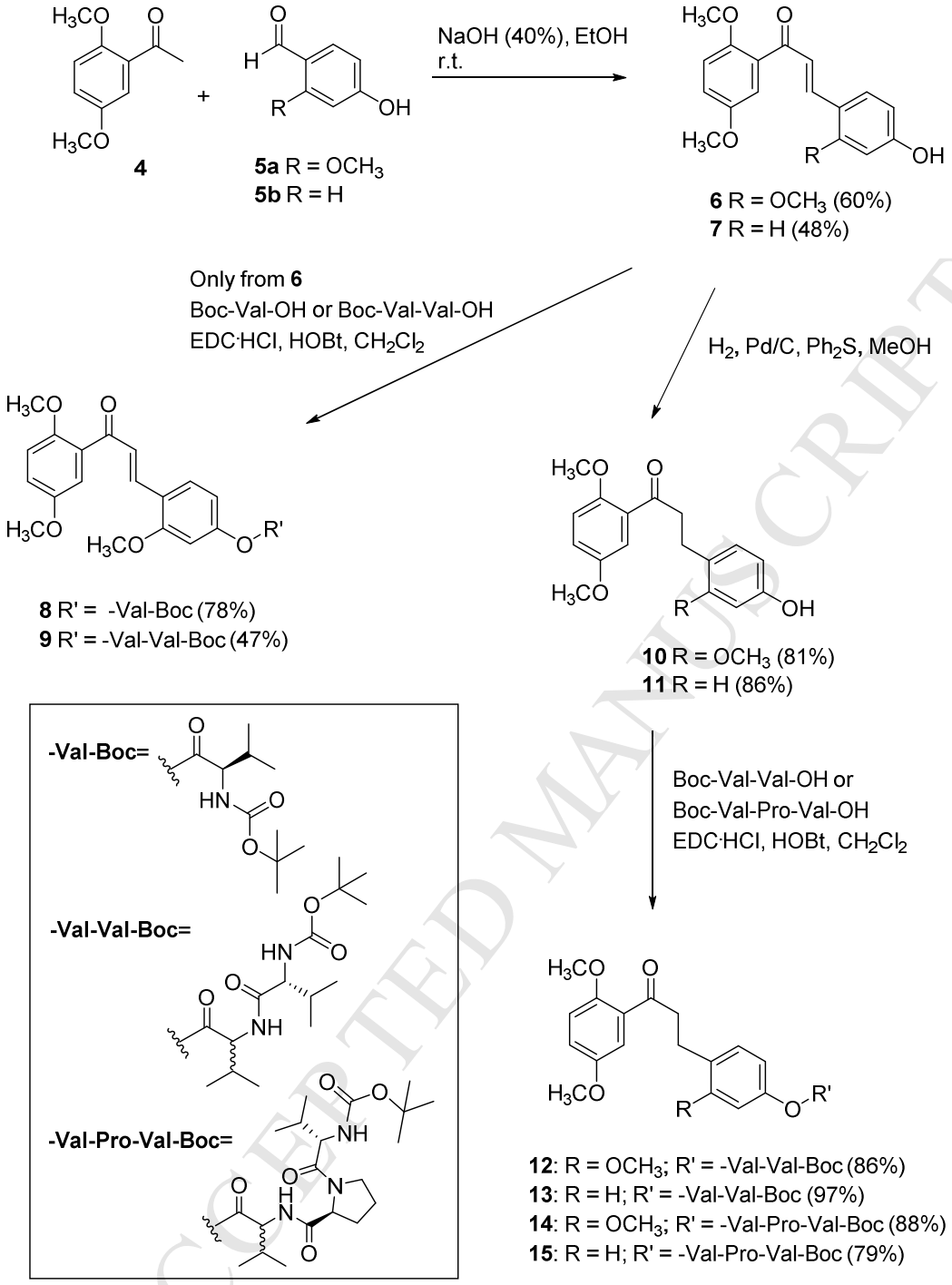
Chemistry.

Valine Derivatives. The valine derivative of chalcone **1** was synthesized as depicted in Scheme 1, by coupling the free 4-hydroxyl group of chalcone **6** with Boc-Val-OH in the presence of EDC/HOBt as coupling system [34]. Subsequent treatment of the so obtained product with boron tribromide to deprotect the methoxy groups led to the expected polyhydroxy chalcone **16**, which was isolated as a bromhydrate salt. The presence of bromhydric acid in the reaction medium also promoted the removal of the Boc protecting group, leading to the free NH_2 of the valine appendage. The results found after studying the chemical stability of this compound in aqueous solution revealed its extremely low stability, liberating the parent drug, compound **1**, in only 30 min (Figure 2). This result prompted us to desist in the preparation of the valine derivatives for compounds **2** and **3**, going directly to the preparation of dipeptide and tripeptide analogues.

Dipeptide Derivatives. Compound **17**, Val-Val derivative of chalcone **1**, was synthesized from chalcone **6** and the dipeptide Boc-Val-Val-OH, following the same procedure previously used for the synthesis of analogue **16**. Compounds **18** and **19**, Val-Val bioprecursors of propanones **2** and **3**, were synthesized following a similar methodology, from compounds **10** and **11**, respectively. These intermediates come from

the selective hydrogenation of the double bond of the corresponding chalcone analogues **6** and **7**, using Pd-C in the presence of Ph_2S that avoid overreduction of the ketone group (Scheme 1) [35].

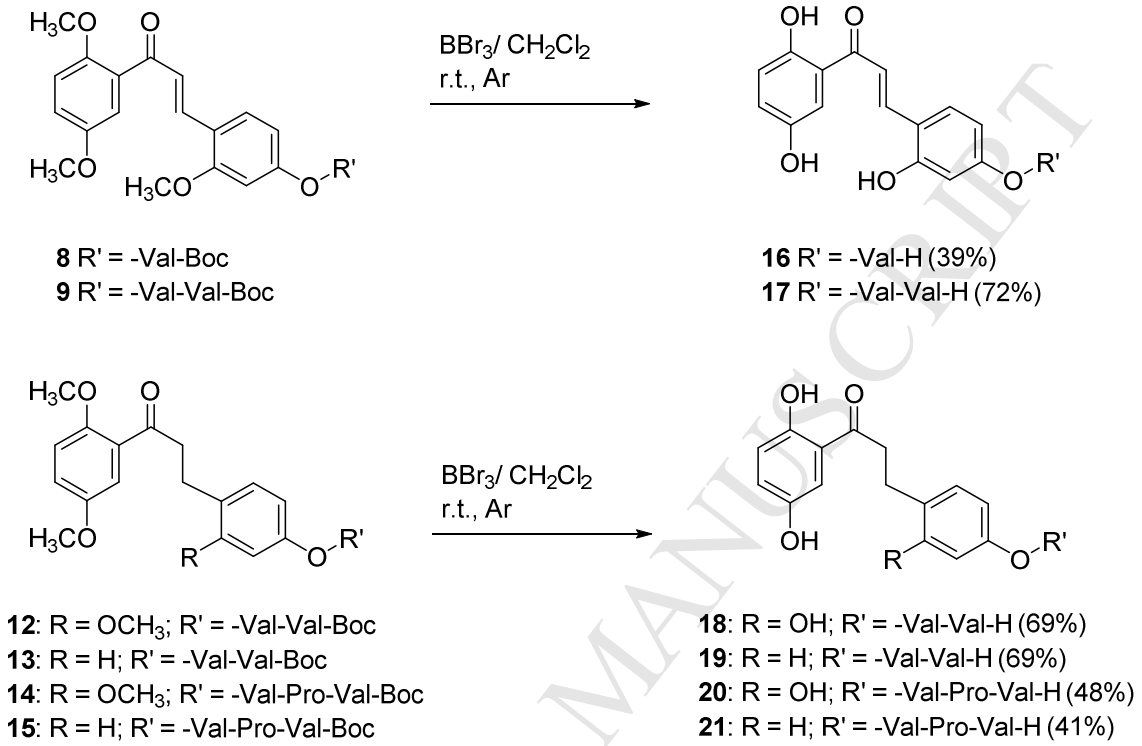
The HPLC-MS chromatograms of compound **17** revealed the presence of two peaks with the same molecular mass. This was indicative of epimerization of the C-terminal valine residue during the coupling reaction, which led to the formation of two diastereoisomers. The presence of two diastereoisomers was also detected in the case of dipeptide analogues **18** and **19**. Moreover, in the case of chalcone **17** a certain amount of the Z-isomer of the linker double bond was also detected. This finding prompted us to prepare only the tripeptide prodrugs of the saturated propanone analogues, because the presence of complex, inseparable isomeric mixtures would made rather complicated to perform the stability studies with these compounds.



Scheme 1. Synthesis of valine, dipeptide and tripeptide methoxy derivatives **8**, **9**, **12**-**15**.

Tripeptide Derivatives. Coupling reaction of compounds **10** and **11** with tripeptide Boc-Val-Pro-Val-OH [32], followed by treatment with BBr_3, led to the desired tripeptide derivatives **20** and **21**, as mixtures of two diastereoisomers in each case, due again to the

epimerization of the C-terminal Val residue, as it was mentioned for dipeptide prodrugs **18** and **19** (Schemes 1 and 2).



Scheme 2. Synthesis of hydroxyl derivatives **16**, **17**, **18**-**21**.

Chemical and enzymatic stability of valine, dipeptide and tripeptide derivatives.

Stability in Aqueous Solutions. The chemical stability of all synthesized prodrugs was determined in PBS (pH = 7.4)/5% DMSO at 25ºC, and monitored by HPLC. As already commented, this study revealed the extremely low chemical stability in aqueous

solution of Val derivative **16**, releasing the parent compound **1** in a very short time (100% transformation in 30 min; Figure 2). This result indicated a high lability of the Val ester bond on this phenol derivative, and therefore the related valine analogues of compounds **2** and **3** were not prepared.

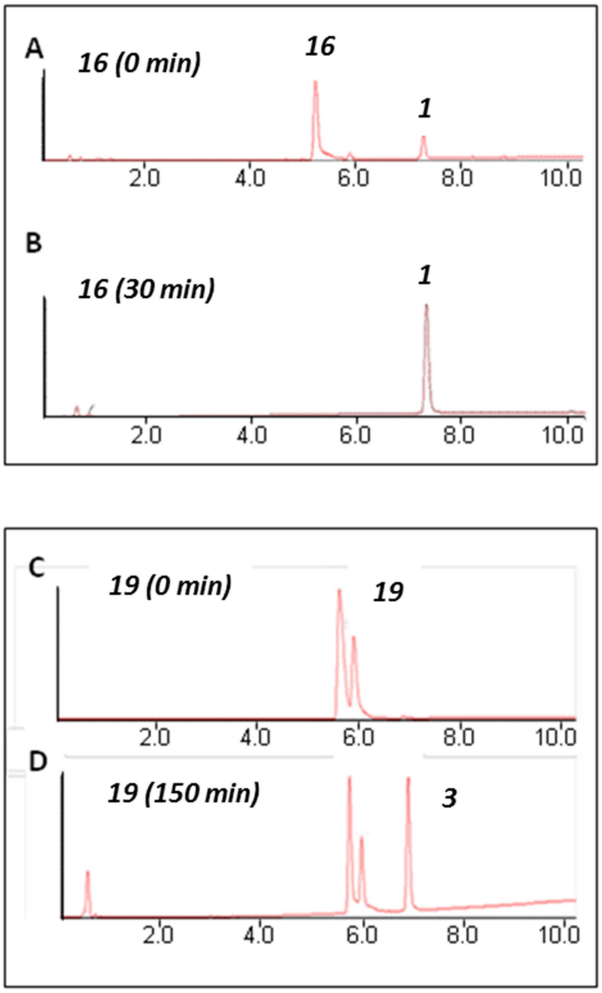


Figure 2. Chemical stability study of compound **16** in PBS/5% DMSO at time 0 (A) and 30 (B) min, and compound **19** at time 0 (C) and 150 (D) min.

Dipeptide derivatives **18** and **19** were expected to release the corresponding parent compounds via the DKP formation (see Figures 2 and 3A). In both cases, after 5 h in solution around 40% release of the active compound was observed (Figures 2 and S1). Therefore, they seem appropriate for further activity studies.

Finally, no significant degradation of tripeptide prodrugs **20** and **21** was observed in PBS (5% DMSO) solution after 5 hours (Figures 3B and S2). This result revealed that, as expected, the presence of the enzyme CD26 (DPP IV) seems to be necessary for their transformation in the corresponding parent drug. As already mentioned, presumably the enzymatic cleavage that liberates the corresponding valine derivative would take place first, which in turn will quickly release the desired compound due to its easiness to suffer hydrolysis in aqueous medium (see Figure 3).

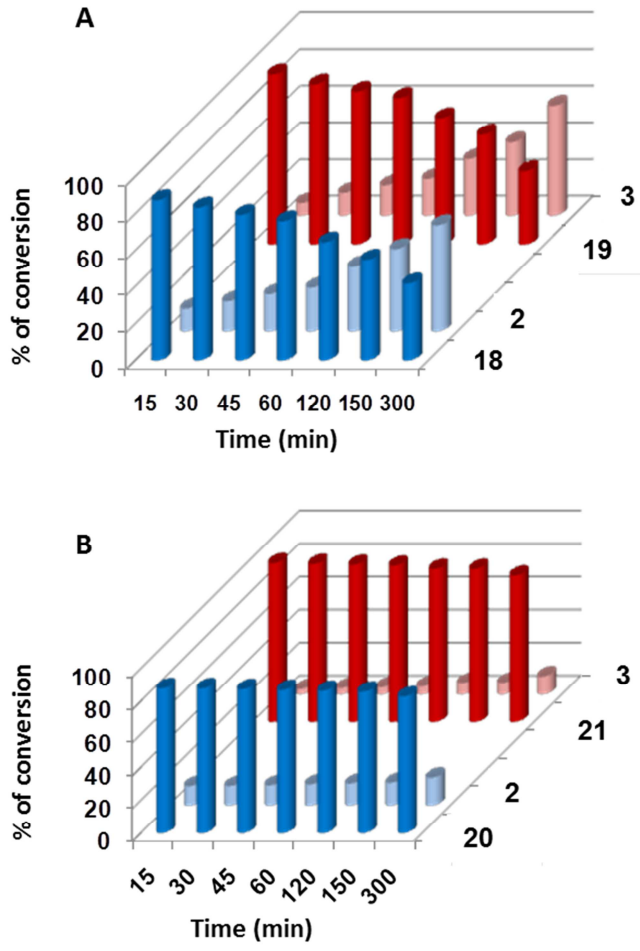


Figure 3. Degradation of dipeptides **18** and **19** (A) and tripeptides **20** and **21** (B) prodrugs in PBS + 5% DMSO, at 25º C. x: time of injection (min); y: conversion (%); z: compounds.

Enzymatic Stability of Tripeptide Prodrugs. In the first series of studies, Val-Pro-Val prodrugs (compounds **20** and **21**) were exposed to purified human recombinant CD26

(Figure S3), with about 70% of parent compounds **2** and **3** released after 15 min, and total disappearance of the prodrug in approximately 1h. Then, the conversion by native CD26 in human serum (HS) (Figure 4) or bovine serum (BS) (Figures S4) was investigated. In human serum, the conversion was slower than with the recombinant enzyme, as expected, but still very high after 60 min incubation (i.e. 78% for **20** and 87% for **21**). Prolonged times were needed to recover the drug from the corresponding prodrug in bovine serum (Figure S4), with total release in about 6 h. The HPLC chromatograms showed that, when incubated with CD26, HS or BS, both peaks of drug diastereoisomers disappeared in favor of free compound. Prodrugs **20** and **21** also showed some spontaneous conversion to the free compound when incubated at 37ºC in PBS, but this conversion was markedly enhanced by the presence of recombinant or native CD26.

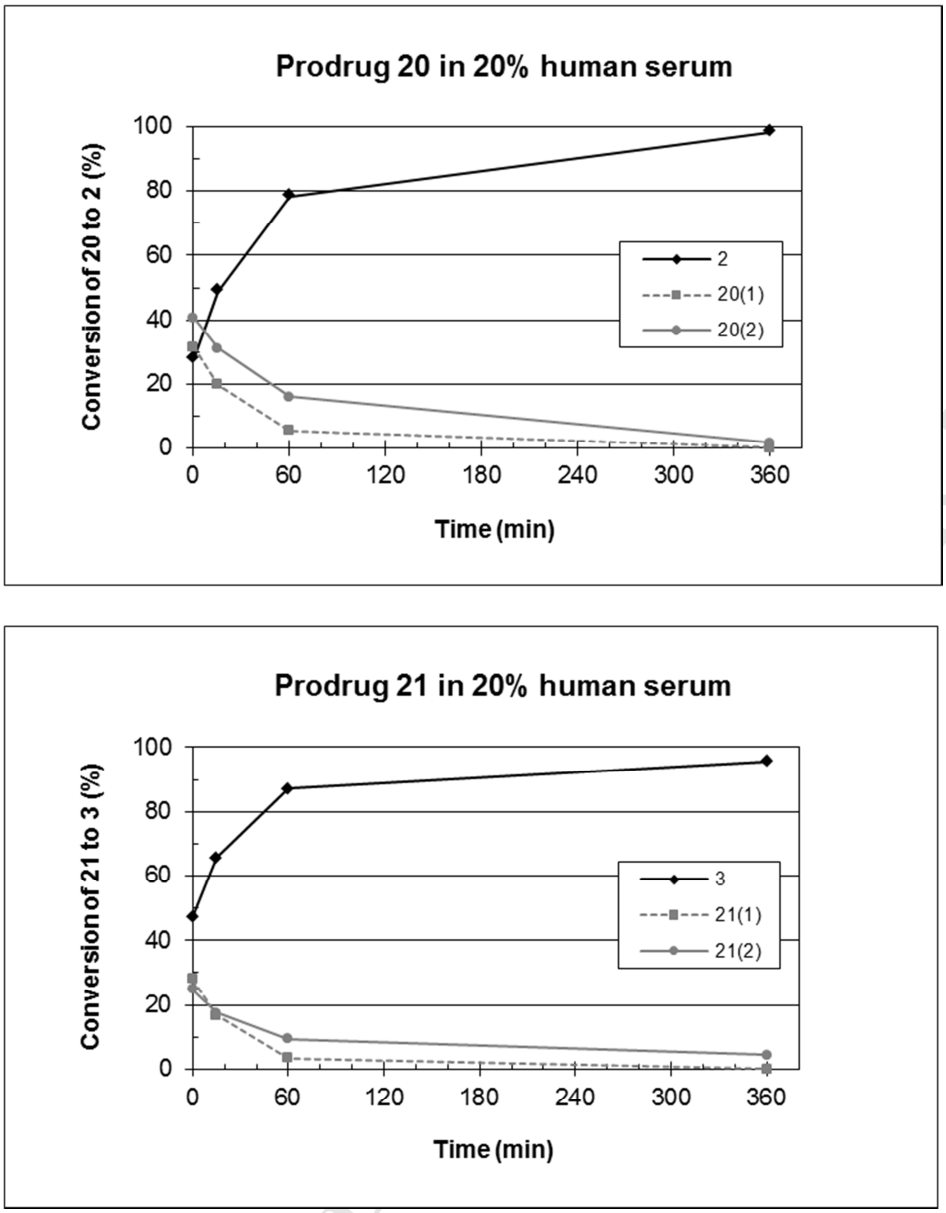


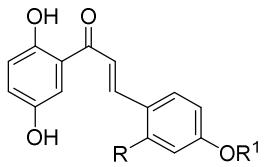
Figure 4. Time-dependent conversion of prodrug **20** into its parent drug **2** (A), and prodrug **21** (B) into its parent drug **3**, in human serum containing 20% PBS, at different times (The red and grey and doted bars correspond to the two diastereoisomers).

Biological Evaluation

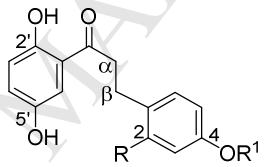
Electrophysiological Studies of New Prodrugs. The new di- and tripeptide derivatives were evaluated for their effect on currents evoked by ACh in *Xenopus* oocytes expressing α7 nAChRs. In contrast to parent compounds **1-3** that behave as PAMs of the α7 nAChR (Table 1, taken from [23, 24] and showing values well over the control value of 100%, which is the current elicited by 200 μM of ACh alone), all peptide

derivatives decreased the ACh-evoked currents significantly (Table 1, compounds **17-21**), therefore acting as inhibitors of the α7 nAChR, probably as negative allosteric modulators. It is interesting to note that the methoxylated analogues precursors of the polyhydroxy parent compounds behave as negative allosteric modulators of the α7 receptors [24]. Taking into account that the amino acid and peptide analogues described here have the same hydroxyl substituents than their parent compounds, except for the 4-OH group that it is blocked, these results may be indicative of a key role of this OH for PAM activity.

Table 1. Percentage Values of Inhibition of α7 nAChR Currents for Prodrugs and Parent Compounds



Compds 1 and 17



Rest of Compds

Compound	R	R^1	% Current vs ACh [200 µM][a]
1	OH	OH	2760±255[23]
2	OH	OH	989±100[24]
3	H	OH	666±83[24]
17	OH	HBr·H_2N-Val-Val-	24.5±0.7
18	OH	HBr·H_2N-Val-Val-	26.7±1.2
19	H	HBr·H_2N-Val-Val-	14.7±5.1
20	OH	HBr·H_2N-Val-Pro-Val-	25.3±11.6
21	H	HBr·H_2N-Val-Pro-Val-	24.7±8.1

[a]Compounds were tested at a unique dose of 10 µM, which was co-applied with 200 µM ACh. Responses were recorded at -80 mV and normalized with respect to that shown by only ACh (200 µM).

Analgesic Activity. It has been described that PAMs of α7 nAChR show analgesic effects. In particular PNU-120596, a well-known type II PAM, has been described to display anti-nociceptive activity in models of persistent and inflammatory pain [13, 36]. In our previous work, compound **3** (10 mg/kg, iv) and PNU-120596 (10 mg/kg, iv), were checked for the reduction of hyperalgesia resulted from inflammation promoted by complete Freund's adjuvant (CFA) in rats. Both compounds showed significant antinociceptive effects in the mechanical hyperalgesia test, but lacked activity in the thermal assay [23, 24]. Compound **3** demonstrated a main peak of activity after 2h from administration, and then a fast decay in the following two hours, just the same behavior than PNU-120596 [24].

To compare the behavior of the new derivatives with that of the parent compound **3**, the mechanical hyperalgesia test was performed after iv administration of dipeptide **19** and tripeptide derivative **21** (10 mg/Kg). As depicted in Figure 5, compound **19** (Figure 5A), the Val-Val derivative of **3**, shows an effect quite similar to that of its parent compound **3**, but it exerts its activity in a shorter time, showing its maximal peak after only 30 min post administration. Then the effect disappears quite fast, decreasing very rapidly, in only 30 min. This quick and short effect suggests either a fast release and metabolization of compound **3** *in vivo* or more probably, a fast liberation just after injection but a sustained release afterward that do not reach effective concentrations of the bioactive compound. In contrast, compound **21** (Figure 5B), the Val-Pro-Val derivative of **3**, shows comparable antinociceptive activity to those of model compounds **3** and PNU-120596, but the effect is statistically significative before, only 30 min after administration, reaching its maximal effect at 1 h. Interestingly, this effect vanishes slowly, lasting for more than 3 h, while **PNU** derivative and compound **3** decay rapidly after 2 h. This behavior invites to think that our initial hypothesis is valid,

suggesting that compound **21** is continuously metabolized through the action of CD26 enzyme in plasma. These results indicate that the presence of compound **3** in the organism at effective concentration is longer when administered in the form of prodrug **21**. Moreover, the fact that compounds **19** and **21**, show analgesic effects in the *in vivo* assay is an evidence of their capacity to really act as carrier prodrugs. In fact, in the *in vitro* electrophysiology tests these compounds display an opposite behavior to that of parent compound **3**, blocking instead of activating the receptor.

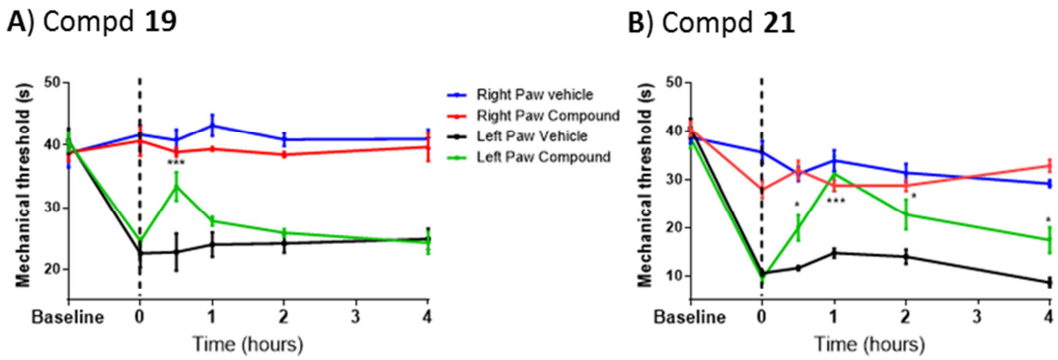


Figure 5. Effect of compounds **19** and **21** in the CFA-induced paw inflammation model. Time course of mechanical hyperalgesia in rats after injection of complete Freund's adjuvant (CFA) 0.5 mg/mL (50 µL) into the right hind paw with and without administration of compound (10 mg/kg i.v.). The diagram shows the paw withdrawal latencies in response to mechanical stimulation ($n \geq 6$ rats/group). Data are given as mean ± SEM n = 6. Two-way ANOVA with Bonferroni post-*hoc* test. **P* <0,05; ***P* <0,01, ****P* <0,001. A) and B) correspond to the model compounds.

Despite an apparent better aqueous solubility of prodrugs **19** and **21**, the in vivo test was performed again by iv administration to be able to compare with the results obtained for the parent compound **3**.[24] As by nature prodrugs should be chemically or enzymatically unstable molecules, the measurement of PK properties was not attempted. It is expected that the permeability of both prodrugs could be better, because it could take place through active transport, involving amino acid/peptide transporters, while the polar parent compound is supposed to permeate only through passive diffusion. On the other

hand, the long-lasting analgesic activity points to a sustained release of the active principle *in vivo* .

CONCLUSIONS

Previous studies that started with the *in vitro* screening of a library of natural compounds, allowed the identification of some chalcones and 1,3-propanones as PAMs of the α7 nACh receptors [23, 24]. Pharmacological studies of these compounds revealed the promising biological properties of some of them, including significant antinociceptive effects in the mechanical hyperalgesia assay performed in rats, and cognitive enhancement in the Morris test. However, their poor pharmacokinetic profile prompted us to prepare some derivatives able to behave as prodrugs, thus releasing the parent compounds *in vivo*. We report here the preparation of the amino acid, di- and tripeptide derivatives of the best compounds of the series, **1**-**3**, which were designed as carrier bioprecursors of the parent compounds. With the exception of the Val derivative that hydrolyzes very quickly in aqueous solution, Val-Val and Val-Pro-Val derivatives showed chemical and enzymatic stabilities compatible with their use as prodrugs. The biological evaluation of these compounds showed that they behave as inhibitors of α7 nAChR currents in electrophysiological assays *in vitro*, in contrast to parent compounds **1-3** that are PAMs. However, the *in vivo* evaluation of selected compounds **19** and **21** revealed that both display analgesic activity, thus indicating the release of the parent compound **3** from the peptide precursors. Compound **21**, the Val-Pro-Val tripeptide derivative, which should release **3** through the action of CD26 and a spontaneous hydrolysis of the Val residue, exhibited comparable analgesic activity than **3** and PNU120596 but with an interesting long-lasting effect, a result good enough to justify further work on this series of compounds.

ACKNOWLEDGEMENTS

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EXPERIMENTAL SECTION

Chemistry. All reagents were of commercial quality. Solvents were dried and purified by standard methods. Analytical TLC was performed on aluminum sheets coated with a 0.2 mm layer of silica gel 60 F254. Silica gel 60 (230-400 mesh) was used for flash chromatography. Some compounds were purified by flash chromatography using SNAP 12g KP-C18-HS cartridges on an ISOLERA ONE (Biotage) apparatus. Mixtures of CH_3CN (solvent A), H_2O + 0.05% TFA (solvent B) were used as mobile phase. Unless indicated, all compounds were isolated in a purity ≥ 95% (HPLC data). Analytical HPLC-MS was performed on a Waters equipment coupled to a single quadrupole ESI-MS (Waters Micromass ZQ 2000) using a reverse-phase SunFire^{TM} C18 4.6 x 50 mm column (3.5 μm) at a flow rate of 1 mL/min and by using a diodo array UV detector. Mixtures of CH_3CN + 0.08% formic acid (solvent A) and H_2O + 0.1% formic acid (solvent B) were used as mobile phase (gradient of 15 to 95% of A in B in 5 or 10 min, as indicated in each case). HRMS (EI+) was carried out in an Agilent 6520 Accurate-Mass Q-TOF LC/MS equipment. NMR spectra were recorded on a Varian-INOVA 300, a Bruker-AVANCE 300, a Varian-MERCURY 400, a Varian-INOVA 400 or a Varian System 500 spectrometer, operating at 300, 400 or 500 MHz for ^1H and at 75, 100, and

125 MHz for ^{13}C recording. To confirm the NMR peak assignments, COSY and HSQC experiments were performed when necessary (For H and C numbering see Table 1 heading formula).

General procedure for the synthesis of chalcones. To a solution of the conveniently substituted benzaldehyde (1.91 mmol) in EtOH (15 mL), the desired acetophenone (1.66 mmol) was added. The solution was cooled at 0ºC, and an aqueous solution of NaOH 40% (1.87 mL) was slowly added. After 15h of stirring at room temperature water was added and the organic solvent was removed at reduced pressure. The organic phase was extracted with EtAcO, and the organic extracts were washed with H_2O and brine. After drying over $MgSO_4$, the solvent was removed to dryness and the resulting residue crystallized from MeOH. Characterization data for all new methoxy-substituted chalcones can be found in the SI.

General procedure for the preparation of 1,3-diphenyl-1-propanones. To a solution of the corresponding chalcone (0,30 mmol) in MeOH (30 mL) and diphenylsulfide (0.003 mmol), Pd/C 10% (10% weight) was added. After hydrogenation, at room temperature and 20 psi during 24h, the catalyst was filtered off, the solvent evaporated under vacuum, and the residue purified by flash chromatography (AcOEt/Hexane).

Typical procedure for the Coupling Reaction of Amino Acids and Peptides. To a solution of the corresponding 4-hydroxy chalcone or 1,3-diphenyl-1-propanone (0.25 mmol) in Cl_2CH_2/THF (4/1 mL), Boc-Val-OH, or Boc-Val-Val-OH or Boc-Val-Pro-Val-OH (0.50 mmol), EDC chlorhydrate (0.50 mmol) and DMAP (0.05 mmol). The reaction was stirred at room temperature for 15 h. The organic phase was successively washed with citric acid 5%, $NaHCO_3$ 10%, and brine. After drying over anhydrous $MgSO_4$, the solvent was removed and the residue chromatographed to give the desired

compound. Characterization data for all the methoxy-substituted derivatives can be found in the SI.

General procedure for the deprotection of methoxy substituted compounds. To a previously cooled solution (0ºC) of the corresponding methoxy substituted compound (0.60 mmol) in dried DCM (15 mL), a 1M solution of BBr_3 in DCM (2 equiv for each MeO- group plus 2 more equiv for every group containing a potentially basic N or O) was slowly added under Ar atmosphere. After stirring 24-48 h at room temperature under Ar, H_2O was added to the reaction mixture. Then DCM was removed under reduced pressure and the residue extracted with EtOAc. The organic extracts were washed with H_2O and brine, dried over Na_2SO_4 and then evaporated. The crude product was purified as indicated in each case.

(E)-1-(2',5'-Dihydroxyphenyl)-3-[2-hydroxy-4-(L-Val)oxy]phenyl-2-propenone (16). Obtained using 10 equiv of BBr_3. Reddish solid (39% yield), mp: 194-197ºC. Purification by reverse phase flash chromatography, (eluent: from 10 to 30% of ACN in H_2O + 0.05% TFA). HPLC (Sunfire): t_R = 5.11 min (10 min gradient from 15 to 95% of A in B). ^{1}H RMN (500 MHz, DMSO-d_6) δ: 1.08, 1.11 (d, 3H, *J* = 6.9 Hz, γ-Val), 2.33 (m, 1H, β-Val), 4.24 (d, 1H, *J* = 4.5 Hz, α-Val), 6.72 (dd, 1H, *J* = 8.5, 2.2 Hz, 5-H), 6.79 (d, 1H, *J* = 2.4 Hz, 3-H), 6.84 (d, 1H, *J* = 8.9 Hz, 3'-H), 7.03 (dd, 1H, *J* = 8.9, 3.0 Hz, 4'-H), 7.43 (d, 1H, *J* = 3.0 Hz, 6`-H), 7.89 (d, 1H, *J* = 15.7 Hz, H_α), 7.97 (d, 1H, *J* = 8.5 Hz, 6-H), 8.03 (d, 1H, *J* = 15.7 Hz, H_β), 8.53 (bs, 3H, NH_3^+), 9.23 (s, 1H, OH), 10.94 (s, 1H, OH), 11.23 (s, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 17.7 (γ-Val), 18.3 (γ-Val), 29.6 (β-Val), 57.4 (α-Val), 109.1 (C-3), 112.60 (C-5), 114.8 (C-6'), 118.4 (C-4'), 120.0 (C-3'), 121.0 (C-1), 121.4 (C_α), 124.3 (C-1'), 130.5 (C-6), 138.87 (C_β), 149.5 (C), 152.0 (C), 154.6 (C), 158.3 (C), 167.47 (CONH), 193.3 (CO) ppm. MS

(ESI$^+$): *m/z* 372.4 $[M + H]^+$. HRMS (ESI+) *m/z* ($[M + H]^+$ calcd for $C_{20}H_{21}NO_6$ 372.14416; found 372.14320

(E)-1-(2',5'-Dihydoxyphenyl)-3-[2-hydroxy-4-(L-Val-L,D-Val)oxy]phenyl-2-propenone (17). Obtained using 20 equiv of BBr_3. Red solid (72% yield), mp. = 189-191ºC. Purification by reverse phase flash chromatography, eluent: from 2 to 30% gradient of ACN in H_2O + 0.05% TFA. HPLC (Sunfire): Mixture of diastereoisomers (47:53) t_R= 5.92 and 6.12 min (10 min gradient from 15 to 95% of A in B). ^{1}H RMN (500 MHz, DMSO-d_6) δ: 0.95, 0.96 (d, 3H, *J* = 7.0 Hz, γ-Val), 0.98, 1.00 (d, 3H, *J* = 6.9 Hz, γ-Val), 1.02, 1.03 (d, 3H, *J* = 6.8 Hz, γ-Val), 1.06, 1.07 (d, 3H, *J* = 6.8 Hz, γ-Val), 2.14 (m, 1H, β-Val), 2.27 (m, 1H, β-Val), 3.75, 3.78 (m, 1H, α-Val), 4.41, 4.50 (dd, 1H, *J* = 7.9, 6.1 Hz, α-Val), 6.62, 6.66 (dd, 1H, *J* = 8.6, 2.3 Hz, 5-H), 6.70, 6.72 (d, 1H, *J* = 2.3 Hz, 3-H), 6.80, 6.84 (d, 1H, *J* = 8.9 Hz, 3'-H), 7.02, 7.04 (dd, 1H, *J* = 8.9, 3.0 Hz, 4'-H), 7.41, 7.42 (d, 1H, *J* = 3.0 Hz, 6'-H), 7.86, 7.87 (d, 1H, *J* = 15.7 Hz, H_α), 7.92, 7.93 (d, 1H, *J* = 8.6 Hz, 6-H), 8.02, 8.03 (d, 1H, *J* = 15.7 Hz, H_β), 8.14 (bs, 3H, NH_3^+), 8.81 (d, 1H, *J* = 6.8 Hz, CONH), 8.93 (d, 1H, *J* = 7.9 Hz, CONH), 9.23 (s, 1H, OH), 10.18 (s, 1H, OH), 10.83, 10.85 (s, 1H, OH), 11.82, 11.83 (s, 1H, OH), ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 17.3, 17.5 (γ-Val), 18.1, 18.25 (γ-Val), 18.2, 18.44 (γ-Val), 18.7, 18.9 (γ-Val), 29. 7, 30.0 (β-Val), 30.0, 31.0 (β-Val), 57.1, 57.2 (α-Val), 57.7, 58.1 (α-Val), 109.1, 109.2 (C-3), 112.7, 112.8 (C-5), 114.7 (C-6'), 118.3, 118.4 (C-4'), 119.5, 119.6 (C-1), 120.9, 120.9 (C-3'), 121.1, 121.12 (Cα), 124.0, 124.3 (C-1'), 130.4, 130.4 (C-6), 139.1 (Cβ), 149.3, 149.5 (C), 152.7, 152.8 (C), 154.6, 154.6 (C), 158.3 (C), 168.6, 168.7 (CONH), 169.3, 169.6 (OCO), 193.3 (CO) ppm. MS (ESI+): *m/z* 471.2 $[M + H]^+$.

1-(2',5'-Dihydoxyphenyl)-3-[2-hydroxy-4-(L-Val-L,D-Val)oxy]phenyl-1-propanone (18). Obtained using 20 equiv of BBr_3. Colorless solid (69% Yield), mp = 162-165ºC). Purification by reverse phase flash chromatography, eluent: 2 to 30% gradient of ACN in H_2O + 0.05% TFA. HPLC (Sunfire): Mixture of diastereoisomers (56:44) t_R= 6.16 and 6.30 min (10 min gradient from 15 to 95% of A in B).). ^{1}H RMN (500 MHz, DMSO-d_6) δ: 0.94, 0.95 (d, 3H, *J* = 6.9 Hz, γ-Val), 0.97, 0.98 (d, 3H, *J* = 6.9 Hz, γ-Val), 1.01, (d, 3H, *J* = 6.8 Hz, γ-Val), 1.04, 1.05 (d, 3H, *J* = 6.8 Hz, γ-Val), 2.11 (m, 1H, β-Val), 2.24 (m, 1H, β-Val), 2.85 (m, 2H, Hz, H_β), 3.25, 3.26 (t, 2H, *J* = 7.5 Hz H_α), 3.74, 3.77 (d, 1H, *J* = 5.3 Hz, α-Val), 4.38, 4.48 (dd, 1H, *J* = 8.1, 6.5 Hz, α-Val) , 6.43, 6.47 (dd, 1H, *J* = 8.3, 2.4 Hz, 5-H), 6.53, 6.55 (d, 1H, *J* = 2.4 Hz, 3-H), 6.81 (d, 1H, *J* = 8.9 Hz, 3'-H), 6.98 (dd, 1H, *J* = 8.9, 3.0 Hz, 4'-H), 7.14, 7.15 (d, 1H, *J* = 8.3 Hz, 6-H), 7.21 (d, 1H, *J* = 3.0 Hz, 6'-H), 8.10 (bs, 3H, NH_3^+), 8.76, 8.89 (d, 1H, *J* = 8.1 Hz, CONH), , 9.16, 9.17 (s, 1H, OH), 9.82, 9.83 (s, 1H, OH), 11.23 (s, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 17.2, 17.5 (γ-Val), 18.1, 18.2 (γ-Val), 18.3, 18.5 (γ-Val), 18.8, 19.0 (γ-Val), 24.2, 24.3 (C_α), 29.7, 30.0 (β-Val), 30.0, 30.2 (β-Val), 38.8, 38.8 (C_β), 57.1, 57.2 (α-Val), 57.6, 58.1 (α-Val), 108.1, 108.1 (C-3), 111.4 (C-4'), 111.6 (C-3'), 114.7 (C-5), 118.4 (C-6'), 120.1, 120.2 (C-1), 124.1 (C-1'), 125.1 (C), 130.2, 130.2 (C-6), 149.4 (C), 153.5 (C), 155.8 (C), 168.6, 168.6 (CONH), 169.7, 169.9 (OCO), 204.9 (CO) ppm. MS (ESI+): *m/z* 473.3 $[M + H]^+$. HRMS (ESI+) *m/z* ($[M + H]^+$ calcd for $C_{25}H_{32}N_2O_7$ 473.22823; found 473.22990.

1-(2',5'-Dihydroxyphenyl)-3-[4-(L-Val-L,D-Val)oxy]phenyl-1-propanone (19). Obtained using 20 equiv of BBr_3, Colorless solid (69% yield), mp = 129-131ºC. Purification by reverse phase flash chromatography, eluent: 2 to 30% gradient of ACN in H_2O + 0.05% TFA. HPLC (Sunfire): Mixture of diastereoisomers (53:47) t_R= 6.00 and 6.21 min (10 min gradient from 15 to 95% of A in B). ^{1}H RMN (500 MHz, DMSO-

d$_6$) δ: 0.93, 0.94 (d, 3H, *J* = 7.0 Hz, γ-Val), 0.96, 0.98 (d, 3H, *J* = 7.0 Hz, γ-Val), 1.02 (d, 3H, *J* = 6.8 Hz, γ-Val), 1.05, 1.06 (d, 3H, *J* = 6.8 Hz, γ-Val), 2.12 (m, 1H, β-Val), 2.26 (m, 1H, β-Val), 2.94 (m, 2H, H$_\beta$), 3.24 (overlapped with H_2O signal; t, 2H, *J* = 7.5 Hz, H$_\alpha$), 3.74, 3.77 (d, 1H, *J* = 5.0 Hz, α-Val), 4.39, 4.48 (dd, 1H, *J* = 7.9, 6.2 Hz, α-Val), 6.81 (d, 1H, *J* = 8.9 Hz, 3'-H), 6.97, 6.99 (dd, 2H, *J* = 8.9, 2.9 Hz, 3-H and 5-H), 7.02 (dd, 1H, *J* = 8.7, 2.0 Hz, 4'-H), 7.21 (d, 1H, *J* = 3.0 Hz, 6'-H), 7.33, 7.35 (d, 2H, *J* = 8.5 Hz, 2-H and 6-H), 8.10 (bs, 3H, NH_3^+), 8.78, 8.90 (d, 1H, *J* = 6.9 Hz, CONH), , 9.18, 9.19 (s, 1H, OH), 11.17, 11.18 (s, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ: 17.3, 17.5 (γ-Val), 18.2, 18.2 (γ-Val), 18.3, 18.4 (γ-Val), 18.9, 19.1 (γ-Val), 28.7, 28.7 (C$_\alpha$), 29.6, 29.7 (β-Val), 30.0, 30.0 (β-Val), 40.5, 40.5 (C$_\beta$), 57.1, 57.2 (α-Val), 57.8, 58.1 (α-Val), 114.7 (C-6'), 118.4 (C-3'), 120.3, 120.3 (C-1), 121.2, 121.3 (C-3, C-5), 124.1 (C-4'), 129.5, 129.6 (C-2, C-6), 139.1 (C), 148.3 (C), 149.4 (C), 153.4 (C), 168.62, 168.7 (CONH), 169.8, 170.1 (OCO), 204.2 (CO) ppm. MS (ESI+): *m/z* 457.3 [M + H]$^+$. HRMS (ESI+) *m/z* [M + H]$^+$ calcd for $C_{25}H_{32}N_2O_6$ 457.23331; found 457.23240.

1-(2',5'-Dihydroxyphenyl)-3-[-2-hydroxy-4-(L-Val-L-Pro-L,D-Val)oxy]phenyl-1-propanone (20). Obtained using 20 equiv of BBr_3. Colorless solid (48% yield), mp = 170-173ºC. Purification by reverse phase flash chromatography, eluent: 2 to 30% gradient of ACN in H_2O + 0.05% TFA. HPLC (Sunfire): Mixture of diastereoisomers (38:62) t_R = 5.85 and 5.99 min (10 min gradient from 15 to 95% of A in B). ^{1}H RMN (500 MHz, DMSO-*d*$_6$) δ: 0.94-1.03 (m, 12H, , γ-Val), 1.87 (m, 2H, γ-Pro), 1.97 (m, 1H, β-Val), 2.13 (m, 2H, β-Pro), 2.20 (m, 1H, β-Val), 2.84 (t, 2H, *J* = 7.5 Hz, H$_\beta$), 3.26 (t, 2H, *J* = 7.5 Hz, H$_\alpha$), 3.50 (m, 1H, δ-Pro), 3.71 (m, 1H, δ-Pro), 3.99 (d, 1H, *J* = 5.6 Hz, α-Val), 4.32, 4.36 (dd, 1H, *J* = 8.0, 6.2 Hz, α-Val), 4.56 (m, 1H, α-Pro), 6.42, 6.44 (dd, 1H, *J* = 8.1, 2.4 Hz, 5-H), 6.53, 6.55 (d, 1H, *J* = 2.4 Hz, 3-H), 6.80 (d, 1H, *J* = 8.9

Hz, 3'-H), 6.98 (dd, 1H, *J* = 8.9, 3.0 Hz, 4'-H), 7.12, 7.13 (d, 1H, *J* = 8.1 Hz, 6-H), 7.21 (d, 1H, *J* = 3.0 Hz, 6'-H), 8.0 (bs, 3H, NH_3^+), 8.45, 8.47 (d, 1H, *J* = 8.0 Hz, CONH), 9.17, 9.18 (s, 1H, OH), 9.79, 9.81 (s, 1H, OH), 11.24 (s, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 17.1, 17.2 (γ-Val), 18.1, 18.3 (γ-Val), 18.4, 18.5 (γ-Val), 19.0, 19.0 (γ-Val), 24.2 (C_α), 24.6, 24.7 (γ-Pro), 29.2, 29.4 (β-Pro), 29.4, 29.6 (β-Val), 30.0, 30.3 (β-Val), 38.8 (C_β), 47.4, 47.5 (δ-Pro), 55.7, 55.7 (α-Val), 57.4, 57.6 (α-Val), 59.2, 59.4 (α-Pro), 108.1, 108.2 (C-3), 111.6 (C-5), 114.7 (C-6'), 118.4 (C-4'), 120.1 (C-3'), 124.1 (C), 124.9, 124.9 (C), 130.1, 130.2 (C-6), 149.1 (C), 149.4 (C), 153.5 (C), 155.8 (C), 166.7, 167.0 (CONH), 170.1, 170.2 (CONH), 171.4, 171.7 (OCO), 204.9 (CO) ppm. MS (ESI+): *m/z* 570.4 $[M + H]^+$, 552.4 $[M - H_2O]^+$. HRMS (ESI+) *m/z* $[M + H]^+$ calcd for $C_{30}H_{39}N_3O_8$ 570.28099; found 570.28226.

1-(2',5'-Dihydroxyphenyl)-3-[-2-hydroxy-4-(L-Val-L-Pro-L,D-Val)oxy]phenyl-1-propanone (21). Obtained using 20 equiv of BBr_3. Colorless solid (41% yield), mp = 130-133ºC). Purification by reverse phase flash chromatography, eluent: 2 to 30% gradient of ACN in H_2O + 0.05% TFA. HPLC (Sunfire): Mixture of diastereoisomers (49:51) t_R= 5.81 and 6.03 min (10 min gradient from 15 a 95% of A in B). ^{1}H RMN (500 MHz, DMSO-d_6) δ: 0.94-1.03 (m, 12H, , γ-Val), 1.81 (m, 2H, γ-Pro), 1.95 (m, 1H, β-Val), 2.12 (m, 2H, β-Pro), 2.21 (m, 1H, β-Val), 2.93 (t, 2H, *J* = 7.5 Hz, H_β), 3. (overlapped with H_2O signal; t, 2H, *J* = 7.5 Hz, H_α), 3.49 (m, 1H, δ-Pro), 3.70 (m, 1H, δ-Pro), 3.98 (s, 1H, α-Val), , 4.36 (m, 1H, α-Val), 4.55 (m, 1H, α-Pro), 6.80 (d, 1H, *J* = 8.9 Hz, 3'-H), 6.96, 6.98 (d, 1H, *J* = 6.6 Hz, 3-H and 5-H) 6.97 (d, 1H, *J* = 8.6 Hz, 4'-H), 7.21 (d, 1H, *J* = 3.00 Hz, 6'-H), 7.31, 7.32 (d, 2H, *J* = 8.5 Hz, 2-H and 6-H), 8.03 (sa, 3H, NH_3^+), 8.47, 8.48 (d, 1H, *J* = 7.7 Hz, CONH), 9.21 (s, 1H, OH), 11.18 (s, 1H, OH) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) δ: 17.6, 17.6 (γ-Val), 18.6, 18.6 (γ-Val), 18.8, 18.8 (γ-Val), 19.5, 19.5 (γ-Val), 25.1, 25.2 (γ-Pro), 29.1 (C_α), 29.6, 29.8 (β-Pro),

29.9, 30.1 (β-Val), 30.4, 30.6 (β-Val), 40.9 (C_β), 47.8, 47.9 (δ-Pro), 56.2 (α-Val), 58.0, 58.1 (α-Val), 59.6, 59.7 (α-Pro), 115.1 (C-6'), 118.8 (C-3'), 120.7, 120.7 (C), 121.7, 121.8 (C-3, C-5), 124.5 (C-4'), 129.8, 129.9 (C-2, C-6), 139.4 (C), 148.8 (C), 149.9 (C), 153.8 (C), 167.2, 167.4 (CONH), 170.7, 170.8 (CONH), 171.9, 172.1 (OCO), 204.7 (CO) ppm. MS (ESI+): *m/z* 554.3 $[M + H]^+$. HRMS (ESI+) *m/z* $[M + H]^+$ calcd for $C_{30}H_{39}N_3O_7$ 554.28608; found 554.28563.

Biological procedures.

Oocyte expression & electrophysiological studies. The human α7 nAChR cDNA was cloned in a derivative of the pSP64T vector containing part of the pBluescript olylinker. Capped mRNA was synthesized in vitro using SP6 RNA polymerase, the mMESSAGEmMACHINE kit from Ambion (Thermo Fisher Scientific, Madrid, Spain) and the pSP64T derivative mentioned above. Defoliculated *Xenopus laevis* oocytes were injected with 5 ng cRNA in 50 nl of sterile water. All experiments were performed within 2–3 days after cRNA injection. Unless otherwise specified, compounds were preapplied in the bath for 2 min and then co-applied with ACh through a pipette held very close to the oocyte for fast application. Functional expression of α7 nAChR was estimated as the peak ionic current evoked by 0.6 s application of 0.2 mM ACh at -80 mV. All experiments were performed at 22°C. Current records were measured with Clampfit 10.0 (MDS Analytical Technologies, Sunnyvale, CA, USA). Normalized peak currents were obtained by dividing the maximum value of the current obtained in the presence of 10 μM compound by the maximum value of the current obtained in control conditions.

Analgesic assay. Male Wistar rats (250–300 g) were obtained from Janvier France. All experiments were approved by the Institutional Animal and Ethical Committee of the Universidad Miguel Hernandez where experiments were conducted and they were in

accordance with the guidelines of the Economic European Community and the Committee for Research (Directive 2010/63/EU) and Ethical Issues of the International Association for the Study of Pain. All parts of the study concerning animal care were performed under the control of veterinarians. CFA emulsion (1:1 oil/saline, 0.5 mg/ml) was injected into the plantar surface (50 μl) of the left hind paw of rats [37]. Compounds were dissolved in DMSO, dilute in physiologic saline (0.9% sodium chloride) and injected IV at a total volume of 0,5 ml. The final DMSO concentration was 2,5%. Compounds were administered at 10 mg/kg i.v. 24 h after CFA injection. The mechanical allodynia was monitored 24 h after CFA injection and up to 4 h after administering the compounds. Paw withdrawal latency to mechanical stimulation was assessed with an automated testing device consisting of a steel rod that is pushed against the plantar surface of the paw with increasing force until the paw is withdrawn (Dynamic Plantar Aesthesiometer; Ugo Basile). The maximum force was set at 50 g to prevent tissue damage and the ramp speed was 2.5 g/s. Rats were placed in test cages with a metal grid bottom. They were kept in the test cages for 30–40 min to allow accommodation. The paw withdrawal latency was obtained as the mean of three consecutive assessments at each time point (at least 10 s between repeated measurements of the same paw).

Conflicts of interest: none.

Authors contribution: Idea: RGM, MJPV; Synthesis and chemical stability: BB, AP; Electrophysiology: JM, SS, FS; In vivo experiments: SGR,RT; Enzymatic stability: LN; Supervision: AF, AFM, MC, MJPV, RGM; This article has been written through contribution of all authors, which have approved the final version.

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Graphical abstract: