Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 741–751

BIOORGANIC & MEDICINAL CHEMISTRY

Anthranilic Acid Derivatives: A New Class of Non-Peptide CCK_1 Receptor Antagonists

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Received 2 August 2002; revised 13 September 2002; accepted 25 September 2002

Abstract—Having successfully obtained new CCK_1 ligands holding appropriate groups on the anthranilic acid dimer used as molecular scaffold we were interested in increasing their micromolar affinity for the CCK_1 receptors by modifying the spatial relationship of the main pharmacophoric groups. Since, we have proposed simplified analogues reducing the anthranilic acid dimer to a monomer. In this stage of our research program we have prepared and tested on CCK receptors a series of *N*-substituted anthranilic acid derivatives keeping a Phe residue at the C-terminal site. The indole-2-carbonyl group imparts the best CCK_1 receptor binding affinity (compound **1**: $IC_{50} = 197.5$ nM) while a sharp decrease in binding affinity is observed for the other indole containing derivatives. Moreover, in order to support the different binding behaviour observed for the synthesized compounds, a conformational investigation was carried out. Finally, on the basis of the main pharmacophoric groups of the obtained new lead compound (**1**) (coded **VL-0395**) a receptor binding hypothesis has been provided.

Introduction

Cholecystokinin (CCK) is an endogenous peptide used as a messenger from both cell chemical signaling systems (endocrine and nervous system).[1] The major hormonal effects of CCK are expressed in the gastrointestinal (GI) tract.[2,3] At the central nervous system (CNS) CCK acts as neurotransmitter/neuromodulator and appears to be involved in different neuropathological situations.[4,5] The entire range of peripheral and central biological actions of CCK is mediated by two distinct receptor types: CCK_1 and CCK_2.[6] The human CCK_1 and CCK_2 receptors have both been cloned and they have shown to belong to the G-protein coupled receptors family.[7,8] Among the different molecular forms of CCK only the C-terminal octapeptide fragment (Asp-Tyr-Met-Gly-Trp-Met-Asp-$PheNH_2$ or CCK-8) binds both receptors with nanomolar affinity. On the other hand, the smallest bioactive fragment of CCK (Trp-Met-Asp-$PheNH_2$ or CCK-4) binds with nanomolar affinity the CCK_2 receptor while its affinity towards the CCK_1 receptor decreases to the millimolar range.[9]

In the last decade a large number of non-peptide compounds known as CCK receptor antagonists have been developed.[10,11] CCK_1 antagonists have been shown to block some GI CCK induced actions such as the gallbladder contraction and the pancreatic secretion.[12,13] CCK_2 receptor antagonists have attracted a major interest because of their anxiolytic effects.[14]

During our ongoing laboratory program aimed at discovering new non-peptide compounds targeted to the CCK receptors, we have proposed a new class of non-peptide CCK_1 ligands holding appropriate pharmacophoric groups on the anthranilic acid dimer used as a molecular scaffold.[15,16] These compounds are endowed with micromolar affinity for the CCK_1 receptors. They have been designed using a strategy which combines the classical disconnection approach with the 'rational design' based on the chemical structure of the endogenous peptide. In fact, the anthranilic acid dimer was obtained as the main synthon of an our disconnection strategy applied to the first natural non-peptide CCK ligand Asperlicin.[17] On the other hand, the 'appended' pharmacophoric groups were selected from the C-terminal tetrapeptide sequence of the endogenous ligand (CCK-4). This part of our work has produced a lead compound

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0968-0896/03/$ - see front matter
PII: S0968-0896(02)00475-3

coded VL-0494 (D,L-{2-[2-(3-1*H*-indol-3-yl-propionyl-amino)-benzoylamino]-benzoyl-amino}-3-phenyl-propionic acid)[18] characterized by the presence of Phe and desaminated Trp residues at the C- and N-termini of anthranilic acid dimer. These two residues represent 'address' type components and were selected from the CCK-4 structure (Fig. 1). Despite these common structural features, the affinity of VL-0494 for the CCK_1 receptor was three orders of magnitude greater than that of CCK-4.

To explain this difference, we undertook a molecular modelling study on VL-0494. This study showed that the main difference concerned the tetrapeptide backbone and the anthranilic acid dimer structure. In particular, the conformational preference of VL-0494 was a staggered U-shape, whereas the CCK-4 assumed an S-like bend conformation. In the proposed graphical model (Fig. 2) of the interaction of VL-0494 at the CCK_1 receptor binding sites compared to that of CCK-4 toward CCK_2 receptor,[19,20] we have hypothesised the presence of two lipophilic pockets in which both ligands interact with the same pharmacophoric groups.

Although VL-0494 and CCK-4 have a similar 'regnylogical type' bioactive conformation, a reversal in selectivity to the CCK receptors has been noticed. This behaviour has already been described in the CCK chemical literature.[21] Nevertheless, in the case of VL-0494, it is possible that the conformational difference of the anthranilic acid dimer in comparison to the central portion of the backbone of CCK-4, could produce the increase of the CCK_1 receptor affinity of the lead compound.

Having successfully obtained new CCK_1 ligands from the native peptide and/or from asperlicin we were interested in increasing the CCK_1 affinity by modifying the spatial relationship of the pharmacophoric groups. Therefore, we have directed our synthetic efforts towards the preparation of simplified analogues of VL-0494 reducing the anthranilic acid dimer to a monomer. In this preliminary stage of our research program we have prepared a series of *N*-substituted anthranilic acid derivatives keeping a Phe residue at the C-terminal site as in the case of the lead compound (Table 1).

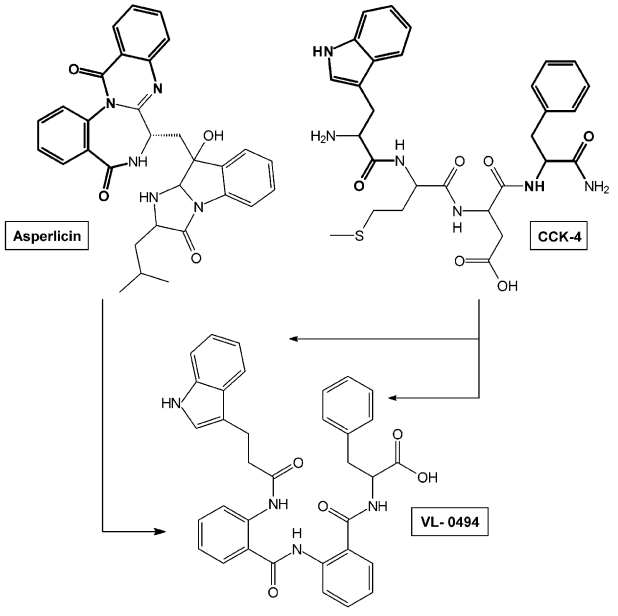


Figure 1. Compound VL-0494 derived from asperlicin as well as from CCK-4.

The substituents selected to be appended via amide bond formation at the N-terminus of anthranilic acid are: bicyclic aromatic or heteroaromatic systems (naphthalene or indole containing substituents) (**1**–**8**), a phenyl ring linked by a short saturated alkyl chain (**9**–**10**) and bulky or branched alkyl groups such as *tert*-butyl and 1-adamantyl groups.

Chemistry

The synthetic pathway to anthranilic acid derivatives **1**–**14** employing standard procedures is shown in Scheme 1. The key intermediate **15** was prepared in good yield (80%) via *ortho*-amino benzoylation of the DL-phenylalanine ethyl ester with isatoic anhydride. Derivatives **19**, **20** were obtained by coupling the intermediate **15** with the corresponding acid that was previously activated by the mixed anhydride method. Compounds **16**, **18** and **29** were obtained via acyl chloride formation. Compounds **21**–**28** were obtained using commercially available acyl chlorides.

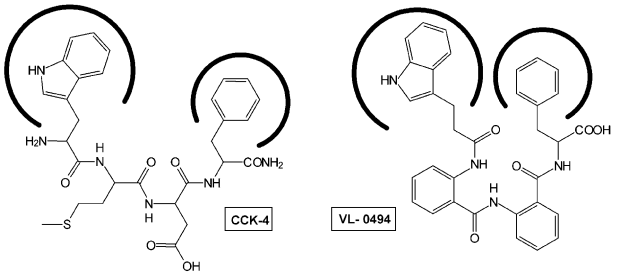
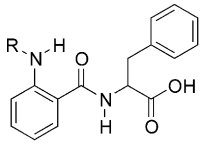


Figure 2. Hypothetic model of the 'regnylogic type' interaction of CCK-4 and VL-0494 at the CCK_2 and CCK_1 receptors, respectively.

Table 1. Structure of the target compounds

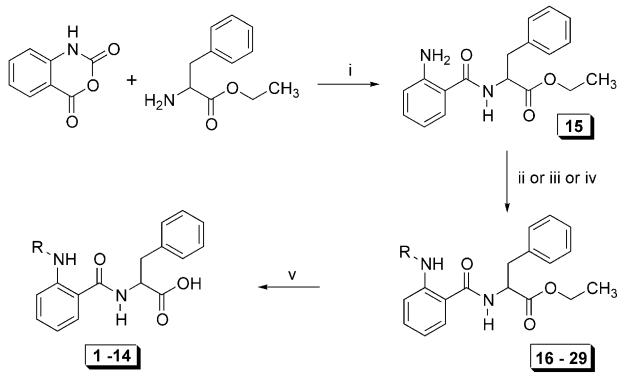


Compd	R	Compd	R
1	2-Indolyl-CO–	**8**	2-Naphthyl–SO_2–
2	5-Indolyl-CO–	**9**	C_6H_5-CH_2–CO–
3	3-Indolyl-CO–	**10**	C_6H_5-$(CH_2)_2$–CO–
4	3-Indolyl-CH_2-CO–	**11**	$C(CH_3)_3$–CO–
5	3-Indolyl-$(CH_2)_2$-CO–	**12**	$C(CH_3)_3$–CH_2–CO–
6	1-Naphthyl-CO–	**13**	1-Adamantyl-CO-
7	1-Naphthyl-SO_2–	**14**	1-Adamantyl-CH_2-CO-

Indole-5-carboxylic acid was condensed with the intermediate **15** using the Mukaiyama reagent[22] to afford compound **17**. The free acids **1**–**14** were obtained in almost quantitative yields by base catalyzed hydrolysis of the corresponding ethyl esters **16**–**29**. Synthetic data of the esters **16**–**29** and of the target compounds **1**–**14** are listed in the Experimental.

Results and Discussion

Table 2 summarizes the results of the CCK_1 receptor affinity of compounds **1**–**14** expressed as IC_{50} or as percentage of inhibition (ISB%) determined at the highest used dose (10 or 30 μM, as indicated). With regard to the affinity with the central CCK_2 receptor, the tested compounds showed low affinity to this receptor subtype. Furthermore, as previously observed for the anthranilic acid dimer derivatives, only few compounds containing the indole moiety exhibited affinity toward CCK_1 receptors. In particular, the indole-2-carbonyl group imparts the best CCK_1 receptor binding affinity and it is therefore the optimum N-terminus group in this series.



Scheme 1. Reagents and reaction conditions: (i) AcOEt, reflux, 2 h; (ii) RCOOH, iBuOCOCl, Et_3N, CH_2Cl_2 dry; (iii) RCOCl, Et_3N, CH_2Cl_2 dry; (iv) RCOOH, 2-chloro-1-methylpyridinium iodide (Mukaiyama reagent), Et_3N, CH_2Cl_2 dry; (v) KOH, MeOH.

In fact, compound **1** (IC_{50} = 197.5 nM) was found to be ten times more potent than the starting lead compound VL-0494 (IC_{50} = 2300 nM).[18] However, a sharp decrease in binding affinity is observed for both indole-5 and indole-3-carbonyl groups (**2** and **3**) (ISB = 41% at 10 and IC_{50} = 1870 nM, respectively). An additional decrease in affinity was observed for the higher homologues of compound **3** (**4** and **5**) (ISB = 22% at 10 and 17,800 nM, respectively). Moreover, it is interesting to note that, among these indole derivatives, the indole-3-propionyl moiety exerts only a weak affinity, whereas it was found to be the optimum group in the *N*-substituted anthranilic acid dimer derivatives (VL-0494).

The introduction of the 1-naphthyl or 2-naphthyl group either by an amidic or sulfonamidic bond to the phenyl ring of the anthranilic acid led to a complete inactivity at the maximum dose tested **6, 7** and **8**). Furthermore, phenyl substituted compounds (**9** and **10**) displayed a very low affinity for CCK_1 receptors compared with the corresponding indolic derivatives (**4** and **5**).

In the same way, the introduction of bulky aliphatic groups led to inactive compounds (**11**–**14**). Nevertheless, while the present investigation does not represent an exhaustive study on the N-terminal optimisation of the anthranilic acid derivatives, the difference in affinity of the compounds containing the indole moiety in comparison to the others, is remarkably consistent.

In order to support the different binding behaviour observed for the synthesized compounds, a conformational investigation was carried out. The anthranilic core of the molecules was first analyzed carefully. When

Table 2. CCK receptors Binding Data

Compd.	R	IC_{50}[a] μM	
		Rat pancreatic acini (CCK_1)	Guinea pig brain cortex (CCK_2[d])
1	2-Indolyl-CO–	0.197 (0.131–0.298)	16.40
2	5-Indolyl-CO–	41% ISB[b]	NT
3	3-Indolyl-CO–	1.87 (1.48–2.38)	30.20
4	3-Indolyl-CH_2-CO–	IN[b]	23% ISB[c]
5	3-Indolyl-$(CH_2)_2$-CO–	17.85 (12.00–26.56)	13.40
6	1-Naphthyl-CO–	IN[b]	6.57 (3.06–14.10)
7	1-Naphthyl-SO_2–	IN[b]	5.90 (3.94–8.84)
8	2-Naphthyl-SO_2–	IN[b]	7.31 (3.90–13.60)
9	C_6H_5–CH_2–CO–	IN[b]	36% ISB[c]
10	C_6H_5–$(CH_2)_2$–CO–	IN[b]	37% ISB[c]
11	$C(CH_3)_3$–CO–	IN[b]	24% ISB[c]
12	$C(CH_3)_3$–CH_2–CO–	IN[b]	37% ISB [c]
13	1-Adamantyl-CO–	IN[b]	14.50
14	1-Adamantyl-CH_2-CO–	51% ISB[c]	15.40

IN, inactive; NT, not tested.
[a]IC_{50}: μM concentration and $p = 0.05$ fiducial limits required to inhibit by 50% the specific binding of 25 pM [^{125}I]-(BH)-CCK8%; ISB: percentage inhibition of specific binding at the maximal concentration tested.
[b]10 μM.
[c]30 μM.
[d]Values without fiducial limits were obtained from not more than two experiments.

this residue is inserted within a pseudopeptidic chain as a non-natural amino acid, it shows the tendency to form an intramolecular hydrogen bond between the anthranilic amide hydrogen and the carbonyl oxygen. On the basis of this structural feature, a number of oligoanthranil amides have been prepared in the past, and used as molecular scaffolds.[23] This tendency is also shown by our ligands, as it can be deduced by their NMR properties, and it is confirmed by the modelling results. Nevertheless, the resulting pseudocyclic structures are quite strained, and low-level semi-empirical methods such as AM1 and PM3 resulted to be prone to compensate steric strain by a rather erratic pyramidalization of one of the two amide nitrogens of the core. This is not confirmed by the known X-ray structures of related compounds,[23] where the anthranilic nitrogen is always full planar, and only a slight piramidalization of the carboxamide nitrogen is observed in a single case. For this reason a reference compound, namely the *N*-acetyl-anthranilic acid *N*-methyl amide was submitted to ab-initio optimization. At the HF/3-21/G(d) level the anthranilic nitrogen is planar, while the other nitrogen is indeed piramidal (sum of bond angles at nitrogen 354.4°); by raising the level of theory up to HF/6-31/G** the whole molecule becomes fully planar.

With this result in mind, the entire set of molecules was resubmitted to an extensive conformational search at the AM1 level, but all the structures not planar at the anthranil nitrogen were discharged, while the phenylalanine nitrogen was allowed to result slightly piramidal in several conformational minima.

The conformational search performed on compound **1** gave two families of very similar U-shaped conformations. The intramolecular hydrogen bond is found in both the conformations (H–O distance 2.13 Å), and both the indole ring and the aromatic ring of phenylalanine lie on the same side of the molecule. The two conformations differ in the orientation of the indole nitrogen, which is in a *trans*-like position with respect to the anthranilic nitrogen in the absolute minimum (Fig. 3).

In the other conformation, which is 1.8 kcal/mol higher in energy, the indole ring is rotated by 180° and its nitrogen is in a *cis*-like position. A better dipole moment is seen to favour the first conformation (2.756 D vs 3.609 D). In the absolute minimum the average distance between the two aromatic ends of the molecule is 9.5 Å, and the angle between the planes containing the two rings is about 114°. Another local minimum can be obtained by rotating the N–Cα bond of Phe by about 60°. This minimum is 3.8 kcal/mol higher in energy, and the two aromatic rings are closer. The average distance is less than 8 Å for this conformation.

The lowest energy conformer of the indole-5-carbonyl derivative (**2**) shows an almost identical geometry to that of compound **1** (Fig. 4), but the intramolecular hydrogen bond of the anthranilic core of the molecule lies on the opposite side of the plane containing the peptide bond with the Phe residue. Nevertheless, a conformation with the hydrogen bond on the same side as in the case of compound **1** is easily accessible, being only 0.1 *K*cal/mol higher in energy.

The distance between the aromatic ends in the two conformations is almost the same, and it is very similar to the distance obtained for the 2-indole carboxylic acid derivative.

Assuming the same binding modalities for compounds **1** and **2**, the higher affinity of the former is probably due to the different spatial position of the indole nitrogen. In particular we have supposed that the hydrogen attached to the indole nitrogen of compound **1** may have a direct interaction with the receptor via a hydrogen bond.

After modelling compound **3** it was possible to find a single conformation family (Fig. 5), which is basically similar to the minimum of the indole-2-carbonyl derivative (**1**). The hydrogen bond is preserved, and the

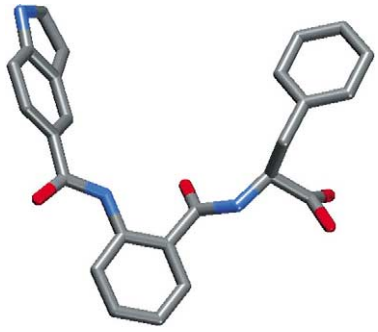


Figure 4. Lowest energy conformer of compound **2** (heavy atoms only).

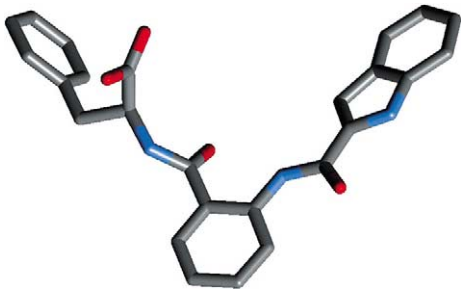


Figure 3. Lowest energy conformer of compound **1** (heavy atoms only).

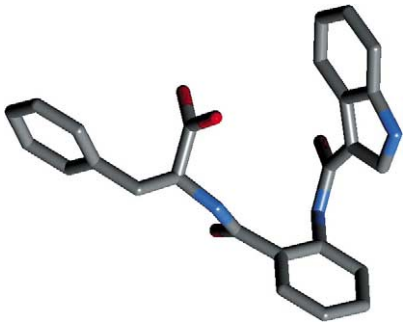


Figure 5. Lowest energy conformer of compound **3** (heavy atoms only).

aromatic rings (indole and Phe) lie on the same side of the molecule at an average distance of about 9.5 Å. The indole ring also, is placed like a 'flag' related to the amido group in the 3 position and its plane is perpendicular to that of the anthranilic acid. In this case the space available to the indole ring by rotation around its C3–CO bond is clearly much larger than in the 2-indole derivative. The difference can be estimated as about 180 $Å^3$ in favour of the 3-indole derivative. In contrast, the indole ring of the 2-derivative reaches a major distance from the anthranilic core of the molecule (8.4 vs 6.3 Å).

From a fitting performed between compound **1** and **3** matching the common anthranoyl-Phe framework of the two molecules it is possible to observe the quite different spatial arrangement of the two indole rings (Fig. 6).

The lower affinity of compound **3** suggests that the indole-3 substituent cannot satisfy completely the conformational restraints imposed by the second hydrophobic pocket of the receptor.

The geometry of the indole-3-propionic derivative (**5**) is very different from the geometries of the carbonyl-indole derivatives (**1**–**3**) (Fig. 7). The intramolecular hydrogen bond in the anthranilic residue is still preserved, but the U-shaped conformation is lost. The indole side of the molecule is bending back towards the Phe residue. This is most likely due to the formation of a new intramolecular hydrogen bond between the indole NH and the Phe carboxylic group, as well as to the π-stacking interactions between the indole and the anthranilic rings, which lie in parallel planes at 3.5 Å. Although the conformational search on this compound was carried out excluding solvent interactions, this conformation seems to be probable in aqueous solution according to the 'hydrophobic collapse' phenomenon.

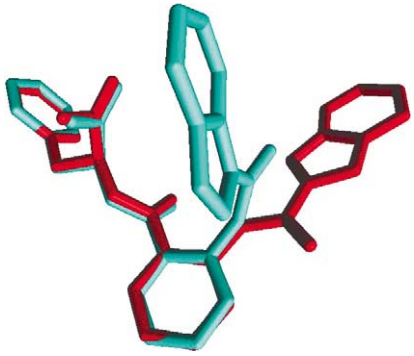


Figure 6. Superimposition (heavy atoms only) of the preferential conformation of compound **1** (red) and compound **3** (green).

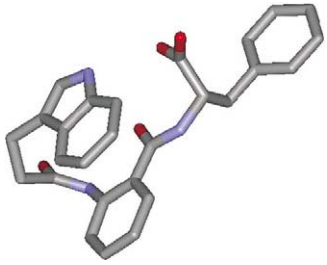


Figure 7. Lowest energy conformer of compound **5** (heavy atoms only).

The conformational analysis of the alkyl group containing derivatives (**11**–**13**) is straightforward. They have a single minimum, with the intramolecular hydrogen bond on the anthranilic residue, and the bulky alkyl group on the same side of the Phe residue, as a consequence of the *trans* geometry of the amide bonds.

Finally, the lowest energy conformations obtained for the 1-naphthyl (**6** and **7**) and 2-naphthyl derivatives (**8**) resemble that of compounds **3** and **1**, respectively. Thus, the conformational effect could not be evoked to explain either the loss in CCK_1 affinity or their reverse selectivity for the CCK_2 receptor subtype. It is possible, therefore, that information on the CCK_2 binding sites may be gained from further studies of these derivatives.

Conclusion

In conclusion, during a program aimed at searching for CCK receptor antagonists, we have found that simplifying the anthranilic acid dimer scaffold to a monomer gives rise to a structurally simple compound endowed with high affinity towards CCK_1 receptors. The new lead compound obtained (compound **1** or **VL-0395**) could represent a new starting point for the development of an innovative class of non-peptide CCK_1 receptor antagonists.

The antagonist nature of **VL-0395** was confirmed by an in vivo functional test. The compound, intravenously administered, inhibited the guinea pig gallbladder contractions induced by CCK8 sulphated (8.8 pmol/kg iv), with an ED_{50} of 0.38 μmol/kg (0.11–1.36; $p = 0.05$ fiducial limits). Its potency was comparable with that exhibited by the reference CCK_1 selective antagonist Loxiglumide (n. 5610 Merck Index, 13th Edition),[24] that was active, under the same experimental condition, at 0.24 μmol/kg (0.13–0.41; $p = 0.05$ fiducial limits).

Moreover, on the basis of the main pharmacophoric groups (indole moiety and phenyl ring of Phe) shared by **VL-0395**, a receptor binding hypothesis, similar to that described for the anthranilic acid dimer derivatives has been provided. In fact, this model of binding (depicted in Fig. 8) requests at least two hydrophobic pockets to accommodate the main pharmacophoric groups. Each binding pocket is about 5 Å deep and the distance

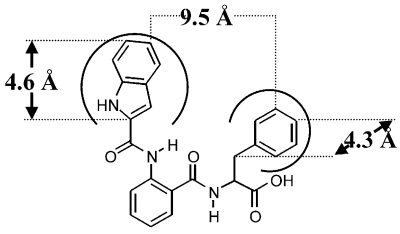


Figure 8. Schematic representation of the interactions of the main pharmacophoric groups of compound **1** with the CCK_1 receptor.

between them is about 9.5 Å. However, the proposed temporary model will be integrated when further experimental data will be available regarding the additional binding contribution due to the anthranilic phenyl ring as well as to the free carboxy terminal group.

Although the importance of one of the pharmacophoric groups considered (2-indole ring) in the interaction with the CCK_1 receptors has already been stressed by the 1,4-benzodiazepine antagonists[25] we have demonstrated that the same receptor, probably with different binding sites, is perfectly able to host anthranilic acid derivatives structurally unrelated to the previous ones.

Finally, in order to obtain more information on the binding modalities of our new lead compound, its main structural features (indole moiety, anthranilic acid, aminoacid side chain, and the free carboxy group) are currently under structural modifications and analysis.

Experimental

Chemical procedures

All chemicals and solvents used in syntheses were reagent-grade products and were used without additional purification. Reaction progress was monitored by ascending thin-layer chromatography (TLC) using precoated silica gel plates (60F-254 Merck). Visualization of the chromatograms was achieved by short wave UV light (254 nm). Melting points were determined on a Büchi 510 melting point apparatus (Büchi, Flawil, Switzerland) and are uncorrected. Preparative medium-pressure chromatography (MPLC) was performed on a Büchi 688 apparatus using silica gel (Merck Kieselgel 60, 15–40 μm). Proton (^{1}H NMR, 200 MHz) and carbon (^{13}C NMR, 50 MHz) nuclear magnetic resonance spectra were recorded on a Varian-Gemini 2000 Fourier Transform spectrometer using $CDCl_3$ or $(CD_3)_2SO$ as solvent. Chemical shifts were reported as parts per million (ppm, δ units) downfield from an internal Me_4Si standard. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and b, broad. ^{13}C NMR spectra were determined using either the Attached Proton Test (APT) or standard ^{13}C pulse sequence parameters. Spectral data are consistent with assigned structures. Mass spectra were recorded on an API-1 Perkin-Elmer SCIEX spectrometer by electrospray ionisation (ES).

Syntheses

2(*R*,*S*)-(2-Amino-benzoylamino)-3-phenyl-propionic acid ethyl ester (15). A suspension of DL-phenylalanine ethyl ester hydrochloride (5.00 g, 21.8 mmol) in 150 mL of ethyl acetate was treated with triethylamine (3.07 mL, 21.8 mmol) followed by isatoic anhydride (3.55 g, 21.8 mmol). The resulting mixture was refluxed under stirring for 2 h, cooled to room temperature and filtered. The organic phase was thoroughly washed with 1 M NaOH (2×50 mL), water (2×50 mL) and brine, dried over anhydrous sodium sulphate and concentrated in vacuo. Trituration with petroleum ether 40–70° afforded the analytically pure title compound in 80% yield. R_f 0.69 (AcOEt/hexane : 1/1); mp 84–85 °C; ^{1}H NMR ($CDCl_3$): δ1.24 (t, 3H, $-CH_3$); 3.21 (m, 2H, $-CH_2-CH<$); 4.18 (q, 2H, $-CH_2-O-$); 4.97 (m, 1H, $>CH-$); 5.45 (s, 2H, $-NH_2$); 6.52 (d, 1H, –NH–); 6.61–7.28 (m, 9H, ar). ^{13}C NMR ($CDCl_3$): δ14.19, 38.05, 53.20, 61.63, 115.41, 116.69, 117.29, 127.15, 127.43, 128.60, 129.43, 132.59, 136.04, 148.84, 168.66, 171.75.

General coupling procedures of anthranoyl-DL-phenylalanine ethyl ester

Method A (19, 20). A solution of 10 mmol of the corresponding acid in 100 mL of dry dichloromethane cooled to −10 °C was treated with triethylamine (1.4 mL, 10 mmol) followed by *iso*butyl chloroformate (1.31 mL, 10 mmol). The resulting mixture was stirred at −10 °C for 20 min and treated dropwise with a solution of compound **15** (3.12 g, 10 mmol) in 50 mL of dry dichloromethane. After the addition was complete, the reaction was stirred at room temperature for 1 h and then refluxed for 3 h. The solvents were evaporated, the residue was dissolved in dichloromethane, the organic layer was washed with diluted aqueous sodium hydroxide solution and with water, dried (sodium sulphate), and evaporated to dryness. Crystallization from MeOH yielded compound **19** while in the case of compound **20** the residue was used without further purification in the next step of the synthesis.

2(*R*,*S*)-[2-(2-1*H*-Indol-3-yl-acetylamino)-benzoylamino]-3-phenyl-propionic acid ethyl ester (19). Yield 72%; Molecular formula: $C_{28}H_{27}N_3O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.43; mp 130–131 °C; ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, $-CH_3$); 3.01 (m, 2H, $-CH_2-CH<$); 3.89 (s, 2H, $-CH_2-CO-$); 4.19 (q, 2H, $-CH_2-O-$); 4.65 (m, 1H, $-CH<$); 6.44 (d, 1H, $-NH-CH<$); 6.96–7.64 (m, 13H, ar); 8.34 (s, 1H, –NH– ind); 8.59 (d, 1H, ar); 10.68 (s, 1H –NH–).; ^{13}C NMR ($CDCl_3$) δ 14.19, 35.33, 37.65, 53.41, 61.77, 108.79, 111.22, 118.86, 119.80, 121.53, 122.31, 122.89, 123.95, 126.49, 127.23, 128.57, 129.38, 132.58, 135.63, 140.20, 143.10, 168.70, 171.50, 172.32.

Method B (16, 18, 21–29). A solution of 10 mmol of the corresponding acyl chloride (indole-2-carbonyl chloride was prepared according to the procedure described by Kermack[26] while indole-3-carbonyl chloride by a standard method using PCl_5 in dry dichloromethane) in 30 mL of dry dichloromethane was gradually added to a solution of compound **15** (3.12 g, 10 mmol) in the same solvent (50 mL). The pH of the reaction was adjusted to 9.5 with triethylamine and the solution stirred for 2 h. The reaction mixture was diluted with 100 mL of dichloromethane and washed in succession with 0.1 N NaOH, water, 0.1 N HCl and water. The dried (sodium sulphate) organic phase was rotary evaporated and the residue was purified as described to yield the titled compounds.

2(*R*,*S*)-{2-[(1*H*-Indole-2-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid ethyl ester (16). Trituration with

hot MeOH afforded the titled compound in 78% yield. Molecular formula: $C_{27}H_{25}N_3O_4$; TLC (AcOEt/hexane: 1/1)R_f:0.69; mp 210–211 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (t, 3H, –CH_3); 3.20 (m, 2H, –CH_2–CH <); 4.11 (q, 2H, –CH_2–O–); 4.79 (m, 1H, –CH <); 6.98 (s, 1H, ind); 7.06–7.82 (m, 12H, ar); 8.64 (d, 1H, ar); 9.30 (d, 1H, –NH—CH <); 11.95 (s, 1H, –NH–ind); 12.15 (s, 1H –NH–); ^{13}C NMR (DMSO-d_6) δ 14.80, 36.83, 55.14, 61.56, 103.32, 113.22, 119.98, 120.63, 120.95, 122.49, 123.30, 124.82, 127.25, 127.62, 128.94, 129.18, 129.81, 132.19, 133.38, 137.81, 138.16, 139.82, 159.77, 169.58, 171.91.

2(*R*,*S*)-{2-[(1H-Indole-3-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid ethyl ester (18). Preparative medium pressure chromatography (MPLC) (silica gel, AcOEt/hexane: 1/3) afforded the titled compound in 20% yield. Molecular formula: $C_{27}H_{25}N_3O_4$; TLC (AcOEt/hexane: 1/1)R_f: 0.30; mp 147–149 °C; ^{1}H NMR (DMSO-d_6) δ 1.11 (t, 3H, –CH_3); 3.14 (m, 2H, –CH_2–CH <); 4.04 (q, 2H, –CH_2–O–); 4.65 (m, 1H, –CH <); 7.07–8.60 (m, 14H, ar); 9.17 (d, 1H, –NH–CH <); 11.46 (s, 1H, –NH– ind); 11.81 (s, 1H –NH–); ^{13}C NMR (DMSO-d_6) δ 13.75, 35.92, 54.06, 60.50, 106.64, 111.09, 119.71, 120.90, 120.99, 121.16, 122.21, 122.67, 125.24, 125.42, 126.21, 127.94, 128.81, 131.65, 133.90, 137.25, 138.40, 160.81, 168.23, 170.99.

2(*R*,*S*)-{2-[(Naphthalene-1-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid ethyl ester (21). Preparative medium pressure chromatography (MPLC) (silica gel, AcOEt/hexane: 1/3) afforded the titled compound as oil in 45% yield. Molecular formula: $C_{29}H_{26}N_2O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.46; ^{1}H NMR ($CDCl_3$) δ 1.15 (t, 3H, –CH_3); 3.16 (m, 2H, –CH_2–CH <); 4.10 (q, 2H, –CH_2–O–); 4.90 (m, 1H, –CH <); 6.93–8.87 (m, 17H, ar and –NH–CH <); 11.54 (s, 1H –NH–); ^{13}C NMR ($CDCl_3$) δ 14.19, 37.87, 53.76, 61.81, 120.36, 121.56, 123.23, 125.00, 125.63, 125.74, 126.50, 127.06, 127.25, 128.45, 128.64, 129.34, 130.58, 131.33, 132.93, 133.98, 134.45, 135.93, 139.86, 167.91, 168.54, 171.39.

2(*R*,*S*)-[2-(Naphthalene-1-sulfonylamino)-benzoylamino]-3-phenyl-propionic acid ethyl ester (22). Crystallization from EtOH 95% afforded the titled compound in 20% yield; Molecular formula: $C_{28}H_{26}N_2O_5S$; TLC (AcOEt/hexane: 1/2) R_f: 0.30; mp 126 °C; ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, –CH_3); 3.10 (m, 2H, –CH_2–CH <); 4.21(q, 2H, –CH_2–O–); 4.72 (m, 1H, –CH <); 6.37 (d, 1H, –NH–CH <); 6.88–8.76 (m, 16H, ar); 11.20 (s, 1H –NH); ^{13}C NMR ($CDCl_3$) δ 14.27, 37.68, 53.48, 61.94, 119.81, 120.30, 123.23, 124.01, 124.78, 126.89, 127.31, 128.07, 128.35, 128.63, 128.94, 129.39, 130.16, 132.92, 134.20, 134.48, 134.62, 135.48, 139.00, 167.54, 171.00.

2(*R*,*S*)-[2-(Naphthalene-2-sulfonylamino)-benzoylamino]-3-phenyl-propionic acid ethyl ester (23). (MPLC) (silica gel, CH_2Cl_2) afforded the titled compound as oil in 52% yield. Molecular formula: $C_{28}H_{26}N_2O_5S$; TLC (AcOEt/hexane: 1/2) R_f: 0.32; mp 48 °C; ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 3H, –CH_3); 3.10 (m, 2H, –CH_2–CH <); 4.17(q, 2H, –CH_2–O–); 4.81 (m, 1H, –CH <); 6.46 (d, 1H, –NH–CH <); 6.94–8.41 (m, 16H, ar); 10.90 (s, 1H –NH–); ^{13}C NMR ($CDCl_3$) δ 14.23, 37.67, 53.50, 61.96, 120.60, 121.17, 122.41, 123.66, 126.93, 127.34, 127.52, 127.93, 128.67, 128.80, 128.87, 129.33, 132.06, 133.08, 134.89, 135.47, 136.53, 139.04, 167.67, 171.01.

3-Phenyl-2(*R*,*S*)-(2-phenylacetylamino-benzoylamino)-propionic acid ethyl ester (24). The residue was used without further purification in the next step of the synthesis.

3-Phenyl-2(*R*,*S*)-[2-(3-phenyl-propionylamino)-benzoylamino]-propionic acid ethyl ester (25). Trituration with petroleum ether 40–70° afforded the titled compound in 82% yield; Molecular formula: $C_{27}H_{28}N_2O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.72; mp 73–75 °C; ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, –CH_3); 2.71 (t, 2H, –CH_2–Ph); 3.06 (t, 2H, –CH_2–CO–); 3.24 (m, 2H, –CH_2–CH <); 4.24 (q, 2H, –CH_2–O–); 4.98 (m, 1H, > CH–); 6.72 (d, 1H, –NH–CH <); 7.03–7.50 (m, 13H, ar); 8.62 (d,1H, ar); 10.93 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$) δ 14.22, 31.42, 37.91, 39.97, 53.60, 61.92, 119.62, 121.48, 122.79, 126.19, 126.66, 127.34, 128.34, 128.41, 128.52, 128.70, 129.38, 132.95, 135.65, 139.67, 140.76, 168.34, 170.97, 171.27.

2(*R*,*S*)-[2-(2,2-Dimethyl-propionylamino)-benzoylamino]-3-phenyl-propionic acid ethyl ester (26). Trituration with hot MeOH afforded the titled compound in 50% yield; Molecular formula: $C_{23}H_{28}N_2O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.64; mp 107 °C; ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 3H, –CH_3); 1.31 (s, 9H, –$C(CH_3)_3$); 3.23 (m, 2H, –CH_2–CH <); 4.22 (q, 2H, –CH_2–O–); 5.03 (m, 1H, > CH–); 6.71 (d, 1H, –NH–CH <); 6.97–8.66 (m, 9H, ar); 11.16 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$) δ 14.20, 27.61, 37.80, 40.19, 53.58, 61.83, 119.88, 121.43, 122.55, 126.63, 127.32, 128.67, 129.35, 132.87, 135.71, 140.15, 168.53, 171.25, 177.73.

2(*R*,*S*)-[2-(3,3-Dimethyl-butyrylamino)-benzoylamino]-3-phenyl-propionic acid ethyl ester (27). Crystallization from EtOH 95% afforded the titled compound in 25% yield; Molecular formula: $C_{24}H_{30}N_2O_4$; TLC (AcOEt/hexane: 1/2) R_f: 0.46; mp 109 °C; ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 9H, –$C(CH_3)_3$); 1.29 (t, 3H, –CH_3); 2.25 (s, 2H, –CO–CH_2–); 3.24 (m, 2H, –CH_2–CH <); 4.22 (q, 2H, –CH_2–O–); 5.01 (m, 1H > CH–); 6.70 (d, 1H, –NH–CH <); 7.02–8.64 (m, 9H, ar); 10.81 (s, 1H –NH–); ^{13}C NMR ($CDCl_3$) δ 14.20, 29.88, 31.29, 37.83, 52.42, 53.58, 61.87, 119.81, 121.43, 122.66, 126.63, 127.33, 128.69, 129.34, 132.87, 135.64, 139.68, 168.41, 170.75, 171.20.

2(*R*,*S*)-{2-[(Adamantane-1-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid ethyl ester (28). Trituration with petroleum ether 40–70° afforded the titled compound as oil in 80% yield; Molecular formula: $C_{29}H_{34}N_2O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.71; ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, –CH_3); 1.70–2.08 (m, 15H, ada); 3.24 (m, 2H –CH_2–CH <); 4.21 (q, 2H, –CH_2–O–); 5.03 (m, 1H, > CH–); 6.94 (d, 1H, –NH–CH <); 7.09–8.65 (m, 9H, ar); 11.02 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$) δ 14.45, 28.53, 36.82, 37.99, 39.38, 42.30, 53.96,

61.96, 120.41, 121.76, 122.71, 127.05, 127.45, 128.86, 129.58, 132.92, 136.22, 140.23, 168.88, 171.56, 177.44.

2(*R*,*S*)-[2-(2-Adamantan-1-yl-acetylamino)-benzoylamino]-3-phenyl-propionic acid ethyl ester (29). MPLC (silica gel, CH_2Cl_2/AcOEt: 4/1) afforded the titled compound in 20% yield. Molecular formula: $C_{30}H_{36}N_2O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.76; mp 142–143 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, –CH_3); 1.65–2.13 (m, 17H, ada and –CH_2–CO–); 3.25 (m, 2H –C<u>H</u>$_2$–CH <); 4.27 (q, 2H, –CH_2–O–); 5.02 (m, 1H, > C<u>H</u>–); 6.65 (d, 1H, –N<u>H</u>–CH <); 7.04–8.66 (m, 9H, ar); 10.77 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$) δ 14.16, 28.60, 33.24, 36.65, 37.75, 42.50, 48.29, 53.55, 61.83, 119.69, 121.29, 122.56, 126.48, 127.23, 128.56, 129.22, 132.79, 135.41, 139.43, 168.23, 169.95, 171.03.

Method C

2(*R*,*S*)-{2-[(1*H*-Indole-5-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid ethyl ester (17). To a solution of compound **15** (1.00 g, 3.20 mmol) and triethylamine (0.45 mL, 3.20 mmol) in 15 mL of dry dichloromethane was added 2-chloro-1-methylpyridinium iodide (1.68 g, 6.40 mmol) and indole-5-carboxylic acid (0.51 g, 3.20 mmol) and the mixture was stirred under reflux for 12 h. After addition of water (20 mL) the reaction mixture was extracted with dichloromethane (3×20 mL). The organic layer was washed in succession with 1 N HCl, water, 1 N NaOH and brine. The dried organic phase was rotary evaporated and the residue was chromatographed on silica gel eluting with CH_2Cl_2/AcOEt (4:1) to afford compound **17** in 35% yield. Molecular formula: $C_{27}H_{25}N_3O_4$; TLC (AcOEt/hexane: 1/1) R_f: 0.45; mp 74–76 °C; ^{1}H NMR (DMSO-d_6) δ 1.12 (t, 3H, –CH_3); 3.18 (m, 2H, –C<u>H</u>$_2$–CH <); 4.10 (q, 2H, –CH_2–O–); 4.72 (m, 1H, –C<u>H</u> <); 6.60–8.68 (m, 14H, ar); 9.26 (d, 1H, –N<u>H</u>–CH <); 11.49 (s, 1H, –NH– ind); 11.91 (s, 1H, –NH–); ^{13}C NMR (DMSO–d_6) δ 14.79, 36.81, 55.12, 61.50, 103.08, 112.29, 120.50, 120.59, 120.65, 120.85, 122.99, 125.95, 127.24, 128.03, 128.92, 129.08, 129.79, 133.15, 138.17, 138.53, 140.28, 166.18, 169.72, 171.92.

General procedure for the synthesis of compounds 1–14

A mixture of 5 mmol of the corresponding ethyl ester (**16**–**29**) in methanol (50 mL) and in the presence of potassium hydroxide (0.56 g, 10 mmol) was gently warmed for 4 h. The solvent was removed under reduced pressure and the residue taken up with water. After cooling, the solution was adjusted to pH 2–3 with diluted HCl to obtain the precipitation of the acid.

2(*R*,*S*)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid (1). Crystallization from MeOH afforded the titled compound in 85% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.61; mp 268–269 °C; ^{1}H NMR (DMSO-d_6) δ 3.27 (m, 2H –C<u>H</u>$_2$–CH <); 4.79 (m, 1H > CH–); 6.97 (s, 1H H ind); 7.06–7.87 (m, 12H, ar); 8.64 (d, 1H, ar); 9.21 (d, 1H, –N<u>H</u>–CH <); 11.93 (s, 1H, –NH– ind); 12.28 (s, 1H, –NH–); ^{13}C NMR (DMSO-d_6) δ 35.94, 53.99, 102.42, 112.32, 118.98, 119.65, 120.01, 121.67, 122.33, 123.88, 126.22, 126.77, 127.98, 128.26, 128.87, 131.35, 132.42, 136.94, 137.82, 139.10, 158.93, 168.55, 172.50. MS (ES) m/z 428 $[M+H]^+$; M_r: 427 (calcd for $C_{25}H_{21}N_3O_4$).

2(*R*,*S*)-{2-[(1*H*-Indole-5-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid (2). Column chromatography (silica gel, AcOEt) afforded the titled compound in 70% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.44; mp 250 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.22 (m, 2H, –C<u>H</u>$_2$–CH <); 4.73 (m, 1H, –C<u>H</u> <); 6.62–8.69 (m, 14H, ar); 9.12 (d, 1H, –N<u>H</u>–CH <); 11.48 (s, 1H, –NH– ind); 12.03 (s, 1H, –NH–); 12.95 (b, 1H, –COOH); ^{13}C NMR (DMSO-d_6) δ 36.91, 54.84, 103.17, 112.31, 120.37, 120.64, 120.75, 122.93, 126.00, 127.10, 127.96, 128.05, 128.86, 129.00, 129.73, 133.08, 138.52, 138.68, 140.40, 166.20, 169.50, 173.41. MS (ES) m/z 428 $[M+H]^+$; M_r: 427 (calcd for $C_{25}H_{21}N_3O_4$).

2(*R*,*S*)-{2-[(1*H*-Indole-3-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid (3). Crystallization from MeOH afforded the titled compound in 41% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.46; mp 223–224 °C; ^{1}H NMR (DMSO-d_6) δ 3.15 (m, 2H, –C<u>H</u>$_2$–CH <); 4.70 (m, 1H, > CH–); 7.06–8.14 (m, 13H, ar); 8.60 (d, 1H, ar); 9.06 (d, 1H, –N<u>H</u>–CH <); 11.58 (s, 1H, –NH– ind); 11.81 (s, 1H, –NH–); 12.90 (b, 1H, –COOH); ^{13}C NMR (DMSO-d_6) δ 36.88, 54.82, 111,71, 113.00, 120.08, 120,72, 121.18, 121,74, 122.42, 123.09, 126.04, 127.10, 128.88, 129.10, 129.71, 132.97, 137.16, 138.66, 140.52, 163.29, 169.54, 173.41. MS (ES) m/z 428 $[M+H]^+$; M_r: 427 (calcd for $C_{25}H_{21}N_3O_4$).

2(*R*,*S*)-[2-(2-1*H*-Indol-3-yl-acetylamino)-benzoylamino]-3-phenyl-propionic acid (4). Trituration with Et_2O afforded the titled compound in 55% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.48; mp 152–153 °C; ^{1}H NMR (DMSO-d_6) δ 3.39 (m, 2H, –C<u>H</u>$_2$—CH <); 3.72 (s, 2H, –CH_2—CO–); 4.61 (m, 1H, > C<u>H</u>-); 6.93–7.62 (m, 13H, ar); 8.37 (d, 1H, ar); 8.92 (d, 1H, –N<u>H</u>–CH <); 10.88 (s, 1H, –NH– ind); 11.00 (s, 1H, –N<u>H</u>–); ^{13}C NMR (DMSO-d_6) δ34.68, 35.96, 53.65, 107.26, 111.22, 118.12, 118.38, 119.93, 120.64, 120.90, 122.20, 124.20, 126.20, 127.85, 127.98, 128.83, 131.63, 135.10, 137.70, 138.45, 167.84, 169.62, 172.59. MS (ES) m/z 442 $[M+H]^+$; M_r: 441 (calcd for $C_{26}H_{23}N_3O_4$).

2(*R*,*S*)-[2-(3-1*H*-Indol-3-yl-propionylamino)-benzoylamino]-3-phenyl-propionic acid (5). Crystallization from EtOH 95% afforded the titled compound in 33% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.46; mp 191–193 °C; ^{1}H NMR (DMSO-d_6) δ 2.80 (m, 2H, –C<u>H</u>$_2$–CH_2–CO–); 3.08 (m, 2H, –C<u>H</u>$_2$–CH <); 3.40 (m, 2H, –CH_2–CO–); 4.67 (m, 1H, > C<u>H</u>–); 6.99–7.66 (m, 13H, ar); 8.38 (d, 1H, ar); 9.01 (d, 1H, –NH–); 10.78 (s, 1H, –NH– ind); 10.87(s, 1H, –NH–); ^{13}C NMR (DMSO-d_6) δ 20.41, 35.96, 37.93, 53.83, 111.17, 113.03, 118.03, 118.09, 120.18, 120.64, 120.78, 122.06, 122.35, 126.22, 126.71, 127.91, 128.00, 128.82, 131.75, 136.00, 137.66, 138.35, 168.10, 170.56, 172.66. MS (ES) m/z 456 $[M+H]^+$; M_r: 455 (calcd for $C_{27}H_{25}N_3O_4$).

2(*R*,*S*)-{2-[(Naphthalene-1-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid (6). Crystallization from

EtOH 50% afforded the titled compound in 65% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.67; mp 199–200 °C; ^{1}H NMR (DMSO-d_6) δ 3.15 (m, 2H, –CH$_2$–CH <); 4.65 (m, 1H, >CH–); 7.10–8.73 (m, 16H, ar); 9.15 (d, 1H, –NH–CH <); 11.77 (s, 1H, –NH–); 12.95 (b, 1H, –COOH); ^{13}C NMR (DMSO-d_6) δ 35.97, 53.80, 120.31, 120.48, 122.92, 124.95, 125.06, 126.16, 126.36, 127.04, 127.92, 128.14, 128.25, 128.83, 129.41, 130.86, 132.14, 133.19, 133.85, 137.71, 138.74, 166.40, 168.19, 172.44. MS (ES) m/z 439 $[M+H]^+$; M_r: 438 (calcd for $C_{27}H_{22}N_2O_4$).

2(*R*,*S*)-[2-(Naphthalene-1-sulfonylamino)-benzoylamino]-3-phenyl-propionic acid (7). Crystallization from EtOH 95% afforded the titled compound in 35% yield. TLC (AcOEt/MeOH: 3/1) R_f: 0.30; mp 196–197 °C; ^{1}H NMR (DMSO-d_6) δ 3.18 (m, 2H, –CH$_2$–CH <); 4.63 (m, 1H, >CH–); 6.97–8.49 (m, 16H, ar); 9.05 (d, 1H, –NH–CH <); 12.06 (s, 1H, –NH–); 12.99 (b, 1H, –COOH); ^{13}C NMR (DMSO-d_6) δ 35.78, 54.07, 116.98, 117.96, 122.24, 123.19, 124.24, 126.26, 126.60, 126.94, 128.05, 128.39, 128.47, 128.77, 129.04, 130.23, 132.67, 133.00, 133.54, 134.77, 137.56, 138.26, 168.07, 172.30. MS (ES) m/z 475 $[M+H]^+$; M_r: 474 (calcd for $C_{26}H_{22}N_2O_5S$).

2(*R*,*S*)-[2-(Naphthalene-2-sulfonylamino)-benzoylamino]-3-phenyl-propionic acid (8). Crystallization from EtOH 95% afforded the titled compound in 45% yield. TLC (AcOEt/MeOH: 3/1) R_f: 0.74; mp 153 °C; ^{1}H NMR (DMSO-d_6) δ 3.10 (m, 2H, –CH$_2$–CH <); 4.62 (m, 1H, >CH–); 6.98–8.51 (m, 16H, ar); 9.34 (d, 1H, –NH–CH <); 11.85 (s, 1H, –NH–).; ^{13}C NMR (DMSO-d_6) δ 35.85, 54.23, 118.55, 119.60, 121.65, 122.42, 126.25, 127.49, 127.58, 128.04, 128.12, 128.58, 128.82, 129.09, 129.46, 131.30, 132.47, 134.06, 135.87, 137.68, 138.83, 168.02, 172.47. MS (ES) m/z 475 $[M+H]^+$; M_r: 474 (calcd for $C_{26}H_{22}N_2O_5S$).

3-Phenyl-2(*R*,*S*)-(2-phenylacetylamino-benzoylamino)-propionic acid (9). Crystallization from EtOH 95% afforded the titled compound in 71% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.50; mp 183–185 °C; ^{1}H NMR (DMSO-d_6) δ 3.27 (m, 2H, –CH$_2$–CH <); 3.64 (s, 2H, –CH$_2$–CO–); 4.66 (m, 1H, >CH–); 7.10–7.65 (m, 13H, ar); 8.34 (d, 1H, ar); 9.00 (d, 1H, –NH–CH <); 10.92 (s, 1H, –NH–); ^{13}C NMR (DMSO-d_6) δ 35.96, 44.20, 53.83, 120.17, 120.89, 122.47, 126.22, 126.57, 127.91, 128.01, 128.26, 128.84, 129.07, 131.68, 134.90, 137.76, 138.29, 167.95, 168.87, 172.63. MS (ES) m/z 403 $[M+H]^+$; M_r: 402 (calcd for $C_{24}H_{22}N_2O_4$).

3-Phenyl-2(*R*,*S*)-[2-(3-phenyl-propionylamino)-benzoylamino]-propionic acid (10). Crystallization from MeOH afforded the titled compound in 67% yield. TLC (AcOEt/MeOH: 3/2) R_f: 0.61; mp 169–171 °C; ^{1}H NMR (DMSO-d_6) δ 2.62 (t, 2H, –CH$_2$–CH$_2$–CO–); 2.90 (t, 2H, –CH$_2$–CO–); 3.21 (m, 2H, –CH$_2$—CH <); 4.66 (m, 1H, >CH–); 7.09–7.67 (m, 13H, ar); 8.37 (d, 1H, ar); 9.00 (d, 1H, –NH–CH <); 10.86 (s, 1H, –NH–); ^{13}C–NMR (DMSO-d_6) δ 30.40, 35.99, 38.55, 53.86, 120.16, 120.59, 122.31, 125.79, 126.21, 127.98, 128.05, 128.11, 128.85, 131.72, 137.75, 138.36, 140.52, 168.03, 169.92, 172.66. MS (ES) m/z 417 $[M+H]^+$; M_r: 416 (calcd for $C_{25}H_{24}N_2O_4$).

2(*R*,*S*)-[2-(2,2-Dimethyl-propionylamino)-benzoylamino]-3-phenyl-propionic acid (11). Crystallization from AcOEt afforded the titled compound in 57% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.49; mp 195–196 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (s, 9H, –C(CH$_3$)$_3$); 3.15 (m, 2H, –CH$_2$–CH <); 4.65 (m, 1H, >CH–); 7.09–8.47 (m, 9H, ar); 9.06 (d, 1H, –NH–CH <); 11.20 (s, 1H, –NH–). 12.95 (b, 1H, –COOH); ^{13}C NMR (DMSO-d_6) δ 26.97, 35.82, 53.83, 119.73, 119.94, 122.04, 126.22, 127.92, 127.99, 128.80, 131.91, 137.82, 139.00, 168.46, 172.47, 176.09. MS (ES) m/z 369 $[M+H]^+$; M_r: 368 (calcd for $C_{21}H_{24}N_2O_4$).

2(*R*,*S*)-[2-(3,3-Dimethyl-butyrylamino)-benzoylamino]-3-phenyl-propionic acid (12). Trituration with petroleum ether 40–70° afforded the titled compound in 62% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.55; mp 149–150 °C; ^{1}H NMR ($CDCl_3$) δ 1.10 (s, 9H, –C(CH$_3$)$_3$); 2.28 (s, 2H, –CH$_2$–CO–); 3.33 (m, 2H, –CH$_2$–CH <); 5.05 (m, 1H, >CH–); 6.70 (d, 1H, –NH—CH <); 7.04–8.58 (m, 9H, ar); 10.83 (s, 1H, –NH–); ^{13}C NMR ($CDCl_3$) δ 29.87, 31.37, 37.38, 52.32, 53.38, 120.10, 121.80, 123.08, 126.68, 127.46, 128.80, 129.36, 132.98, 135.43, 139.24, 168.58, 171.49, 174.77. MS (ES) m/z 383 $[M+H]^+$; M_r: 382 (calcd for $C_{22}H_{26}N_2O_4$).

2(*R*,*S*)-{2-[(Adamantane-1-carbonyl)-amino]-benzoyl-amino}-3-phenyl-propionic acid (13). Trituration with hot MeOH afforded the titled compound in 75% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.53; mp 140–141 °C; ^{1}H NMR (DMSO-d_6) δ 1.68–2.01 (m, 15H, ada); 3.15 (m, 2H, –CH$_2$–CH <); 4.71(m, 1H, >CH–); 7.09–8.49 (m, 9H, ar); 9.04 (d, 1H, –NH–CH <); 11.08 (s, 1H, –NH–); ^{13}C NMR (DMSO-d_6) δ 27.38, 35.79, 38.37, 41.12, 53.73, 119.88, 120.00, 122.00, 126.19, 127.93, 128.82, 131.84, 137.82, 138.97, 168.33, 172.49, 175.55. MS (ES) m/z 447 $[M+H]^+$; M_r: 446 (calcd for $C_{27}H_{30}N_2O_4$).

2(*R*,*S*)-[2-(2-Adamantan-1-yl-acetylamino)-benzoylamino]-3-phenyl-propionic acid (14). Crystallization from MeOH afforded the titled compound in 40% yield. TLC (AcOEt/MeOH: 2/1) R_f: 0.45; mp 195 °C; ^{1}H NMR (DMSO-d_6) δ 1.57–1.91 (m, 15H, ada); 1.99 (s, 1H, –CH$_2$–CO–); 3.17 (m, 2H –CH$_2$–CH >); 4.67 (m, 1H, >CH–); 7.08–8.42 (m, 9H, ar); 8.97 (d, 1H, –NH–CH <); 10.75 (s, 1H, –NH–); ^{13}C NMR (DMSO-d_6) δ 28.75, 33.42, 36.89, 37.08, 42.76, 53.33, 54.82, 120.81, 121.20, 123.07, 127.10, 128.88, 129.71, 132.66, 138.68, 139.27, 168.97, 169.44, 173.39. MS (ES) m/z 461 $[M+H]^+$; M_r: 460 (calcd for $C_{28}H_{32}N_2O_4$).

Biological evaluations

Male Hartley guinea pigs (300–350 g) and male Sprague Dawley rats (250–300 g) were used. For binding assays to isolated rat pancreatic acini, animals were fasted, but allowed free access to water, for 18–24 h prior to the experiment.

[^{125}I]-BH-CCK-8 (CCK_8(sulphated), [^{125}I]Bolton and Hunter labelled-specific activity 2000 Ci/mol) was purchased from Amersham Pharmacia Biothech (Buckinghamshire, UK). All other drugs and reagents were obtained from commercial sources.

The binding parameters for the substances under investigation (IC_{50} values and $p = 0.05$ fiducial limits) were determined by regression analysis of competition curves.

[^{125}I]BH-CCK-8 receptor binding assay in isolated rat pancreatic acinar cells. Isolated pancreatic acini were prepared by enzymatic digestion of pancreas as previously described by Makovec et al.[27] Drug displacing experiments were carried out by incubating acinar cells, [^{125}I]BH-CCK-8 (25 pM final concentration) and competitors in 0.5 mL total volume at 37 °C for 30 min, in shaking bath. At the end of incubation 1 mL of ice-cold Hepes–Ringer buffer (10 mM Hepes, 118 mM NaCl, 1.13 mM $MgCl_2$, 1.28 $CaCl_2$, 1% BSA, 0.2 mg/mL Soybean trypsin inhibitor, pH 7.4) was added and the tubes were centrifuged 5 min at 12,500*g*. The supernatant was aspirated and the radioactivity associated to the pellet measured. The non-specific binding was estimated in the presence of 1 μM CCK-8, accounting 15% of total binding.

[^{125}I]BH-CCK-8 receptor binding assay in guinea pig cerebral cortices. Membranes from guinea pig cerebral cortices, were prepared as previously described.[27] Protein concentration was determined according to Bradford,[28] using bovine serum albumin (BSA) as standard. The binding experiments were performed in assay buffer containing 10 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 5.0 mM $MgCl_2$, 1.0 mM EGTA, pH 6.5 and supplemented with 0.2 mg/mL bacitracin. The incubation of membranes suspension with labelled ligand and inhibitors was carried out in a microtiter 96-well filter plate (Multiscreen, Millipore Inc, Bedford, MA, USA) with integral Whatman GF/B membrane filters. Aliquot of membranes (0.5 mg of protein/mL) were added to each well, containing [^{125}I]BH-CCK8 (25 pM), in a final volume of 250 μL. The non-specific binding of iodinated peptide was defined in the presence of 1 μM CCK-8, accounting of 20% of total binding. Nonspecific binding of [^{125}I]BH-CCK-8 to membrane filters (blank), measured in wells containing an equal amount of labelled ligand, but no membranes, was 0.2% of total radioligand added. After 120 min at 25 °C, the 96-well plate was placed on the vacuum filtration apparatus (Millipore Inc.). The integral membrane filters were rinsed with 0.25 mL of ice cold assay buffer, dried, punched into polycarbonate tubes and counted in a COBRA-5002 γ-counter (Packard Biosciences).

Molecular modelling

A first set of optimized conformations for all the compounds was obtained by a simple Monte Carlo search. All the rotatable bonds, including the amide bonds, were allowed to rotate in order to generate the starting set of geometries. Each bond was twisted by 10° torsional increments randomly and the the initial set was thus obtained. The geometries were optimized first using molecular mechanics calculations with the Amber forcefield;[29] the optimizations were carried out with the Polak–Ribiere conjugate gradient algorithm at a gradient of 0.001 kcal/Å mol. The first ten conformations obtained at this first step were then submitted to a further refinement, and their geometries were reoptimized with a semiempirical calculation using the AM1[30] hamiltonian as implemented in SYBYL 6.8.[31] The SCF convergence limit for the UHF calculation was set to full accuracy. All the calculations were carried out on a Silicon Graphics O2 workstation.

Acknowledgements

We are grateful to prof. Vinicio Galasso for the ab initio optimizations of the anthranoyl amide model.

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