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New Anthranilic Acid Based Antagonists with High Affinity and Selectivity for the Human Cholecystokinin Receptor 1 (hCCK$_1$-R)

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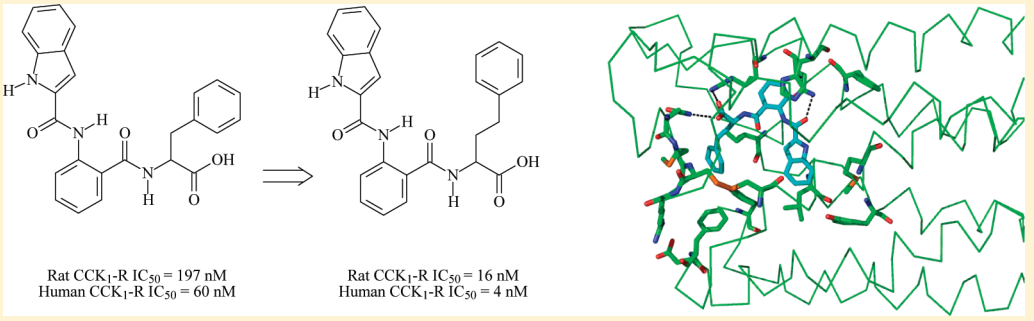
Supporting Information

ABSTRACT:



The anthranilic acid diamides represent the most recent class of nonpeptide CCK$_1$ receptor (CCK$_1$-R) antagonists. Herein we describe the second phase of the anthranilic acid C-terminal optimization using nonproteinogenic amino acids containing a phenyl ring in their side chain. The *Homo*-Phe derivative **2** (VL-0797) enhanced 12-fold the affinity for the rat CCK$_1$-R affinity and 15-fold for the human CCK$_1$-R relative to the reference compound **12** (VL-0395). The eutomer of **2** (**6**) exhibited a nanomolar range affinity toward the human CCK$_1$-R and was at least 400-fold selective for the CCK$_1$-R over the CCK$_2$-R. Molecular docking in the modeled CCK$_1$-R and its validation by site-directed mutagenesis experiments showed that the **6** binding site overlaps that occupied by the C-terminal bioactive region of the natural agonist CCK. Owing to their interesting properties, new compounds provided by this study represent a solid basis for further advances aimed at synthesis of clinically valuable CCK$_1$-R antagonists.

INTRODUCTION

Cholecystokinin (CCK) is a gut−brain peptide engaged as endocrine messenger in the gastrointestinal (GI) tract and as neurotransmitter/neuromodulator at the central nervous system (CNS).[1−4] The biological actions of CCK are mediated by two distinct G-protein coupled receptors: CCK$_1$ and CCK$_2$.[5−8] CCK circulates in the blood in different molecular forms, and only the C-terminal octapeptide (Asp^{26}-Tyr(SO$_3$H)^{27}-Met^{28}-Gly^{29}-Trp^{30}-Met^{31}-Asp^{32}-Phe^{33}-NH$_2$ or CCK-8S) binds both receptors with subnanomolar affinity. The smallest bioactive fragment of CCK (Trp^{30}-Met^{31}-Asp^{32}-Phe^{33}-NH$_2$ or CCK-4) binds with nanomolar affinity only the CCK$_2$ receptor (CCK$_2$-R), while its affinity toward the CCK$_1$ receptor (CCK$_1$-R) decreases to the micromolar range.[9]

In view of the importance of CCK in different physiopathological processes as irritable bowel syndrome (IBS), chronic and acute pancreatitis, gastric ulcer, anxiety, panic disorder, eating disorders etc., over the past 15 years, great efforts have been made to develop clinically useful small nonpeptide molecules known as CCK receptor antagonists.[10−15]

In an earlier report, we described an innovative class of nonpeptide CCK$_1$-R antagonists keeping appropriate pharmacophoric groups on the anthranilic acid unit employed as a molecular scaffold.[16] The lead compound obtained 2(*R*,*S*)-{2-[(1*H*-indole-2-carbonyl)-amino]-benzoylamino}-3-phenyl-propionic acid (VL-0395) (Figure 1) is characterized by the presence of Phe and 2-indole moiety at the C- and N-termini of anthranilic acid, respectively, and is endowed with submicromolar affinity (IC$_{50}$ = 197.5 nM) toward CCK$_1$-R, demonstrating also an antagonist nature similar to that exhibited by the reference CCK$_1$ selective

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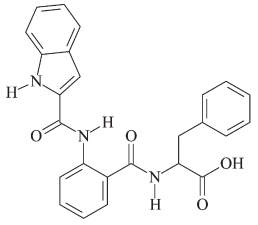
antagonist Loxiglumide.[16] A possible mode of interaction of compound **12** (VL-0395) with the CCK_1-R has already been proposed and involves at least two hydrophobic pockets in order to accommodate the main pharmacophoric groups (2-indole ring and Phe side chain).

Previously, a structure−affinity relationship study (SAfR) enabled us to demonstrate that the N-terminal substituent highly modulates the affinity and that the 2-indole moiety behaves as a "needle" because any substitution of the indole group with at least 50 different other residues produces a loss in affinity.[17−19]

The only modification allowed in this first optimization step is the substitution on the indole ring with groups characterized by minimal steric hindrance, a parameter that deeply influences the affinity. In addition, the replacement of the phenyl ring of the indole moiety with a saturated one (cyclohexyl or cyclopentyl) is detrimental for the binding affinity, indicating the essential role of the planar aromatic ring.[18]



Comp. 12 (VL-0395)

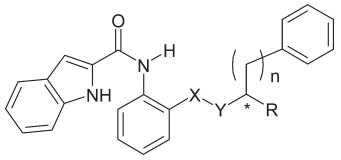
Figure 1. Structure of the anthranilic acid based lead antagonist **12**.

On the contrary, the results obtained from a preliminary SAR study regarding the C-terminal optimization of the anthranilic acid of compound **12** suggested that the hypothesized receptor pocket, which accommodates the aminoacidic residue, allows many more degrees of freedom than those imposed to the N-terminal group.[20] In fact, the substitution on the phenyl ring of Phe or its saturation was tolerated by the receptor pocket, as well as replacement of the Phe residue of **12** by other proteinogenic amino acids of the C-terminal sequences of CCK led to compounds endowed with comparable receptor affinity and selectivity as the lead one.[21]

So, to optimize compound **12**, we synthesized compounds reported in Table 1 and tested their affinity and activity toward CCK receptors.

First, to investigate the tolerance of the hydrophobic pocket engaged by the Phe side chain of **12**, we introduced, on the C-terminus of anthranilic acid, nonproteinogenic amino acids with different degrees of homology to Phe (compounds **1**−**4**). Then, the amino acidic side chain which confers the higher affinity has been utilized in order to explore the topographic tolerance of the receptor binding subsite associated to the amino acidic C-terminal free carboxy group (compounds **7**−**10**). In addition to the above aims, compound **11** was designed in order to highlight the importance of the anthranilic acid scaffold for the correct spatial orientation of the two main pharmacophoric groups (indole

Table 1. CCK Receptors Binding Data



						IC_{50} (μM)[a]			
compd	X	Y	*n*	R	stereo *	rat pancreatic acini (CCK_1)	guinea pig brain cortex (CCK_2[e])	SI	1/ER
12	CO	NH	1	−COOH	RS	0.197 ± 0.107	16.40	83[f]	
1	CO	NH	0	−COOH	RS	0.317 ± 0.066	NT		
2	CO	NH	2	−COOH	RS	0.0157 ± 0.0008	5.1 ± 0.3	324[f]	
3	CO	NH	3	−COOH	RS	0.107 ± 0.048	0.6 ± 0.1	6[f]	
4	CO	NH	4	−COOH	RS	39% ISB [c]	NT		
5	CO	NH	2	−COOH	S	0.313 ± 0.100	7.9 ± 1.5	25	33
6	CO	NH	2	−COOH	R	0.0095 ± 0.001	3.8 ± 0.6	400	
7	CO	NH	2	$-COOC_2H_5$	RS	24% ISB [b]	NT		
8	CO	NH	2	$-CONH_2$	RS	IN [b]	IN [c]		
9	CO	NH	2	$-CH_3$	RS	36% ISB [c]	NT		
10	CO	NH	2	−CONHOH	RS	0.419 ± 0.026	37% ISB [d]		
11	NH	CO	2	−COOH	RS	0.717 ± 0.110	6.2 ± 0.4	8[f]	

[a] IC_{50} ± standard error (ALLFIT analysis); % ISB, percentage inhibition of specific binding of 25 pM [^{125}I]-(BH)-CCK-8 at the maximal concentration tested. [b] 3 μM. [c] 10 μM. [d] 30 μM. [e] Values without standard errors were obtained from not more than two experiments. IN: inactive (ISB less than 20%). NT: not tested. SI: selectivity index. [f] Apparent selectivity index. ER: eudismic ratio.

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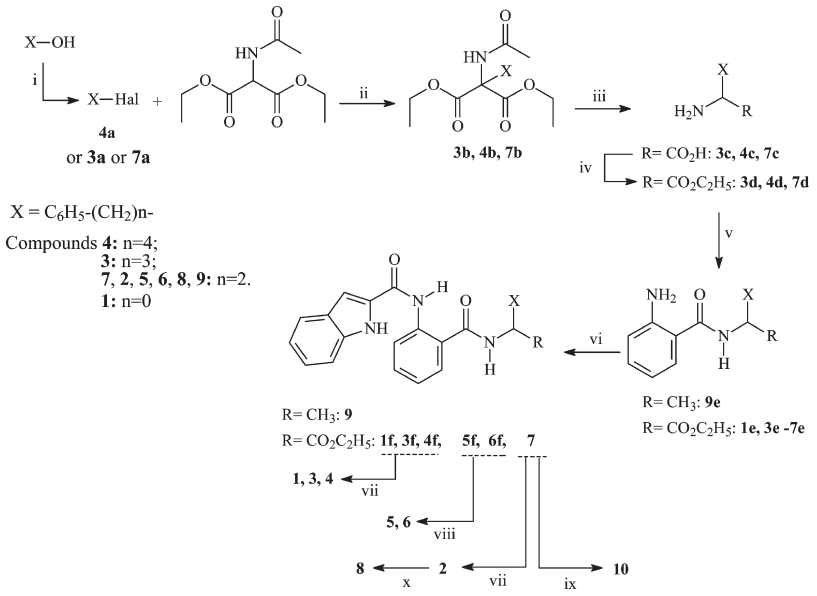
Scheme 1. Synthesis of Compounds 1−10[a]



[a] Reagents and conditions: (i) ((a) MsCl, TEA, CH_2Cl_2, b) NaI, acetone; (ii) EtONa, abs EtOH, reflux; (iii) 6N HCl, 1,4-dioxane, reflux; (iv) HCl (g), abs EtOH; (v) isatoic anhydride, TEA, AcOEt, reflux; (vi) indole-2-carboxylic acid, PCl_5, dry CH_2Cl_2, pyridine; (vii) KOH, THF/H_2O 1:1; (viii) LiOH·H_2O, THF/H_2O 1:1; (ix) hydroxylamine hydrochloride, MeONa, MeOH; (x) TEA, PyBOP, NH_4Cl, DMF.

and amino acidic side chain) of this class of CCK_1 antagonists. In the final step, we extended the study on the binding effect in relation to the receptor subsite stereospecificity, determining the eutomer and the eudismic ratio (ER) of the two pure enantiomers of the most active compound in this new series (compounds **5**, **6**).

CHEMISTRY

Derivatives **1**−**10** were synthesized according to the synthetic pathway reported in Scheme 1. The ethyl esters of the racemic phenyl-glycine (**1d**) and those of the pure enantiomers of Homo-Phe (**5d**, **6d**) were commercially available, while the ethyl esters of Homo-Phe (**7d**) and those of its higher homologues, introduced in compounds **3** and **4** (**3d**, **4d**), were obtained in three subsequent synthetic steps as follows. Alkylation of diethyl acetamidomalonate with the corresponding alkyl halide afforded the intermediates **3b**, **4b**, and **7b**. In the case of intermediate **4b**, the alkyl halide used (**4a**) was obtained starting from the corresponding alcohol, whereas the other two alkyl halides (**3a** and **7a**) were commercially available. Acidic hydrolysis in HCl 6N of **3b**, **4b**, and **7b** afforded the unnatural amino acids **3c**, **4c**, and **7c** as racemates, which were converted successively, by treatment with gaseous HCl in absolute EtOH, in the corresponding ethyl esters hydrochloride **3d**, **4d**, and **7d**.

o-Aminobenzoylation of 2-methyl-3-phenyl propylamine and of the C-protected aminoacids including the commercially available ones (**1d**, **5d**, **6d**) with isatoic anhydride in ethyl acetate afforded the anthranilic intermediates **9e**, **1e**, and **3e**−**7e**, respectively.

Further coupling with indole-2-carboxylic acid, via acyl chloride formation using PCl_5 in dry CH_2Cl_2, provided the final compounds **7** and **9** as well as the indole intermediates **1f**, and **3f**−**6f**.

The optically active final compounds **5** and **6** were obtained by base catalyzed hydrolysis with LiOH of the corresponding esters **5f** and **6f**. Base catalyzed hydrolysis of the ethyl ester **7** and of the intermediates **1f**, **3f**, and **4f**, using a KOH aqueous solution, afforded the final compounds **2**, **1**, **3**, and **4** respectively as racemates.

The optical purity of the enantiomers **5** and **6** was determined by analytical reverse phase enantioselective HPLC (RP-eHPLC) using a chiral stationary phase (CSP) containing native teicoplanin chemically bonded on silica matrix according to a recently published procedure.[22,23]

All analytical data were obtained by using UV, chiro-optical (circular dichroism, CD), and evaporative light scattering (ELSD) detectors.

Ethyl ester **7** was treated with MeONa in MeOH followed by hydroxylamine hydrochloride, yielding hydroxamic acid **10** as mixture of *E*/*Z* isomers while treatment of the final compound **2** with PyBop and ammonium chloride in DMF provided primary amide **8**.

Compound **11** was synthesized according to the synthetic route reported in Scheme 2.

Alkylation of diethyl malonate with 2-bromo-ethyl benzene afforded the intermediate **11b**, which was converted successively to **11c** by base catalyzed monohydrolysis. The *o*-phenylendiamine derivative **11d** was obtained by TBTU mediated condensation of **11c** with diamine. Indolation of **11d** and successive base catalyzed hydrolysis of **11e** using the same protocols of the anthranilic acid derivatives of Scheme 1 afforded the final *o*-phenylendiamine based compound **11**.

RESULTS AND DISCUSSION

The binding affinities of the synthesized compounds **1**−**11** for CCK_1 and CCK_2-Rs were evaluated according to established protocols and are expressed as IC_{50} or as percentage inhibition of specific binding of 25 pM [^{125}I]-(BH)-CCK-8 (ISB%) determined at the highest used dose (3, 10, and 30 μM as indicated).[24] When the ISB% values were lower than 20%, the compounds

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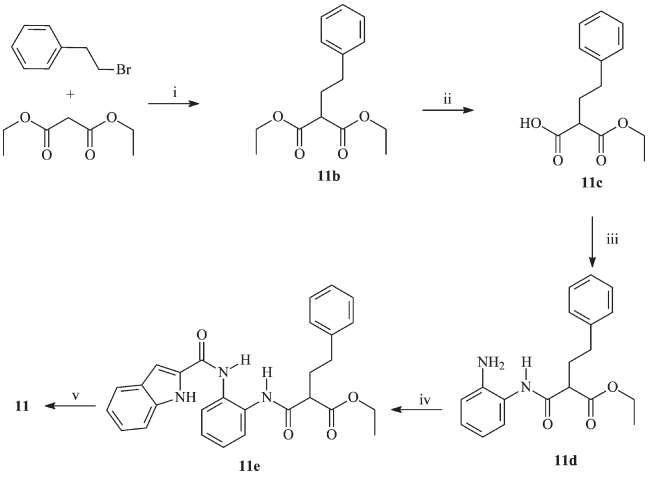
Scheme 2. Synthesis of Compound 11[a]



[a] Reagents and conditions: (i) EtONa, abs EtOH, reflux; (ii) KOH, abs EtOH; (iii) TEA, TBTU, *o*-phenylenediamine, CH_2Cl_2; (iv) indole-2-carboxylic acid, PCl_5, dry CH_2Cl_2, pyridine; (v) KOH, THF/H_2O 1:1.

were considered inactive. Values without standard errors are those obtained from no more than two experiments.

Binding data, selectivity indices (SI, expressed as CCK_2/CCK_1 ratio), and the eudismic ratio (ER) for the optically active compounds, along with those of the lead **12**, all useful for the following discussion of SAR, are reported in Table 1.

The synthesized compounds were divided into four groups (Table 1) and will be discussed separately according to the prefixed targets (importance of the amino acid side chain, of the chiral center configuration, of C-terminal free carboxy group and that of anthranilic acid scaffold).

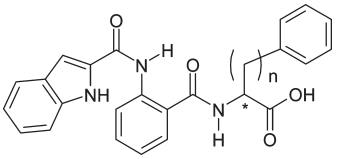
1. SAR Study. As expected, all the tested compounds showed low affinity toward the CCK_2-R, confirming their preference for the CCK_1-Rs.

The analysis of the obtained data shows that the binding affinity of compounds **1**−**4** appears clearly influenced by the number of the methylene units of the amino acidic side chain. In fact, the receptor affinity displays a biphasic profile in relation to the number of the methylene units which are interposed between the amino acidic backbone and the phenyl ring. In the absence of methylene units, as in the case of the phenylglycine derivative (compound **1**), the receptor affinity resulted approximately 2-fold lower than that of the lead compound and comparable to that of compound **3** characterized by the presence of 3 methylene units. The smallest binding affinity for the CCK_1-R was achieved with 4 methylene units, while the optimum was presented by the Homo-Phe side chain (compound **2**) bearing 2 methylene units.

The Homo-Phe derivative (compound **2**), coded VL-0797, presented a 12-fold higher affinity for the CCK_1-R and 4-fold higher apparent selectivity than the reference compound **12**. Thus, a simple elongation, by one methylene unit, of the Phe side chain in compound **12** yielded the most potent anthranilic acid based CCK_1-R ligand **2**, which displays a comparable affinity to that of the best benzodiazepine derivative Devazepide ((±)-L 364,718) in its racemic form (IC_{50}, 10.4 nM ± 1.5 and 169 nM ± 41.7 for the CCK_1 and CCK_2-R, respectively).

Compounds **5** and **6**, coded VL-0797L and VL-0797D, respectively, were synthesized in order to check the influence of the

Table 2. CCK Receptors Binding Data from Human CCK_1 and CCK_2 Receptors Expressed on Cos-7 Cells



			human CCK_1-R	human CCK_2-R		
compd	*n*	stereo *	K_i (nM, SEM)	K_i (μM, SEM)	SI[a]	1/ER
12	1	RS	59.8 ± 4.7	22.0 ± 3.6	368 [a]	
2	2	RS	3.9 ± 0.2	15.3 ± 1.1	3900 [a]	
5	2	S	973 ± 196	1−10	1−10	423
6	2	R	2.3 ± 0.2	1−10	435−4348	

[a] Apparent selectivity index; ER: eudismic ratio.

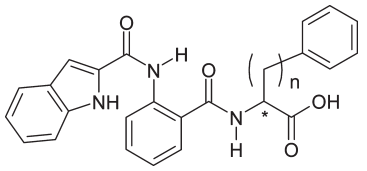
stereochemistry of the amino acidic chiral center of the more active racemate of this series (compound **2**) on the receptor affinity. Accordingly, we determined the eutomer and the distomer of **2** and their selectivity indices (SI) as well as the eudismic ratio (ER) of these two pure enantiomers. The binding data reported in Table 1 establish that the pharmacologically active enantiomer is that in which the absolute configuration of the single chiral center is *R* (rectus). In fact, compound **6** exhibited a nanomolar range affinity (IC_{50} = 9.5 ± 1.0 nM) and good selectivity (SI = 400), resulting in about 33-fold (1/ER ≈ 33) increase of activity relative to the distomer (compound **5**).

In view to interspecies difference polymorphisms in G-protein-coupled receptors that can alter drug affinity and/or activity, a binding and activity studies were also carried out on cells expressing human CCK receptors. For this purpose, Cos-7 cells were transiently transfected with plasmids containing cDNA encoding either human CCK_2 or CCK_1-R cloned from human pancreas and gallbladder, respectively.[25,26]

As illustrated in Table 2, all derivatives inhibited the binding of [^{125}I]BH-(Thr,-Nle)-CCK-9 to the CCK_1-R with higher affinity than they did to the CCK_2-R. The lead compound, **12**, and the new one, **2**, both in their racemic form, showed near 3- or 4-fold increased affinity for the human CCK_1-R in comparison to that for the rat receptors and 4- or 12-fold enhancement, respectively, of their apparent indices of selectivity, thus denoting a deteriorate human CCK_2-R recognition. Accordingly, the eutomer (compound **6**) showed a nanomolar range affinity (IC_{50} of 2.3 nM) with 400-fold enhancement of potency relative to the distomer (compound **5**) and at least 400-fold improvement of selectivity toward the human CCK_1 over the CCK_2-R.

In addition to the above derivatives, compounds **7**−**10** were synthesized in order to elucidate the role of the amino acidic free carboxy group in receptor recognition. The binding affinity data for compounds **7**−**10** demonstrate clearly that the free carboxy group of the amino acid is essential for the receptor binding. In fact, the remarkable drop in receptor affinity of the ethyl ester derivative (compound **7**) in addition to the inactivity of the primary amide (compound **8**) supports the view that the free carboxy group is engaged in the receptor binding through ionic or reinforced ionic bond formation rather than in a double hydrogen bond interaction. Nevertheless, because the double hydrogen bond formation can be established even by the primary amide,

Table 3. Functional Test: Inhibition of Guinea Pig Gallbladder CCK-8-Induced Contractions in Vitro and in Vivo



compd	*n*	stereo *	in vitro IC_{50} (nM, SEM)	in vivo ID_{50} (μmol/kg, SEM)
2	2	RS	48.9 ± 10.4	NT[a]
5	2	S	420.3 ± 2.6	NT
6	2	R	43.9 ± 1.7	0.35 ± 0.13

[a] NT: not tested.

the dramatic decrease of the binding affinity of substituted primary amide compound relative to compound **2** excludes this type of interaction. Further investigations in order to replace the hypothesized ionic bond between compound **2** and the receptor binding pocket with a double hydrogen bond interaction was accomplished by designing the hydroxamate derivative **10**, which is considered as alternative bioisoster of the carboxylic acid functionality. The compound having an hydroxamic acid moiety (compound **10**) showed, as expected, a 27-fold lower affinity relative to compound **2**, thus confirming the crucial importance of the ionic interaction.

The reduction of the free carboxylic group of **2** to a methyl group (compound **9**) confirms the losses of the affinity observed for the ester and amide derivatives.

Finally, compound **11** was designed and synthesized in order to highlight the importance of the anthranilic acid scaffold for the correct spatial orientation of the main pharmacophoric groups (indole and amino acidic side chain) during the ligand−receptor interactions. The 45-fold lower affinity of compound **11** toward CCK_1-Rs reinforces the role of the anthranilic acid template for the receptor subtype recognition. In fact, compound **11** exhibited a binding affinity to the CCK_2-R similar to that of **2**, confirming the critical function of the anthranilic acid for the CCK_1-R selectivity.

2. In Vitro and in Vivo Functional Studies. The antagonist nature of compounds reported in Table 3 was checked by in vitro and/or in vivo functional tests according to established protocols reported in Experimental Section. The compounds inhibited the (Thr,Nle)-CCK-9-induced production of inositol phosphate, with potencies in close agreement with their affinity values. Moreover, isolated guinea pig gallbladder was used to verify antagonism of these compounds toward CCK_1-R both on in vitro and in vivo models. None of these compounds showed any intrinsic contractile effect in the gallbladder preparations, while in both models compound **6** was a potent inhibitor of CCK-8 induced contraction, with an IC_{50} value around 40 nM in vitro and an ID_{50} of 0.35 μmol/kg in vivo. In comparison, compound **6** in vitro was about 10-fold more potent than distomer **5** and 30 times more active relative to Dexloxiglumide (IC_{50} = 1230 ± 20) used as the reference compound.

3. Molecular Modeling Study. *Ground States.* We have carried out first a conformational analysis on the ligands, and the main structural parameters for their ground state conformations are reported in Table 4.

The lead compound **12** was included as reference in the modeling study, and its minimum energy conformation resulting from the present analysis is closely similar to that derived from NOE NMR data recently described by ourselves (Table 4, Figure 2).

The molecule is U-shaped, and the two aromatic indole and phenylalanine rings are found on the same side at a centroid distance of 7.6 Å. The indole and the anthranilic acid rings lie almost on the same plane, while the plane angle between the aromatic ends of the molecules is 141°. The centroid distance between the anthranilic and the phenylalanine rings is 8.1 Å. This conformation is stabilized also by two intramolecular hydrogen bonds at the core anthranilic residue and between the carboxylate group and hydrogen 15 (Scheme 3a).

A similar conformation is shared by all the compounds containing the anthranilic core, with the exception of the hydroxamic derivative, as it can be seen from the overlay in Figure 3.

For this reason, the distances between the pharmacophoric groups and the surface and volume of the phenylalanine one are mainly dependent upon the length of the phenylalanine side chain and are optimal in compound **2** (Table 4).

The presence of the negatively charged carboxy group plays a key role for the activity. This is clearly seen within the series of C2 side chain compounds, which show very similar surfaces and volumes but very large difference in activities. This is most likely due to a field effect, but also the centroid distance between the ends is somewhat affected by the lack of the negative charge and is higher in the neutral compounds. This is due to the fact that in the negatively charged compounds, torsion angle $H_{15}N_{14}C_{16}C_{17}$ is constrained also by the hydrogen bond interaction between H_{15} and one of the oxygens of the carboxy group.

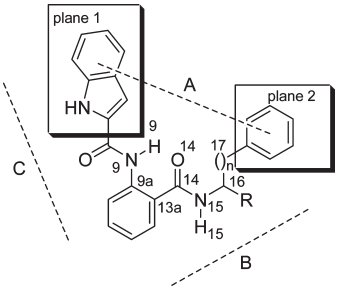
When the carboxylic group is replaced by the hydroxamic acid moiety, the ground state conformation is different from that of the other compounds. The hydroxamic group is stabilized by a strong intramolecular hydrogen bond between the carbonyl oxygen and the terminal hydrogen (Scheme 3b, Figure 4). The formation of this 5-membered pseudocycle is well-known and makes the terminal hydrogen less acidic than the N-hydrogen.[27,28] In our case, also the N-hydrogen is involved in a hydrogen bond with the central anthranilic core, and for this reason the molecule adopts a conformation that moves both the hydroxamic group itself and the phenyl ring away from the positions adopted by the other ligands (Figure 4). The distance between the aromatic ends of the molecule is thus the largest of the series.

Moreover, the phenylenediamine derivative **11** shows a different conformation (Scheme 3c, Figure 5), and the amino acidic side chain is turned in order to allow the carboxylate group to establish strong interactions with both the core N-hydrogens. The distance between the ends of the molecule is thus somewhat higher, and the carboxylate seems not available for intermolecular interactions, at least if the molecule is bound in its ground state conformation.

*Dynamic Behavior of Compounds **2** and **12**.* The longer chain connecting the phenyl ring with the core of the molecule in compound **2** allows a higher conformational freedom to this molecule in comparison with the phenylalanine derivative **12**. This effect could also give a contribution to the better activity of **2** because the ligand could reach also conformational states that are less populated in the shorter lead compound. To investigate this possibility, we have submitted the two molecules to several molecular dynamics runs.

The centroid distance between the indole and the phenyl ring was monitored during the simulations, and Figure 6 reports the typical behavior of the two compounds.

Table 4



compd	n	A[a] Å	B[b] Å	C[c] Å	Pl. angle[d] deg	$H_9N_9C_{9a}C_{13a}$ deg	$C_{9a}C_{13a}C_{14}O_{14}$ deg	$H_{15}N_{15}C_{16}C_{17}$ deg	S_{phe}[e] Å^2	V_{phe}[f] Å^3
1	0	7.4	6.9	7.5	92.0	347.4	44.3	95.6	89.0	84.0
12	1	7.6	8.1	7.5	141.4	346.9	43.5	114.9	98.4	120.4
2	2	8.8	9.5	7.5	84.8	346.2	43.6	99.2	123.4	125.5
10	2	12.1	9.6	7.5	85.0	340.5	44.7	24.2	125.3	128.2
7	2	10.0	9.5	7.5	87.9	340.1	44.6	62.7	126.3	129.0
8	2	11.5	9.6	7.5	88.6	340.6	44.8	28.6	121.4	125.8
9	2	9.4	9.2	7.4	86.9	340.3	45.6	62.3	125.3	127.1
11[g]	2	9.8	9.9	7.4	110.9	347.4	57.5[h]	21.0[i]	123.1	129.6
3	3	10.8	11.0	7.6	9.4	357.8	7.7	111.0	143.1	136.9
4	4	11.5	11.8	7.6	63.9	357.6	8.0	110.4	161.1	151.9

[a] Distance between centroids defined on the aromatic systems of indole and phenylalanine. [b] Distance between centroids defined on the aromatic systems of phenylalanine and anthranilic acid. [c] Distance between centroids defined on the aromatic systems of anthranilic acid and indole. [d] Plane angle between plane 1, containing the indole ring, and plane 2, containing the phenylalanine ring. [e] Connoly surface calculated with a spherical probe of 0.51 Å radius on all the atoms from C_{16} to the end of the phenylalanine side chain. [f] Volume under surface *S*. [g] Phenylelediamine derivative (compd **11**), the positions of O_{14}—H_{15} and C_{14}—N_{15} are inverted. [h] $C_{9a}C_{13a}N_{15}H_{15}$. [i] $O_{14}C_{14}C_{16}C_{17}$.

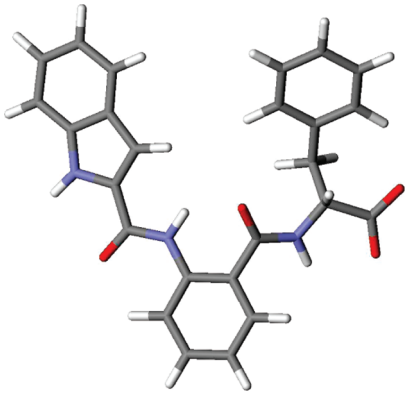
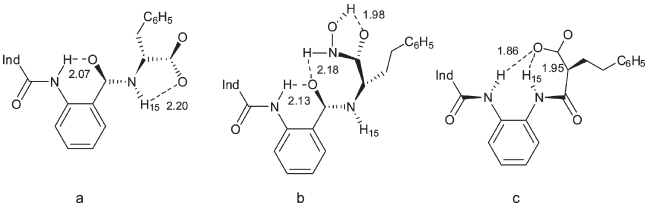


Figure 2

Scheme 3. Schematic Representation of the Ground State Minimum Energy Conformation of Compound 12 (a), Compound 10 (b), and Compound 11 (c)



It can be seen that compound **2** can reach during the simulation both larger and shorter distances than the equilibrium one much more frequently than **12**. A population analysis of such distances is reported in Figure 7.

The distances available span was in the range 4.5−12.5 Å for compound **12** and between 3.5 and 14.5 Å for compound **2**. During the runs, the central hydrogen bond at the anthranilic residue has never been lost by both the molecules. Compound **12** is found in its ground state conformation in about the 80% of the population, while only a 40% of the population of compound **2** conformers is represented by its ground state, and there is a second populated conformation at a distance of about 11 Å, with loss of the hydrogen bond between H15 and the carboxylate residue. Similar but less populated conformations are found in compound **12** at a distance of about 9.5 Å.

4. Molecular Docking of Compounds 2 and 12 in the Modeled CCK_1-R. To identify the key amino acids involved in the CCK_1 receptor recognition process, a molecular docking study and its validation by site-directed mutagenesis experiments has been accomplished. In fact, we previously mapped the CCK_1-R binding site for CCK using site-directed mutagenesis and molecular modeling and found that the binding sites of nonpeptide agonists and antagonists most likely occupy a region of CCK_1-R, which interacts with the C-terminal amidated tripeptide of CCK, i.e. Met-Asp-Phe-NH_2.[29]

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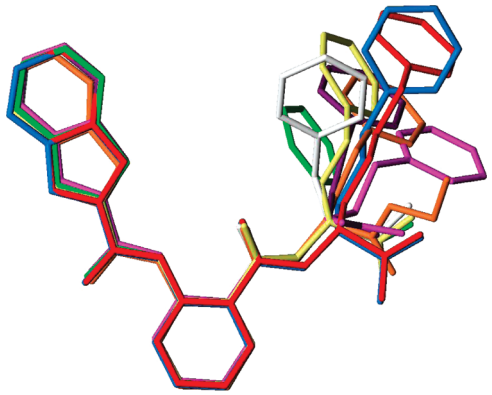

Figure 3. Overlay of the ligands optimized structures: compd **12**, white; compd **2**, yellow; compd **3**, cyano; compd **1**, green; compd **8**, magenta; compd **7**, orange; compd **9**, purple; compd **4**, red.

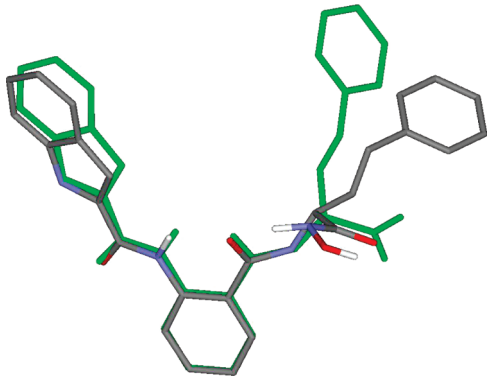

Figure 4. Overlay of the optimized structures of **2** (green) and of its hydroxamic acid derivative **10**.

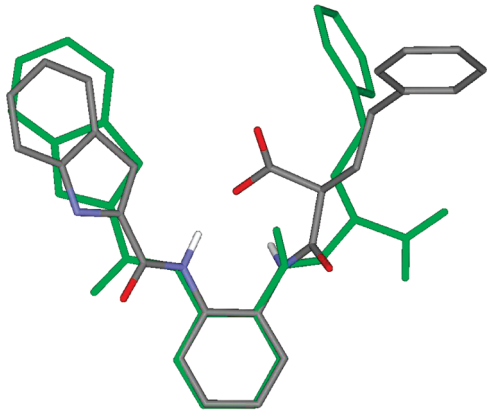

Figure 5. Overlay of the optimized structures of compound **2** (green) and of compound **11**.

Initially, two docking orientations of compound **12** in the binding pocket of the CCK_1-R were generated. In orientation A, compound **12** overlapped with the N-terminal moiety of CCK, Asp-Tyr-Thr-Gly-Trp (Figure 8a), whereas in orientation B, the antagonist overlapped with the amidated C-terminus of CCK, namely Trp-Met-Asp-Phe-NH_2 (Figure 8b).

In orientation A, the binding site is situated between Trp39 and Tyr48 in helix I, Phe107 in the first extracellular loop, Tyr176

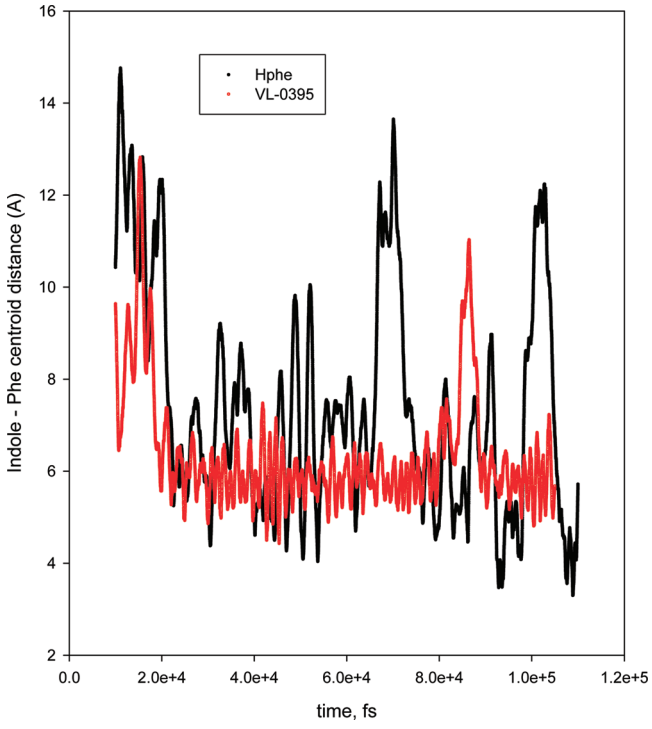

Figure 6. Compound **2** (black) and compound **12** (red).

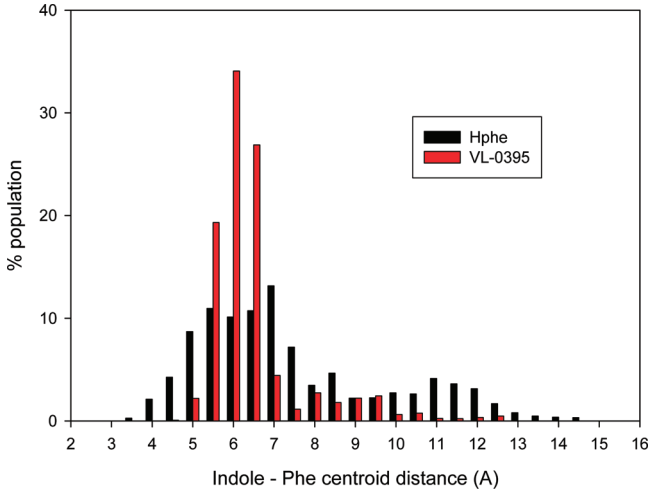

Figure 7. Population analysis of low energy conformations of compound **12** (red) and of compound **2** (black) after molecular dynamics at 300 K.

in helix IV, Met195 and Arg197 in the second extracellular loop, Thr117 and Met121 in helix III, Arg336 in helix VI, and Ile352, Leu356, and Tyr360 in helix VII (Figure 9). In this orientation, the indole moiety of the ligand occupies the same region as that occupied by the Trp indole ring of CCK-9 between helices II, III, and VII. The phenyl moiety of the ligand approximates Trp39 in helix I, Phe107 in the first extracellular loop and Met195 in the second extracellular loop, mimicking in this way the sulfated tyrosine of CCK. The carboxylate group of the ligand can interact with two arginines, Arg197 in the second extracellular loop and Arg336 in the helix VI. The same interactions were observed in the inactive state of CCK_1-R.

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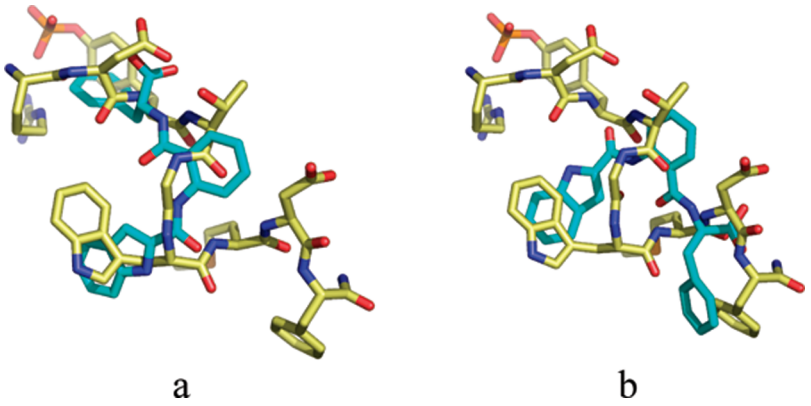

Figure 8. Superposition of **CCK-9** (yellow) and compound **12** (blue) in orientations (a) and (b).

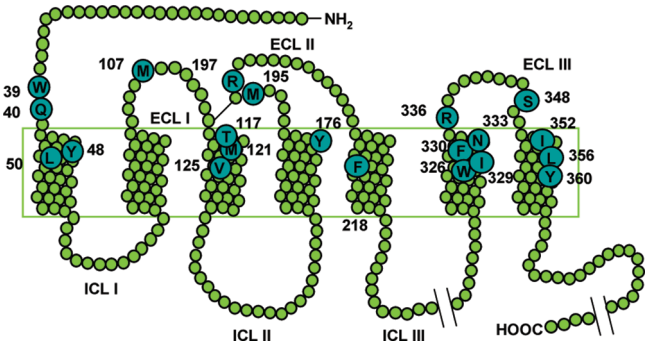

Figure 9. Serpentine representation of the CCK_1 receptor. Numbered amino acids are those which are part of the binding site of CCK and/or compound **12**.

In orientation B, the binding site for the ligand is formed by Thr117, Thr118, and Met121 in helix III, Tyr176 in helix IV, Met195 in the second extracellular loop, Asn333 and Arg336 in helix VI, Ile352, Leu356, and Tyr360 in helix VII (Figure 9). In this case, the indole ring of the ligand engages the same region occupied by the Trp indole ring of CCK. The carboxylate group accepts two hydrogen bonds one from Arg336 and the other from the amide of Asn330 in helix VI. The phenylalanine aromatic ring is sited between helix III and helix VI. This positioning is observed in the inactive state of CCK_1-R.

5. Validation of the Docking Orientation through Site-Directed Mutagenesis. To discriminate between both orientations we have performed site directed mutagenesis and competition binding assays in the presence of two known CCK_1-R antagonists: SR-27897 and JMV-179. We have focused our attention on the residues Trp39, Phe107, Met195, Arg197, Asn333, and Arg336 (Table 5).

The two most important residues for the binding of compound **6** are Arg336 and Asn333 because Arg336A and Asn333A mutants bind the ligand with 101.6, 16.2-fold lower affinity than the wild-type receptor, respectively. The Arg197Ala mutant also shows lower affinity, but its F_{mut} is only 3. Met195Ala and Trp39Ala mutations do not affect ligand binding, indicating that eventual interactions with these residues are not significant.

In this manner, we have three important interactions Arg336 > Asn333 > Arg197. Interactions with Arg336 and Asn333 are present in orientation B of docking and interactions with Arg197, and Arg336 are present in orientation A.

Table 5. Effects of CCK_1 Receptor Mutations on the Receptor Binding

CCK_1 receptor	K_d ^{3}H-SR-27897 (nM)[a]	K_d ^{125}I-BH-JMV-179 (nM) [a]	K_i compd **6** (nM) [a]	F_{mut} [b] (compd **6**)
WT		21.9 ± 1.2	0.32 ± 0.04	1
WT	3.4 ± 0.1		5.8 ± 0.3	1
W39A	2.4 ± 0.2		3.7 ± 0.4	0.6
F107A	5.0 ± 0.3		3.8 ± 0.8	0.8
M195A	2.9 ± 0.1		7.6 ± 0.7	1.3
R197A	5.6 ± 0.7		19.6 ± 2.8	3.8
R336A	47.1 ± 4.5		522 ± 25	101.6
F330A	13.2 ± 1.9		4.9 ± 0.1	1.1
I352A	6.0 ± 0.4		39.1 ± 5.3	7.4
N333A		23.7 ± 2.8	5.0 ± 0.6	16.2

[a] Results are the mean ± SEM from at least three experiments performed in duplicate. [b] The mutation factors (F_{mut}) were calculated as K_i (mutated receptor)/K_i (wild-type-CCK_1R).

Because the initial docking was performed in the CCK_1-R taken from the CCK_1-R/CCK complex and the binding experiments have been performed in the presence of antagonist which bind to the inactive state of the CCK_1-R, we decided to carry out the docking in the inactive conformation of the receptor. In this case, orientation B is not found but instead, an orientation very similar to A was observed, with a small displacement of the ligand toward Asn333 in a manner that its amide can interact with the indole-2-carbonyl group of the ligand.

In this disposition, the carboxylate group of **6** interacts with Arg197 and Arg336 and the indole carbonyl interacts with Asn333. The interactions of **2** with the receptor amino acids affect only to a minor extent its conformation when compared with the ground state one. The indole and anthranyl sides of the molecule are almost perfectly superimposable in the free and bound molecules. On the other side, the intramolecular hydrogen bond between H15 and the carboxylate end is lost in favor of the interactions with Arg197 and Arg336. This leads also to a slight rotation of the phenyl end of the molecule, and the bound conformation is somewhat intermediate between the two populations of conformers found by MD. The slight movement of the phenyl ring might improve the interactions of this aromatic end with Phe107, Met195, and Ile 352.

Moreover, we have studied the stability of the complex by means of molecular dynamics simulations in an explicit lipid

Table 6. Calculated van der Waals and Electrostatic Interaction Energies between Compound 6 and CCK_1 Receptor[a]

CCK_1 receptor	van der Waals	electrostatic	CCK_2 receptor
Trp39	−0.2		Glu
Phe107	−0.8		Phe120
Thr117	−1.1		Val130
Thr118	−0.4		Ser131
Met121	−1.2		Met134
Gln194	−2.4	−11.4	**Leu203**[b]
Met195	−3.3	−1.3	**Gln204**[b]
Arg197	−0.1	−2.1	**Val206**[b]
His210	−3.6		**Ser219**[b]
Phe330	−0.2		Try350
Asn333	−2.0	−5.8	Asn353
Arg336	−3.8	−21.8	Arg356
Ile352	−1.7		Ile372
Leu356	−3.2		**His376**[b]
Tyr360	−0.7		Tyr380

[a] Calculated with ANAL program of AMBER. [b] Residues marked in bold are those important for the binding, as revealed by energetic analysis decomposition, and also different between CCK_1 and CCK_2 receptors. The selectivity can be explained by these interactions.

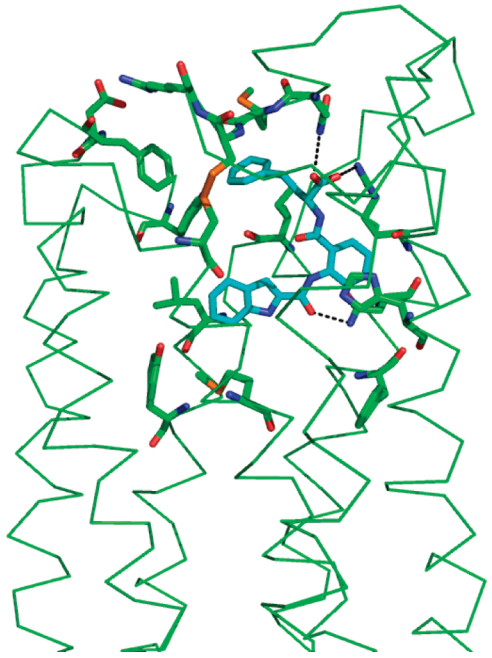

Figure 10. Docking of compound **6** to CCK_1 receptor in the inactive conformation after molecular dynamic simulation in an explicit membrane. Residues relevant for compound **6** binding are displayed as sticks.

environment. The interaction with Arg197 is lost, but an electrostatic analysis revealed that there is still an electrostatic interaction (Table 6). A long-range scale electrostatic interaction between the carboxylate group of **6** and Arg197 may be the reason of the observed loss of affinity with Arg197Ala mutant.

Nevertheless, as the interaction with Ag197 is very small and site directed mutagenesis does not reveal contacts with Met195 and Trp39, we constructed additional mutants to better discriminate A and B. To this purpose, we mutated Ile352 and Phe330. Ile352Ala mutant binds **6** with 7.4 lower affinity that the WT receptor, but the Phe330Ala mutation does not affect the binding (F_{mut}: 1.1). This last result rules out orientation B.

In summary, both site-directed mutagenesis findings and in silico docking showed that the binding site of compound **6** overlaps that occupied by the C-terminal bioactive region of the natural agonist CCK as well as that of other nonpeptide antagonists as the benzodiazepine derivative Devazepide; the thiazole derivative SR-27897 and of the pyridopyrimidine-based ligands.[29]

Description of the Binding Site after Molecular Dynamics Simulations. The binding site for compound **6** comprises Phe107 in the first extracellular loop, Gln194 and Met195 of the second extracellular loop, Thr117, Thr118, and Met121 in helix III, His210 in helix V, Asn333 and Arg336 in helix VI, and in helix VII, Ile352, Leu356, and Tyr360.

The indole-2-carbonyl group is located between helices II, III, and VII and occupies the same region that is occupied by the Trp indole ring of CCK-9. Asn333 donates a hydrogen bond to the indole-2-carbonyl oxygen. The phenylalanine ring interacts with Phe107 in the first extracellular loop, Met195 in the second extracellular loop, and Ile352 in helix VII. The carboxylate group interacts with Arg336 side chain and possibly with Gln194. In the ester and amide derivatives (compounds **7** and **8**), it is not possible to have these interactions (Figure 10).

CONCLUSION

In this second phase of the development of this new class of CCK_1-R antagonists dedicated to the C-terminal optimization of the anthranilic acid of **12**, we synthesized new compounds bearing unnatural amino acids instead of Phe present in the lead. The *Homo*-Phe derivative (compound **2**) showed a 12-fold increased binding affinity for the CCK_1-R and 4-fold increased apparent selectivity over the CCK_2-R relative to the reference compound **12**. We confirm the receptor subsite stereospecificity defining the eutomer of **2** (compound **6**) in which the absolute configuration of the single chiral center is *R* (rectus). Compound **2** and its eutomer exhibited a nanomolar range affinity toward the human CCK_1-R, and compound **6** was at least 400-fold more selective for the human CCK_1 over the CCK_2-Rs. The antagonist nature of **6** was validated by an in vitro and in vivo functional test using guinea pig gallbladder preparations. In both models, compound **6** confirmed to be a potent inhibitor of CCK-8-induced contraction of gallbladder and was 30 times more active than reference compound Dexloxiglumide in vitro.

The lower affinity observed for compounds **7**−**10** ascertains that the free carboxy group of the *Homo*-Phe is essential for the receptor recognition, suggesting its engagement through ionic bond formation rather than in a double hydrogen bond interaction.

Moreover, data with compound **11** underlines the importance of the anthranilic acid scaffold for the correct spatial orientation adopted by the main pharmacophoric groups of **2** in receptor subtype recognition.

The molecular modeling study elucidate that the higher receptor affinity of **2** is due to the higher conformational freedom of the *Homo*-Phe side chain in comparison with the Phe derivative **12**.

Finally, identification of key amino acids of CCK_1-R involved in the receptor desmodynamic process of **6** has been accomplished via molecular docking study and successive validation by site-directed mutagenesis experiments.

Such results provide suitable information for our research project, and we are convinced that further efforts should be accomplished

in order to establish with precision the receptor subsite tolerance in relation to the variation of the aminoacidic side chain.

Nevertheless, compound **6**, owing to its chemical features (simple structure, low molecular weight, number of synthetic steps, overall yield and low cost synthesis), represents an attractive opportunity for the pharmaceutical industry. Thus, the anthranilic acid based derivative **6** is actually under further biological investigation in order to establish its drug-like properties.

EXPERIMENTAL SECTION

Chemistry. All chemicals and solvents used in the syntheses were reagent grade and were used without additional purification. The progress of all reactions was monitored by thin-layer chromatography (TLC) using precoated silica gel plates (60F-254 Merck) and visualized by UV light (254 nm). Melting points were determined on a Büchi 510 melting point apparatus (Büchi, Flawil, Switzerland) and are uncorrected. Silica gel (Merck Kieselgel 60, 40−63 μm) was used for flash chromatography. Specific rotations were measured with a Perkin-Elmer 241 polarimeter at 589 nm (Na D-line) and 25 °C. Enantiomeric excess determinations were performed on a Jasco HPLC chromatograph on CSP-TE-SP-100 (250 mm × 4.0 mm ID) column by using an ammonium acetate solution (20 mM) in methanol/water 85/15 (v/v) as the mobile phase at flow rate of 1.00 mL/min with UV detection at 254 nm. 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 for $CDCl_3$ or $(CD_3)_2SO$ solutions, using Me_4Si as internal standard. Splitting patterns are designated as: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad. Coupling constants (J values) are given in hertz (Hz). Spectral data are consistent with assigned structures. Mass spectra were recorded an API-1 Perkin-Elmer SCIEX spectrometer by electrospray ionization (ES). The purities (>95%) of all compounds tested in biological systems were established by elemental analyses. Elemental analyses were performed by the Microanalyses Laboratory of the Department of Chemical and Pharmaceutical Sciences of the University of Trieste and were within ±0.4% of the theoretical values calculated for C, H and N.

The following abbreviations are used: TEA, triethylamine; MeOH, methanol; MeONa, sodium methoxide; EtOH, ethanol; AcOEt, ethyl acetate; AcOH, acetic acid; *n*-BuOH, 1-butanol; THF, tetrahydrofuran; DMF, dimethylformamide; MsCl, methanesulfonyl chloride; TBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

4-Phenyl-1-iodobutane (4a). To a 4-phenyl-butan-1-ol (10.00 g, 66.60 mmol) and TEA (14.10 mL, 101.33 mmol) solution in CH_2Cl_2 (150 mL), MsCl (5.84 mL, 75.20 mmol) was added dropwise at 0 °C. After 2 h stirring at room temperature, the solution was washed with ice−water (2 × 50 mL), cold 5N HCl (2 × 50 mL), saturated aqueous $NaHCO_3$ (2 × 50 mL), and brine (2 × 50 mL), dried over anhydrous Na_2SO_4, and concentrated. The mesylate product was mixed with NaI (43.30 g, 283.05 mmol) in acetone (250 mL), and the mixture was refluxed until TLC indicated full conversion of the starting material. Water was added (200 mL), and the solution was extracted with diethyl ether (3 × 100 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (2 × 50 mL) and concentrated.[30] The product was purified by flash chromatography (pentane) to yield (**4a**) as a colorless oil. Spectroscopic data were consistent with data described elsewhere.[31]

General Procedure for Synthesis of Diethyl α-Alkyl-acetamidomalonic Derivatives (3b, 4b, 7b). To a solution of 20.00 g (92.00 mmol) of diethyl acetamidomalonate in 90 mL of abs EtOH, EtONa 96% (6.94 g, 98.00 mmol) was added. After stirring under reflux for 30 min, 90.0 mmol of the corresponding alkyl halide was added dropwise and the reflux was continued overnight. The reaction mixture was then poured into 500 mL of 0.05 M $KHSO_4$ and 250 mL of *n*-hexane under vigorous stirring at 0 °C. The product precipitated at the interface was filtered and recrystallized from AcOEt/*n*-hexane.

*2-Acetylamino-2-(3-phenyl-propyl)-malonic Acid Diethyl Ester (**3b**).* The product was obtained in 64% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.45; mp 85 °C [lit. 86−87 °C].[32] ^{1}H NMR ($CDCl_3$) δ 1.21 (t, 6H, $-CH_2-CH_3$), 1.43 (m, 2H, $-CH_2-CH_2-CH_2-$), 2.02 (s, 3H, $-CO-CH_3$), 2.43 (m, 2H, $-CH_2-(CH_2)_2-C_6H_5$), 2.60 (t, 2H, $-CH_2-C_6H_5$), 4.21 (m, 4H, $-CH_2-O-$), 6.79 (s, 1H, −NH−), 7.11−7.26 (m, 5H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.06, 23.15, 25.78, 31.92, 35.52, 62.60, 66.53, 125.98, 128.34, 128.42, 141.82, 168.16, 169.08.

*2-Acetylamino-2-(4-phenyl-butyl)-malonic Acid Diethyl Ester (**4b**).* The product was obtained in 80% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.52; mp 56−57 °C [lit. 83−84 °C].[33] ^{1}H NMR ($CDCl_3$) δ 1.10−1.29 (m, 8H, $-CH_2-CH_3$ and $-CH_2-(CH_2)_2-C_6H_5$), 1.64 (m, 2H, $-CH_2-CH_2-C_6H_5$), 2.01 (s, 3H, $-CO-CH_3$), 2.32−2.41 (m, 2H, $-CH_2-(CH_2)_3-C_6H_5$), 2.60 (t, 2H, $-CH_2-C_6H_5$), 4.22 (m, 4H, $-CH_2-O-$), 6.82 (s, 1H, −NH−), 7.12−7.30 (m, 5H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.09, 23.02, 23.13, 30.93, 31.97, 35.43, 62.53, 66.58, 125.73, 128.27, 128.39, 142.07, 168.14, 168.95.

*2-Acetylamino-2-phenylethyl-malonic Acid Diethyl Ester (**7b**).* The product was obtained in 75% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.55; mp 116 °C (lit. 111−114 °C).[34] ^{1}H NMR ($CDCl_3$) δ 1.24 (t, 6H, $-CH_2-CH_3$), 1.97 (s, 3H, $-CO-CH_3$), 2.45−2.75 (m, 4H, $-(CH_2)_2-$), 4.17 (m, 4H, $-CH_2-O-$), 6.79 (s, 1H, −NH−), 7.12−7.26 (m, 5H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.08, 23.05, 30.23, 33.40, 62.65, 66.45, 126.16, 128.44, 128.51, 140.61, 168.10, 169.14.

General Procedure for the Synthesis of Amino Acids Hydrochloride (3c, 4c, 7c). A suspension of 25.00 mmol of the corresponding diethyl α-alkyl-acetamidomalonic derivative **3b**, **4b**, or **7b** in 150 mL of a solution 1:1 of 6N HCl and 1,4-dioxane was stirred under reflux. After completion (TLC monitoring), the solvent was removed in vacuo and the residue triturated with cold diethyl ether. The product was collected by filtration.

*2-(R,S)-Amino-5-phenyl-pentanoic Acid Hydrochloride (**3c**).* The product was obtained in 77% yield. TLC (*n*-BuOH/AcOH/H_2O 3:1:1); R_f 0.66; mp 228 °C. ^{1}H NMR (DMSO-d_6) δ 1.60−1.85 (m, 4H, $-(CH_2)_2-$), 2.55 (m, 2H, $-CH_2-C_6H_5$), 3.84 (m, 1H, −CH<), 7.15−7.30 (m, 5H, Ar), 8.57 (b, 3H, NH_3^+). ^{13}C NMR (DMSO-d_6) δ 26.06, 29.47, 34.42, 51.81, 125.62, 128.08, 141.22, 170.67.

*2-(R,S)-Amino-6-phenyl-hexanoic Acid Hydrochloride (**4c**).* The product was obtained in 95% yield. TLC (*n*-BuOH/AcOH/H_2O 3:1:1); R_f 0.66; mp 168−170 °C. ^{1}H NMR (DMSO-d_6) δ 1.32−1.61 (m, 4H, $-(CH_2)_2-CH_2-C_6H_5$), 1.84 (m, 2H, $-CH_2-CH<$), 2.54 (m, 2H, $-CH_2-C_6H_5$), 3.84 (m, 1H, −CH<), 7.13−7.31 (m, 5H, Ar), 8.50 (b, 3H, NH_3^+). ^{13}C NMR (DMSO-d_6) δ 23.89, 29.89, 30.50, 34.78, 51.77, 125.57, 128.13, 128.16, 141.79, 170.78.

*2-(R,S)-Amino-4-phenyl-butyric Acid Hydrochloride (**7c**).* The product was obtained in 53% yield. TLC (*n*-BuOH/AcOH/H_2O 3:1:1); R_f 0.64; mp 272 °C [lit. 260−272 °C]. ^{1}H NMR (DMSO-d_6) δ 2.10 (m, 2H, $-CH_2-CH<$), 2.68 (m, 2H, $-CH_2-C_6H_5$), 3.87 (m, 1H, −CH<), 7.22−7.35 (m, 5H, Ar), 8.66 (b, 3H, NH_3^+). ^{13}C NMR (DMSO-d_6) δ 30.23, 31.70, 51.41, 125.97, 128.08, 128.28, 140.24, 170.55.

General Procedure for the Synthesis of Amino Acids Ethyl Esters Hydrochloride (3d, 4d, 7d). Gaseous HCl is bubbled for 30 min into a stirred suspension of 20.00 mmol of the corresponding aminoacid hydrochloride **3c**, **4c**, or **7c** in 150 mL of abs EtOH and cooled to 0 °C. After solvent removal, the residue is triturated with cold diethyl ether and filtered.

*2-(R,S)-Amino-5-phenyl-pentanoic Acid Ethyl Ester Hydrochloride (**3d**).* The product was obtained in 88% yield. TLC (AcOEt/MeOH 1:1); R_f 0.73; mp 126 °C. ^{1}H NMR (DMSO-d_6) δ 1.20 (m, 3H, $-CH_3$), 1.62−1.82 (m, 4H, $-(CH_2)_2-$), 2.56 (m, 2H, $-CH_2-C_6H_5$), 3.97

(m, 1H, −CH<), 4.18 (m, 2H, −O-CH_2−), 7.18−7.28 (m, 5H, Ar), 8.73 (b, 3H, NH_3^+). ^{13}C NMR (DMSO-d_6) δ 13.78, 25.85, 29.37, 34.27, 51.55, 61.43, 125.66, 128.09, 141.11, 169.20.

*2-(R,S)-Amino-6-phenyl-hexanoic Acid Ethyl Ester Hydrochloride (**4d**).* The product was obtained in 66% yield. TLC (AcOEt/MeOH 1:1); R_f 0.79; mp 126−27 °C. ^{1}H NMR (DMSO-d_6) δ 1.16 (t, 3H, −CH_3). 1.22−1.46 (m, 4H, −(CH_2)$_2$-CH_2-C_6H_5), 1.58 (m, 2H, −CH_2-CH<), 2.54 (m, 2H, −CH_2-C_6H_5), 3.89 (m, 1H, −CH<), 4.14 (q, 2H, −O-CH_2−), 7.14−7.27 (m, 5H, Ar), 8.67 (b, 3H, NH_3^+). ^{13}C NMR (DMSO-d_6) δ 13.88, 23.69, 29.72, 30.30, 34.64, 51.68, 61.52, 125.55, 128.09, 128.14, 141.67, 169.30.

*2-(R,S)-Amino-4-phenyl-butyric Acid Ethyl Ester Hydrochloride (**7d**).* The product was obtained in 84% yield. TLC (AcOEt/MeOH 1:1); R_f 0.65; mp 137 °C [lit. 135−136 °C]. ^{1}H NMR (DMSO-d_6) δ 1.23 (t, 3H, −CH_3), 2.14 (m, 2H, −CH_2-CH<), 2.71 (m, 2H, −CH_2-C_6H_5), 3.96 (m, 1H, −CH<), 4.18 (q, 2H, −O-CH_2−), 7.21−7.31 (m, 5H, Ar), 8.65 (b, 3H, −NH_3^+). ^{13}C NMR (DMSO-d_6) δ 13.78, 30.07, 31.67, 51.32, 61.54, 125.98, 128.11, 128.24, 140.09, 169.06.

2-Phenethyl-malonic Acid Diethyl Ester (11b). To a solution of EtONa (2.30 g, 32.90 mmol) in abs EtOH (150 mL), diethyl malonate was added dropwise during 10 min. After stirring under reflux for 45 min, 4.50 mL (32.90 mmol) of (2-bromo-ethyl)-benzene were added dropwise and the reaction mixture was refluxed overnight. The solution was concentrated under reduced pressure, and the residue, taken up in 100 mL of water, was extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with water (3 × 25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, and concentrated. Distillation (60−80 °C and 0.04 mmHg) of the residual reactants afforded a pale-yellow oil in 42% yield. TLC (AcOEt/*n*-hexane 1:3); R_f 0.67. ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 6H, −CH_3), 2.22 (m, 2H, −CH_2-CH<), 2.66 (t, 2H, −CH_2-C_6H_5), 3.34 (t, 1H, −CH<), 4.19 (q, 4H, −O-CH_2−), 7.17−7.32 (m, 5H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.25, 30.50, 33.45, 51.37, 61.47, 126.24, 128.50, 128.59, 140.69, 169.35.

2-(*R,S*)-Phenethyl-malonic Acid Monoethyl Ester (11c). A mixture of **11b** (2.53 g, 9.57 mmol) and KOH (0.54 g, 9.57 mmol) in abs EtOH was stirred overnight at room temperature. After solvent removal, the residue was dissolved in H_2O and extracted with diethyl ether (3 × 50 mL). Aqueous layer was adjusted to pH 2−3 with 1N HCl and extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude residue was used without further purification in the next step.

2-(*R,S*)-(2-Amino-phenylcarbamoyl)-4-phenyl-butyric Acid Ethyl Ester (11d). To a solution of **11c** (1.75 g, 7.41 mmol), TEA (1.15 mL, 8.15 mmol), and TBTU (2.62 g, 8.15 mmol) in CH_2Cl_2, *o*-phenylenediamine (3.06 g, 14.82 mmol) was added.[37] The resulting mixture was stirred at room temperature overnight and then concentrated in vacuo. The residue, dissolved in 100 mL of AcOEt, was washed twice with 1N HCl, 1N NaOH, water, and brine, dried over anhydrous Na_2SO_4, and rotary evaporated. Trituration with a solution CH_2Cl_2/*n*-hexane 1:1 afforded a white solid in 79% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.39; mp 110 °C. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, −CH_3), 2.32 (m, 2H, −CH_2-CH<), 2.72 (m, 2H, −CH_2-C_6H_5), 3.39 (m, 1H, −CH<), 3.82 (b, 2H, −NH_2), 4.21 (q, 2H, −O-CH_2−), 6.74−7.33 (m, 9H, Ar), 8.30 (s, 1H, −NH−). ^{13}C NMR ($CDCl_3$) δ 14.29, 32.34, 33.48, 52.60, 62.00, 117.84, 119.36, 123.69, 125.36, 126.36, 127.38, 128.60, 140.54, 140.76, 166.93, 171.97.

General Procedure for the Synthesis of Anthranoyl Derivatives (1e, 3e−7e, 9e). A suspension of 20.00 mmol of the amino acid ethyl ester hydrochloride in 100 mL of AcOEt was treated with TEA (2.78 mL, 20.00 mmol) followed by isatoic anhydride (3.26 g, 20.00 mmol). The resulting mixture was refluxed under stirring for 4 h, cooled to room temperature, and filtered. The organic phase was washed with 1 N NaOH (3 × 50 mL), water (1 × 50 mL), and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified as described to yield the titled compounds.

Compound (**9e**) was obtained in the same way as described above, except no TEA was added to the reaction.

*(R,S)-(2-Amino-benzoylamino)-phenyl Acetic Acid Ethyl Ester (**1e**).* Trituration with cold petroleum ether 40−60° afforded the titled compound in 42% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.71; mp 81−83 °C. ^{1}H NMR ($CDCl_3$) δ 1.23 (m, 3H, −CH_3), 4.20 (m, 2H, −O-CH_2−), 5.03 (b, 2H, −NH_2), 5.70 (m, 1H, >CH-C_6H_5), 6.64 (m, 2H, >CH-NH− and Ar), 7.00−7.43 (m, 8H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.07, 56.71, 62.07, 116.61, 117.36, 127.24, 127.56, 128.49, 128.85, 128.99, 132.75, 136.83, 149.06, 168.44, 171.11.

*2-(R,S)-(2-Amino-benzoylamino)-5-phenyl-pentanoic Acid Ethyl Ester (**3e**).* Purification by flash chromatography (AcOEt/*n*-hexane) afforded the titled compound in 60% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.81; mp 53 °C. ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, −CH_3). 1.32−2.00 (m, 4H, −(CH_2)$_2$−), 2.67 (m, 2H, −CH_2-C_6H_5), 4.22 (q, 2H, −O-CH_2−), 4.80 (m, 1H, −CH<), 5.49 (b, 2H, −NH_2), 6.66 (m, 3H, >CH-NH− and Ar), 7.16−7.33 (m, 6H, Ar), 7.40 (d, 1H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.32, 27.20, 32.29, 35.46, 52.21, 61.63, 115.45, 116.70, 117.38, 126.02, 127.55, 128.48, 132.66, 141.74, 148.92, 168.95, 172.80.

*2-(R,S)-(2-Amino-benzoylamino)-6-phenyl-hexanoic Acid Ethyl Ester (**4e**).* Purification by flash chromatography (CH_2Cl_2/AcOEt) afforded the titled compound in 50% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.86; mp 63−64 °C. ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 3H, −CH_3), 1.42 (m, 2H, −CH_2−), 1.46−2.02 (m, 4H, −(CH_2)$_2$−), 2.61 (m, 2H, −CH_2-C_6H_5), 4.21 (q, 2H, −O-CH_2−), 4.74 (m, 1H, −CH<), 5.46 (b, 2H, −NH_2), 6.66 (m, 3H, >CH-NH− and Ar), 7.13−7.38 (m, 7H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.21, 24.73, 30.97, 32.45, 35.55, 52.16, 61.50, 115.35, 116.56, 117.19, 125.65, 127.36, 128.21, 128.29, 132.47, 142.06, 148.69, 168.68, 172.64.

*(+)-2-(S)-(2-Amino-benzoylamino)-4-phenyl-butyric Acid Ethyl Ester (**5e**).* Purification by flash chromatography (CH_2Cl_2/AcOEt) followed by trituration with cold *n*-hexane afforded the titled compound in 62% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.64; $[\alpha]_D^{25}$ = +40.6 (*c* = 2.0, CH_2Cl_2); mp 95 °C. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, −CH_3), 2.06−2.40 (m, 2H, −CH_2-CH<), 2.73 (m, 2H, −CH_2-C_6H_5), 4.22 (q, 2H, −O-CH_2−), 4.82 (m, 1H, −CH<), 5.50 (b, 2H, −NH_2), 6.56−6.69 (m, 3H, >CH-NH− and Ar), 7.18−7.33 (m, 7H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.37, 31.85, 34.24, 52.35, 61.70, 115.36, 116.64, 117.31, 126.24, 127.47, 128.47, 128.60, 132.62, 140.85, 148.87, 168.83, 172.49.

*(−)-2-(R)-(2-Amino-benzoylamino)-4-phenyl-butyric Acid Ethyl Ester (**6e**).* Purification by flash chromatography (CH_2Cl_2/AcOEt) followed by trituration with cold *n*-hexane afforded the titled compound in 58% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.64; $[\alpha]_D^{25}$ = −40.8 (*c* = 2.0, CH_2Cl_2); mp 95 °C. ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, −CH_3), 2.05−2.34 (m, 2H, −CH_2-CH<), 2.72 (m, 2H, −CH_2-C_6H_5), 4.21 (q, 2H, −O-CH_2−), 4.81 (m, 1H, −CH<), 5.50 (b, 2H, −NH_2), 6.59−6.67 (m, 3H, >CH-NH− and Ar), 7.17−7.32 (m, 7H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.38, 31.88, 34.21, 52.37, 61.69, 115.36, 116.61, 117.32, 126.25, 127.50, 128.48, 128.61, 132.61, 140.87, 148.90, 168.88, 172.51.

*2-(R,S)-(2-Amino-benzoylamino)-4-phenyl-butyric Acid Ethyl Ester (**7e**).* Trituration with cold petroleum ether 40−60° followed by crystallization from MeOH afforded the titled compound in 56% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.64; mp 97 °C. ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, −CH_3), 2.05−2.38 (m, 2H, −CH_2-CH<), 2.72 (m, 2H, −CH_2-C_6H_5), 4.21 (q, 2H, −O-CH_2−), 4.81 (m, 1H, −CH<), 5.14 (b, 2H, −NH_2), 6.59−6.67 (m, 3H, >CH-NH− and Ar), 7.17−7.28 (m, 7H, Ar). ^{13}C NMR ($CDCl_3$) δ 14.34, 31.84, 34.16, 52.35, 61.70, 115.37, 116.65, 117.35, 126.28, 127.54, 128.51, 128.65, 132.66, 140.90, 148.92, 168.95, 172.60.

*2-(R,S)-Amino-N-(1-methyl-3-phenyl-propyl)-benzamide (**9e**).* Purification by trituration with cold petroleum ether 40−60° afforded the

titled compound in 62% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.71; mp 100 °C. ^{1}H NMR ($CDCl_3$) δ 1.27 (d, 3H, $-CH_3$), 1.87 (m, 2H, $-CH_2$-CH<), 2.72 (m, 2H, $-CH_2$-C_6H_5), 4.23 (m, 1H, −CH<), 5.81 (b, 2H, $-NH_2$), 5.90 (m, 1H, −NH−), 6.62−7.33 (m, 9H, Ar). ^{13}C NMR ($CDCl_3$) δ 21.14, 32.59, 38.69, 45.28, 116.54, 117.26, 125.95, 127.02, 128.38, 128.52, 132.12, 141.80, 148.66, 168.72.

General Procedure for the Synthesis of Derivatives (1f, 3f−6f, 7, 9, and 11e). To a suspension of 0.81 g (5.00 mmol) of indole-2-carboxylic acid in 20 mL of acetyl chloride, 1.04 g (5.00 mmol) of PCl_5 was added in portions at 0 °C over a period of 0.5 h. After the mixture turned into a clear solution, stirring was continued at room temperature for 3 h. The solution was concentrated under reduced pressure, and the residue, taken up in 5 mL of dry CH_2Cl_2, was added dropwise at 0 °C to a solution of 4.00 mmol of the corresponding derivatives (**1e**, **3e**−**7e**, **9e**, and **11d**) in 4 mL of pyridine. After the addition was completed, the reaction mixture was stirred at room temperature overnight. Then 150 mL of CH_2Cl_2 were added and the organic layer washed twice with 40 mL of 1N HCl, 1N NaOH, H_2O, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated, and the mixture was purified as described to yield the titled compounds. Compound (**1f**) was collected by filtration from the reaction mixture.

*(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-phenyl Acetic Acid Ethyl Ester (**1f**).* Trituration with cold petroleum ether 40−60° afforded the titled compound in 61% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.72; mp 264−266 °C. ^{1}H NMR (DMSO-d_6) δ 1.17 (t, J = 6.8 Hz, 3H, $-CH_3$), 4.17 (m, 2H, $-O-CH_2-$), 5.74 (d, J = 6.3 Hz, 1H, >CH-C_6H_5), 7.07−7.26 (m, 4H, Ar), 7.39−7.62 (m, 7H, Ar), 7.71 (m, 1H, Ar), 7.97 (d, J = 7.8 Hz, 1H, Ar), 8.62 (d, J = 8.1 Hz, 1H, Ar), 9.57 (d, J = 6.2 Hz, 1H, >CH-NH−), 11.95 (s, 1H, −NH−), 12.15 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 13.82, 57.01, 60.92, 102.47, 112.35, 119.43, 119.89, 120.05, 121.57, 122.40, 123.92, 126.78, 128.21, 128.37, 129.02, 131.39, 132.47, 135.32, 136.97, 138.87, 159.00, 168.73, 170.02.

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-5-phenyl-pentanoic Acid Ethyl Ester (**3f**).* Trituration with hot MeOH afforded the titled compound in 28% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.75; mp 173 °C. ^{1}H NMR (DMSO-d_6) δ 1.19 (t, J = 7.1 Hz, 3H, $-CH_3$), 1.74−1.90 (m, 4H, $-(CH_2)_2-$), 2.63 (m, 2H, $-CH_2$-C_6H_5), 4.15 (q, J = 7.0 Hz,2H, $-O-CH_2-$), 4.45 (m, 1H, −CH<), 7.06 (s, 1H, Ar), 7.11−7.28 (m, 8H, Ar), 7.50 (d, J = 8.1 Hz, 1H, Ar), 7.63−7.71 (m, 2H, Ar), 7.94 (d, J = 7.8 Hz, 1H, Ar), 8.65 (d, J = 8.1 Hz, 1H, Ar), 9.20 (d, J = 6.7 Hz,1H, >CH-NH−), 11.96 (s, 1H, −NH−), 12.27 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 13.93, 27.35, 29.68, 34.43, 52.71, 60.44, 102.39, 112.33, 119.38, 119.84, 120.03, 121.58, 122.44, 123.90, 125.53, 126.77, 128.05, 128.57, 131.39, 132.42, 136.96, 138.94, 141.47, 158.97, 168.97, 171.59.

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-6-phenyl-hexanoic Acid Ethyl Ester (**4f**).* Trituration with hot MeOH followed by flash chromatography (from CH_2Cl_2/petroleum ether 40−60° 1:1 to CH_2Cl_2 to CH_2Cl_2/AcOEt 20:1) afforded the titled compound in 91% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.69; mp 167−168 °C. ^{1}H NMR (DMSO-d_6) δ 1.17 (t, J = 7.1 Hz, 3H, $-CH_3$), 1.44 (m, 2H, $-CH_2-CH_2-CH<$), 1.61 (m, 2H, $-CH_2-CH_2-C_6H_5$), 1.86 (m, 2H, $-CH_2-CH<$), 2.56 (m, 2H, $-CH_2-C_6H_5$), 4.11 (q, J = 7.1 Hz, 2H, $-O-CH_2-$), 4.47 (m, 1H, −CH<), 7.03−7.30 (m, 9H, Ar), 7.47 (m, 1H, Ar), 7.57−7.70 (m, 2H, Ar), 7.88 (d, J = 7.6 Hz,1H, Ar), 8.62 (d, J = 8.1 Hz, 1H, Ar), 9.09 (m, 1H, >CH-NH−), 11.91 (s, 1H, −NH−), 12.20 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 14.06, 25.27, 29.98, 30.40, 34.86, 52.81, 60.54, 102.46, 112.40, 119.49, 119.91, 120.11, 121.66, 122.51, 123.98, 125.48, 126.82, 128.06, 128.09, 128.62, 131.42, 132.49, 136.99, 138.84, 141.87, 159.00, 168.97, 171.69.

*(+)-2-(S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-4-phenyl-butyric Acid Ethyl Ester (**5f**).* Trituration with hot MeOH followed by flash chromatography (from CH_2Cl_2 to CH_2Cl_2/AcOEt 4:1) afforded the titled compound in 82% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.63; $[\alpha]_D^{25}$ = +54.5 (c = 0.51, CH_2Cl_2); mp 243−244 °C. ^{1}H NMR (DMSO-d_6) δ 1.19 (t, J = 6.8 Hz, 3H, $-CH_3$); 2.15 (m, 2H, $-CH_2$-CH<), 2.75 (m, 2H, $-CH_2-C_6H_5$), 4.13 (q, J = 6.8 Hz,2H, $-O-CH_2-$), 4.45 (m, 1H, −CH<), 7.03−7.97 (m, 13H, Ar), 8.64 (d, J = 8.1 Hz, 1H, Ar), 9.25 (d, J = 6.6 Hz,1H, >CH-NH−), 11.94 (s, 1H, −NH−), 12.18 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 14.00, 31.53, 31.81, 52.27, 60.58, 102.42, 112.35, 119.59, 119.88, 120.05, 121.56, 122.49, 123.92, 125.81, 126.77, 128.16, 128.23, 128.59, 131.37, 132.43, 136.94, 138.87, 140.58, 158.94, 169.05, 171.53.

*(−)-2-(R)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-4-phenyl-butyric Acid Ethyl Ester (**6f**).* Trituration with hot MeOH followed by flash chromatography (from CH_2Cl_2 to CH_2Cl_2/AcOEt 4:1) afforded the titled compound in 81% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.63; $[\alpha]_D^{25}$ = −54.4 (c = 0.54, CH_2Cl_2); mp 243−244 °C. ^{1}H NMR (DMSO-d_6) δ 1.19 (t, J = 6.7 Hz, 3H, $-CH_3$), 2.15 (m, 2H, $-CH_2$-CH<), 2.75 (m, 2H, $-CH_2-C_6H_5$), 4.13 (q, J = 6.7 Hz, 2H, $-O-CH_2-$), 4.45 (m, 1H, −CH<), 7.03−7.97 (m, 13H, Ar), 8.64 (d, J = 8.0 Hz, 1H, Ar), 9.25 (d, J = 6.5 Hz, 1H, >CH-NH−), 11.93 (s, 1H, −NH−), 12.18 (s, 1H, −NH−), ^{13}C NMR (DMSO-d_6) δ 14.00, 31.53, 31.82, 52.27, 60.59, 102.42, 112.35, 119.59, 119.88, 120.06, 121.56, 122.50, 123.92, 125.81, 126.77, 128.17, 128.23, 128.59, 131.37, 132.44, 136.94, 138.87, 140.58, 158.94, 169.05, 171.53.

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-4-phenyl-butyric Acid Ethyl Ester (**7**).* Trituration with hot MeOH afforded the titled compound in 36% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.63; mp 214 °C. ^{1}H NMR (DMSO-d_6) δ 1.19 (t, J = 6.9 Hz, 3H, $-CH_3$), 2.17 (m, 2H, $-CH_2-CH<$), 2.75 (m, 2H, $-CH_2-C_6H_5$), 4.14 (q, J = 6.9 Hz, 2H, $-O-CH_2-$), 4.45 (m, 1H, −CH<), 7.04−7.98 (m, 13H, Ar), 8.65 (d, J = 8.1 Hz, 1H, Ar), 9.27 (d, J = 6.7 Hz, 1H, >CH-NH−), 11.96 (s, 1H, −NH−), 12.21 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 13.91, 31.46, 31.74, 52.21, 60.53, 102.40, 112.34, 119.56, 119.87, 120.05, 121.56, 122.49, 123.91, 125.81, 126.77, 128.16, 128.23, 128.60, 131.38, 132.45, 136.96, 138.89, 140.59, 158.97, 169.10, 171.57. MS (ES) m/z 470.2 $[MH]^+$; MW 469.53 (calcd for $C_{28}H_{27}N_3O_4$).

*(R,S)-1H-Indole-2-carboxylic Acid [2-(1-Methyl-3-phenyl-propylcarbamoyl)-phenyl]-amide (**9**).* Trituration with hot CH_2Cl_2 followed by crystallization from EtOH afforded titled compound in 50% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.68; mp 242 °C. ^{1}H NMR (DMSO-d_6) δ 1.21 (d, J = 6.2 Hz, 3H, $-CH_3$), 1.87 (m, 2H, $-CH_2-CH<$), 2.64 (t, J = 6.4 Hz, 2H, $-CH_2-C_6H_5$), 4.14 (m, 1H, −CH<), 7.06−7.89 (m, 13H, Ar), 8.62−8.73 (m, 2H, >CH-NH− and Ar), 11.93 (s, 1H, −NH−), 12.61 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 20.42, 31.87, 37.20, 44.62, 102.35, 112.33, 119.81, 120.02, 120.16, 121.62, 122.35, 123.85, 125.47, 126.84, 128.03, 128.08, 128.17, 131.55, 131.95, 136.94, 139.00, 141.57, 159.00, 167.85. MS (ES) m/z 412.2 $[MH]^+$; MW 411.50 (calcd for $C_{26}H_{25}N_3O_2$).

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-phenylcarbamoyl}-4-phenyl-butyric Acid Ethyl Ester (**11e**).* Trituration with hot MeOH followed by flash chromatography (from CH_2Cl_2 to CH_2Cl_2/AcOEt 4:1) afforded titled compound in 71% yield. TLC (AcOEt/*n*-hexane 1:1); R_f 0.53; mp 191 °C. ^{1}H NMR (DMSO-d_6) δ 1.05 (t, J = 6.8 Hz, 3H, $-CH_3$), 2.14 (m, 2H, $-CH_2-CH<$), 2.54 (m, 2H, $-CH_2-C_6H_5$), 3.60 (m, 1H, −CH<), 4.02 (m, 2H, $-O-CH_2-$), 7.05−7.77 (m, 14H, Ar), 9.78 (s, 1H, −NH−), 10.15 (s, 1H, −NH−), 11.84 (s, 1H, −NH−), ^{13}C NMR (DMSO-d_6) δ 14.62, 31.21, 33.46, 52.64, 61.62, 104.02, 113.17, 120.75, 122.37, 124.58, 125.90, 126.13, 126.29, 126.61, 127.70, 128.90, 129.00, 131.09, 131.47, 131.83, 137.61, 141.53, 160.26, 168.44, 170.12.

General Procedure for the Synthesis of Compounds (1−4, 11). A mixture of the corresponding ethyl ester **1f**, **3f**−**6f**, **7**, and **11e** (4.0 mmol) and KOH (0.22 g, 4.00 mmol) in water (25 mL) and THF (25 mL) was stirred at room temperature until hydrolysis completion (TLC monitoring). After organic solvent removal, the aqueous solution

was adjusted to pH 2−3 with diluted HCl at 0 °C, yielding precipitation of the titled compound.

*(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-phenyl Acetic Acid (**1**).* Trituration with hot MeOH afforded the titled compound in 77% yield. TLC (AcOEt/MeOH 3:1); R_f 0.34; mp 290−291 °C. ^{1}H NMR (DMSO-d_6) δ 5.64 (d, J = 5.4 Hz, 1H, −CH<), 7.07−7.40 (m, 7H, Ar), 7.48−7.64 (m, 4H, Ar), 7.73 (m, 1H, Ar), 8.00 (d, J = 7.6 Hz, 1H, Ar), 8.64 (d, J = 8.1 Hz, 1H, Ar), 9.35 (d, J = 6.4 Hz, 1H, >CH-NH−), 11.96 (s, 1H, −NH−), 12.29 (s, 1H, −NH−), 12.80 (b, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 57.09, 102.53, 112.33, 119.60, 119.91, 120.01, 121.68, 122.46, 123.89, 126.80, 127.57, 127.91, 128.13, 128.82, 131.42, 132.35, 136.96, 137.04, 138.96, 159.04, 168.19, 171.29. MS (ES) m/z 414.2 $[MH]^+$; MW 413.43 (calcd for $C_{24}H_{19}N_3O_4$).

*2-(R,S)-{2-[(1H-Indole-2-carbonyl) -amino]-benzoylamino}-4-phenyl-butyric Acid (**2**).* The target compound was obtained by hydrolysis of **7**. Trituration with hot MeOH afforded the titled compound in 75% yield. TLC (AcOEt/MeOH 2:1); R_f 0.48; mp 259−260 °C. ^{1}H NMR (DMSO-d_6) δ 2.17 (m, 2H, −CH$_2$-CH<), 2.75 (m, 2H, −CH$_2$-C$_6$H$_5$), 4.42 (m, 1H, −CH<), 7.07−8.01 (m, 13H, Ar), 8.68 (d, J = 8.1 Hz, 1H, Ar), 9.16 (d, J = 7.0 Hz, 1H, >CH-NH−), 11.98 (s, 1H, −NH−), 12.39 (s, 1H, −NH−), 12.83 (b, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 31.65, 31.88, 52.05, 102.41, 112.33, 119.50, 119.80, 120.03, 121.65, 122.43, 123.89, 125.74, 126.79, 128.13, 128.23, 128.55, 131.42, 132.36, 136.95, 139.03, 140.76, 158.99, 168.89, 173.16. MS (ES) m/z 442.2 $[MH]^+$; MW 441.48 (calcd for $C_{26}H_{23}N_3O_4$)

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-5-phenyl-pentanoic Acid (**3**).* Trituration with hot MeOH afforded the titled compound in 50% yield. TLC (AcOEt/MeOH 2:1); R_f 0.54; mp 256 °C. ^{1}H NMR (DMSO-d_6) δ 1.72−1.91 (m, 4H, −(CH$_2$)$_2$−), 2.63 (t, J = 7.0 Hz, 2H, −CH$_2$-C$_6$H$_5$), 4.53 (m, 1H, −CH<), 7.08 (s, 1H, Ar), 7.10−7.29 (m, 8H, Ar), 7.50 (d, J = 8.1 Hz, 1H, Ar), 7.62 (t, J = 7.7 Hz, 1H, Ar), 7.74 (d, J = 7.9 Hz, 1H, Ar), 7.97 (d, J = 7.6 Hz, 1H, Ar), 8.68 (d, J = 8.3 Hz, 1H, Ar), 9.10 (d, J = 7.3 Hz, 1H, >CH-NH−), 11.96 (s, 1H, −NH−), 12.43 (s, 1H, −NH−). 12.82 (s, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 27.54, 29.81, 34.47, 52.43, 102.39, 112.32, 119.27, 119.80, 120.01, 121.68, 122.38, 123.89, 125.51, 126.80, 128.05, 128.55, 131.43, 132.38, 136.95, 139.09, 141.54, 159.00, 168.81, 173.12. MS (ES) m/z 456.2 $[MH]^+$; MW 455.51 (calcd for $C_{27}H_{25}N_3O_4$).

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-6-phenyl-hexanoic Acid (**4**).* Trituration with hot MeOH afforded the titled compound in 74% yield. TLC (AcOEt/MeOH 1:1); R_f 0.86; mp 237−238 °C. ^{1}H NMR (DMSO-d_6) δ 1.45 (m, 2H, −CH$_2$-CH$_2$-CH<), 1.58 (m, 2H, −CH$_2$-CH$_2$-C$_6$H$_5$), 1.87 (m, 2H, −CH$_2$-CH<), 2.59 (m, 2H, −CH$_2$-C$_6$H$_5$), 4.47 (m, 1H, −CH<), 7.06−7.30 (m, 9H, Ar), 7.46−7.74 (m, 3H, Ar), 7.92 (d, J = 7.9 Hz, 1H, Ar), 8.65 (d, J = 8.3 Hz, 1H, Ar), 9.05 (d, J = 7.3 Hz, 1H, >CH-NH−), 11.86 (s, 1H, −NH−), 12.41 (s, 1H, −NH−), 12.81 (b, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 25.46, 30.10, 30.47, 34.90, 52.55, 102.45, 112.40, 119.38, 119.85, 120.08, 121.76, 122.46, 123.97, 125.46, 126.85, 128.06, 128.09, 128.61, 131.48, 132.46, 137.00, 139.11, 141.93, 159.04, 168.80, 173.23. MS (ES) m/z 470.2 $[MH]^+$; MW 469.53 (calcd for $C_{28}H_{27}N_3O_4$).

*2-(R,S)-{2-[(1H-Indole-2-carbonyl)-amino]-phenylcarbamoyl}-4-phenyl-butyric Acid (**11**).* Trituration with hot MeOH followed by AcOEt afforded the titled compound in 26% yield. TLC (AcOEt/MeOH 2:1); R_f 0.57; mp 196 °C. ^{1}H NMR (DMSO-d_6) δ 2.11 (m, 2H, −CH$_2$-CH<), 2.55 (m, 2H, −CH$_2$-C$_6$H$_5$), 3.50 (m, 1H, −CH<), 7.03−7.81 (m, 14H, Ar), 9.79 (s, 1H, −NH−), 10.15 (s, 1H, −NH−), 11.85 (s, 1H, −NH−), 12.90 (b, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 31.31, 33.59, 52.79, 104.10, 113.14, 120.67, 122.51, 124.55, 125.78, 126.02, 126.54, 127.75, 128.88, 128.96, 131.03, 131.43, 131.83, 137.59, 141.67, 160.27, 169.08, 171.83. MS (ES) m/z 442.2 $[MH]^+$; MW 441.48 (calcd for $C_{26}H_{23}N_3O_4$).

General Procedure for the Synthesis of Compounds (5, 6). To 0.60 mmol of the corresponding ethyl ester (compounds **5f** and **6f**) in 50 mL of a solution of H_2O/THF 1:1, 25 mg (0.60 mmol) of LiOH·H_2O were added. After stirring 24 h at room temperature, the organic solvent was evaporated and 30 mL of $NaHCO_3$ satd and AcOEt were added. The mixture was then vigorously stirred; the salt formed was filtrated and taken up in THF.

The pH of the solution was adjusted to 2−3 with 1N HCl, the organic solvent evaporated under reduced pressure, and the white solid collected by filtration. Trituration with hot AcOEt and then with abs EtOH afforded the titled compounds.

*(−)-2-(S)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-4-phenyl-butyric Acid (**5**).* The product was obtained in 66% yield. TLC (AcOEt/MeOH 2:1); R_f 0.48; $[\alpha]_D^{25}$ = −34.8 (c = 0.65, DMF); ee > 99.9%, mp 267−268 °C. ^{1}H NMR (DMSO-d_6) δ 2.16 (m, 2H, −CH$_2$-CH<), 2.74 (m, 2H, −CH$_2$-C$_6$H$_5$), 4.42 (m, 1H, −CH<), 7.05−8.00 (m, 13H, Ar), 8.66 (d, J = 8.0 Hz, 1H, Ar), 9.14 (d, J = 7.1 Hz, 1H, >CH-NH−), 11.94 (s, 1H, −NH−), 12.35 (s, 1H, −NH−), 12.83 (b, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 31.77, 31.94, 52.05, 102.48, 112.42, 119.52, 119.89, 120.14, 121.74, 122.55, 124.01, 125.85, 126.86, 128.24, 128.32, 128.66, 131.47, 132.50, 137.01, 139.08, 140.77, 159.04, 168.99, 173.18. MS (ES) m/z 442.2 $[MH]^+$; MW 441.48 (calcd for $C_{26}H_{23}N_3O_4$).

*(+)-2-(R)-{2-[(1H-Indole-2-carbonyl)-amino]-benzoylamino}-4-phenyl-butyric Acid (**6**).* The product was obtained in 60% yield. TLC (AcOEt/MeOH 2:1); R_f 0.48; $[\alpha]_D^{25}$ = +35.4 (c = 0.65, DMF); ee > 99.7%, mp 267−268 °C. ^{1}H NMR (DMSO-d_6) δ 2.16 (m, 2H, −CH$_2$-CH<), 2.74 (m, 2H, −CH$_2$-C$_6$H$_5$), 4.42 (m, 1H, −CH<), 6.95 (s, 1H, Ar), 7.06−7.83 (m, 12H, Ar), 8.63 (d, J = 8.1 Hz, 1H, Ar), 9.17 (d, J = 7.0 Hz, 1H, >CH-NH−), 11.92 (s, 1H, −NH−), 12.25 (s, 1H, −NH−), 12.81 (b, 1H, −OH). ^{13}C NMR (DMSO-d_6) δ 31.74, 31.93, 52.04, 102.44, 112.38, 119.48, 119.85, 120.09, 121.71, 122.50, 123.95, 125.81, 126.82, 128.19, 128.29, 128.61, 131.44, 132.45, 136.97, 139.05, 140.74, 159.00, 168.94, 173.13. MS (ES) m/z 442.2 $[MH]^+$; MW 441.48 (calcd for $C_{26}H_{23}N_3O_4$).

(*R,S*)-1*H*-Indol-2-carboxylic Acid 2-(1-Hydroxycarbamoyl-3-phenyl-propylcarbamoyl)-phenyl-amide (10). Ethyl ester **7** (0.30 g, 0.64 mmol) was dissolved in MeOH (15 mL). To the solution hydroxylamine hydrochloride (0.09 g, 1.3 mmol) was added, followed by MeONa, freshly prepared from sodium (0.10 g, 3.19 mmol) dissolved in MeOH (2 mL). The reaction mixture was stirred for 36 h at room temperature and then worked up by partitioning between diluted hydrochloric acid and AcOEt. The aqueous phase was extracted with AcOEt, the combined organic layers were dried on anhydrous Na_2SO_4, and the solvent was evaporated.[38] The product was crystallized from diethyl ether/MeOH and collected by filtration (0.14 g, 48% yield). TLC (AcOEt); R_f 0.66; mp 218−219 °C (dec). ^{1}H NMR (DMSO-d_6) δ 2.07 (m, 2H, −CH$_2$-CH<), 2.62 (m, 2H, −CH$_2$-C$_6$H$_5$), 4.41 (m, 1H, −CH<), 7.04−7.26 (m, 9H, Ar), 7.46 (d, J = 8.2 Hz, 1H, Ar), 7.58 (m, 1H, Ar), 7.68 (d, J = 7.9 Hz, 1H, Ar), 7.98 (d, J = 7.7 Hz, 1H, Ar), 8.60 (d, J = 8.2 Hz, 1H, Ar), 8.90, 9.35 (2 s, 1H, −OH), 9.01 (d, J = 7.8 Hz, 1H, >CH-NH−), 10.86, 11.17 (2 s, 1H, −NH-OH), 11.91 (s, 1H, −NH), 12.34, 12.36 (2 s, 1H, −NH) (doubling of resonance is due to *Z*- and *E*-amide conformers). ^{13}C NMR (DMSO-d_6) δ 31.69, 33.06, 51.09, 102.52, 112.39, 119.72, 119.84, 120.07, 121.63, 122.41, 123.93, 125.71, 126.81, 128.16, 128.82, 131.52, 132.29, 136.97, 139.03, 140.90, 159.02, 167.86, 168.62. MS (ES) m/z 457.3 $[MH]^+$; MW 456.49 (calcd for $C_{26}H_{24}N_4O_4$).

(*R,S*)-1*H*-Indole-2-carboxylic Acid [2-(1-Carbamoyl-3-phenyl-propylcarbamoyl)-phenyl]-amide (8). To a solution of **2** (0.88 g, 2.00 mmol), TEA (1.10 mL, 7.90 mmol), and PyBOP (1.54 g, 3.00 mmol) in 150 mL of DMF, 0.21 g (4.00 mmol) of NH_4Cl were added. After overnight mixture stirring, DMF was evaporated in vacuo and the residue dissolved in 50 mL of AcOEt. The organic layer was washed twice with 50 mL of 1N HCl, 1N NaOH, water, and brine and then dried over anhydrous Na_2SO_4.[39] After solvent removal, the resulting residue was purified by flash chromatography (CH_2Cl_2/AcOEt 3:2) and triturated with *n*-hexane to give the titled compound in 50%

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yield. TLC (AcOEt/MeOH 1:1); R_f 0.40; mp 295−296 °C. ^{1}H NMR (DMSO-d_6) δ 2.09 (m, 2H, −C$\underline{H}_2$-CH<), 2.63 (m, 2H, −C$\underline{H}_2$-C_6H_5), 4.43 (m, 1H, −CH<), 7.06−7.14 (m, 8H, Ar), 7.21−7.73 (m, 4H, Ar), 7.99 (d, J = 7.8 Hz, 1H, Ar), 8.61 (d, J = 8.3 Hz, 1H, Ar), 8.92 (d, J = 7.8 Hz, 1H, >CH-N$\underline{H}$−), 11.91 (s, 1H, −NH−), 12.38 (s, 1H, −NH−). ^{13}C NMR (DMSO-d_6) δ 31.84, 32.91, 53.052 102.45, 112.34, 119.86, 120.02, 121.62, 122.41, 123.87, 125.62, 126.80, 128.09, 128.70, 131.48, 132.15, 136.91, 138.92, 141.06, 158.99, 168.61, 173.04. MS (ES) m/z 441.0 $[MH]^+$; MW 440.49 (calcd for $C_{26}H_{24}N_4O_3$).

Molecular Modeling and Docking. The geometries of all the ligands were optimized at different levels of theory, including MM calculations carried out with the AMBER 8 force field 99, and AM1 and DFT calculations carried out with the parallel version of the Gaussian 03 suite[40] running on an Intel Core 2 CPU 6600@2.40 GHz, 3.50 GB RAM. All the ab initio and semiempirical calculations on ligands were carried using the Berny algorithm for full optimizations with the default Gaussian SCF and convergence criteria.

Conformational analysis: our previous work on similar molecules ensured us that, for anthranilic acid derivatives, a Monte Carlo search carried out with the AM1 semiempirical Hamiltonian[41] yields results comparable with those obtained at higher ab initio levels.[16,20] All the optimizations and searches were thus obtained from AM1 calculations. The only exception is represented by the hydroxamic derivative **10** (Table 4): the AM1 method is in fact unable to model the structural features of the hydroxamic group, and the optimizations were carried out using the multilayer ONIOM method[42] which has proven by Remko to peform well with hydroxamic acids.[27] According to this protocol, the high layer was represented by the hydroxamic group and by carbon 16 (Table 4), and its geometry was optimized with a DFT calculation at the B3LYP/6.311+G (d,p)[43−45] level, while the low layer, containing all the remaining atoms of the molecule, was optimized at the AM1 level. All the molecules containing a carboxylic group at carbon 16 were considered as carboxylate anions, while the hydroxamic acid derivatives were kept in their neutral protonated form, because the pK_a experimental values for such acids are found in the 8.5−8.7 range.[28]

The molecular dynamics runs were carried out with the SANDER module of AMBER 8 and the Sybyl7.3 suite running on SGI Octane2-MIPS R12000 workstations or on a RedHat linux Intel Pentium 4 2.53 GHz machine. After relaxing the complexes, the structures were allowed to reach thermal equilibrium at 300 K by a multistep molecular dynamic run in the NTV ensemble (5000 fs at 100 K and 200 K and 5000 fs at 300 K). The behavior of the two ligands was analyzed at 300 K, after a slow equilibration, by collecting dynamics in the NTV ensemble for 1 μs at 0.2 fs steps. The Tripos forcefield was used to run such simulations.[46]

Docking of Compound 2 inside the Homology Model of CCK$_1$R. Because antagonists are able to bind both the inactive and the active conformations of the receptor, we have selected the solutions that satisfy this assumption and thus performed docking on both the inactive and active conformations of CCK$_1$R. A previously described model of CCK1R*[29] was used as starting point to dock compound **2**. The starting three-dimensional conformation of the ligands was obtained using Corina software.[47] Electrostatic potential derived charges were obtained with the restrained electric potential methodology[48] using a 6-31G* basis set as implemented in the Gaussian 98 program. The Lamarckian genetic algorithm77 implemented in Auto-Dock 3.0 was used to generate docked conformations of **2** in a putative pocket within the transmembrane helices of CCK$_1$R by randomly changing the overall orientation of the molecules as well as torsion angles. Default settings were used, except for the number of runs, population size, and maximum number of energy evaluations, which were fixed at 100, 100, and 250000, respectively. Rapid intra- and intermolecular energy evaluation of each configuration was achieved by having the receptor atomic affinity potentials for carbon, oxygen, and hydrogen atoms precalculated in a three-dimensional grid with a spacing of 0.375 Å. The selected docking solutions were refined by means of molecular dynamics simulations in an explicit lipid bilayer. The simulation was carried out at neutral pH, and the positive charges inside the receptor structure were neutralized by the addition of 15 chloride ions. The receptor model was placed in a rectangular box (78 × 80 × 78 in size) containing a lipid bilayer (153 molecules of palmitoyl-oleoyl-phosphatidylcholine and 8708 molecules of water in addition to the receptor structure), resulting in a final density of 1.0 g/cm^3. The initial steric clashes were eliminated by means of 1000 steps of steepest descent energy minimization, followed by 2000 steps of conjugate gradient energy minimization of all the system with the exception of Cα carbons of helices I, II, and III that were harmonically restrained to their initial positions. Molecular dynamic (MD) simulations were then run at 300 K and constant pressure (1 atm) with anisotropic scaling were then run for 1 ns using the SANDER module in AMBER 8. The conditions of the MDs and the simulation protocol were the same as previously described.[49] The resulting complexes were then studied by MDs in an explicit lipid bilayer under the same conditions as those described above.

Biological Evaluation. 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 labeled-specific activity 2000 Ci/mol) was purchased from Amersham Pharmacia Biothech (Buckinghamshire, UK). All other drugs and reagents were obtained from commercial sources.

Cumulative curves analysis (log nM or log μM concentration of test compound vs % specific residual binding B/Bo) was performed by using the Allfit program, which calculates lower and upper plateau, slope, and antagonist IC$_{50}$.[50]

[^{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.[51] Drug competition experiments were carried out 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 12500g. The supernatant was aspirated and the radioactivity associated to the pellet measured. The nonspecific 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.[51] Protein concentration was determined according to Bradford, using bovine serum albumin (BSA) as standard.[52]

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 labeled ligand and inhibitors was carried out in a microtiter 96-well filter plate (Multiscreen, Millipore Inc., Bedford, MA) 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−CCK-8 (25 pM), in a final volume of 250 μL. The nonspecific 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 labeled 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 (Canberra Packard) with 85% efficiency.

Functional Studies. *Isolated Muscular Strips of Guinea Pig Gallbladder Stimulated by CCK-8.* The assay was based on the method described by Bishop et al.[53] Male Hartley guinea pigs weighing 400−450 g (Charles River, Calco, Italy) were sacrificed by cervical dislocation and exsanguination after slight ethereal anesthesia. The abdomen was opened, and the gallbladder was removed and transferred to a Petri dish containing gassed Krebs−Henseleit solution of the following composition (mM): NaCl 118.9, KCl 4.69, $CaCl_2.2H_2O$ 3.33, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1. The neck and the fundus of the gallbladder was cut away; then the tissue was cut transversally, obtaining two rings, each about 3 mm high. From these rings two strips of about 3 mm × 15 mm were obtained, containing mainly the circular smooth muscle fibres. The strips were suspended with silk thread in 20 mL organ baths containing Krebs−Henseleit solution, maintained at 32 °C, bubbled with 95% O_2−5% CO_2. Contractions were measured with an isometric transducer (model 7003; Basile U, Comerio, Italy). A resting tension of 0.5 g was applied. The isometric contraction was monitored using a pen recorder (model 7070; Basile U, Comerio, Italy). The strips were washed with fresh buffer every 15 min over a 60 min equilibration period.

Submaximal-effect (70−80%) concentration of CCK-8 was chosen to test the inhibitory effects of the substances under study. Various concentrations of the antagonists or vehicles were added and allowed 5 min contact with the tissues before adding the agonist to the bathing fluid. The antagonist activity was expressed as percentage of inhibition of the agonist contractions. The regression line was calculated and the concentration effective in inhibiting by 50% the effect of the agonist (IC_{50}) were obtained from the regression line by using Allfit program.

In Vivo Guinea Pig Gallbladder Contractions Induced by CCK-8. The assay was based on the method described by Chang and Lotti.[54] Male Hartley guinea pigs weighting 400− 450 g (Charles River, Calco, Italy) fasted for 48 h were anesthetized with urethane 1.5 g/kg ip (6 mL/kg of 25% w/v solution in NaCl 0.9%). The external jugular vein was exposed and cannulated with a PE 50 polyethylene tubing, filled with saline for iv drug administration. The trachea was exposed and cannulated with a PE 240 polyethylene tubing to facilitate breathing. Then 10 ng/kg (0.0087 nmol/kg) of CCK-8 was administered iv to induce gallbladder emptying. A midline incision 2 cm long was made in the superior part of the abdomen, the gallbladder was gently separated from the adhering liver tissue and was lifted cautiously, and the fundus was tied with a silk thread. The thread was passed in a caudal direction at about 45° over a freely movable pulley and was connected to an isotonic transducer (model 7006; Basile U, Comerio, Italy). To minimize the possible interference of the respiratory movements, the abdomen was kept open using an adjustable diverter; the peritoneal cavity and the gallbladder were covered with paraffin oil. A resting tension of 1 g was applied and the gallbladder contractions were recorded using a pen recorder (model Unirecord 7050; Basile U, Comerio, Italy). An equilibration period of 15 min was allowed before the administration of the substances. CCK-8 10 ng/kg caused contractions that developed slowly and reached their peak after 2 min. The relaxation was slowed, and a 30 min delay period was allowed to recover the basal tension. CCK-8 was administered repeatedly until reproducible contractions were obtained.

The substances under study were administered iv at different doses 2 min before CCK-8 in order to test their capacity to antagonize CCK-8 induced contractions. The antagonizing effects were expressed as percentage of reduction of the area of the CCK-8 contractions. The percentages of inhibition were plotted against the logarithm of the antagonists doses. The regression line was calculated and the concentration effective in inhibiting by 50% the effect of the agonist (IC_{50}) were obtained from the regression line by using Allfit program.

Site-Directed Mutagenesis and Transfection of COS-7 Cells. All mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Quick-Change site-directed mutagenesis kit, Stratagene, France) using the human CCK_1R cDNAs cloned into pRFENeo vector as template. The presence of the desired and the absence of undesired mutations were confirmed by automated sequencing of both cDNA strands (Applied Biosystem).

COS-7 cells (1.5×10^6) were plated onto 10 cm culture dishes and grown in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum (complete medium) in a 5% CO_2 atmosphere at 37 °C. After overnight incubation, cells were transfected with 2 μg/plate of pRFE-Neo vectors containing the cDNA for the wild-type or mutated CCK receptors, using a modified DEAE-dextran method. Cells were transferred to 24-well plates at a density of 20000−80000 cells/well 24 h after transfection, depending on transfected mutant and experiment to be performed.

Receptor Binding Assay. Approximately 24 h after the transfer of transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline pH 6.95, 0.1% BSA, and then incubated for 60 min at 37 °C in 0.5 mL of Dublecco's Modified Eagle's Medium, 0.1% BSA with either 71 pM ^{125}I-BH-(Thr, Nle)CCK-9,[55] or 1.83 nM [^{3}H]SR-27,897 (tritiated 1-[2-(4-(2-chlorophenyl)thiazol-2-yl)aminocarbonyl-indoyl]acetic acid, specific activity 31 Ci/mmol, Sanofi-Synthelabo, Toulouse, France) in the presence or in the absence of competing agonists or antagonists. The cells were washed twice with cold phosphate-buffered saline pH 6.95 containing 2% BSA, and cell associated radioligand was collected with NaOH 0.1N added to each well. The radioactivity was directly counted in a γ counter (Auto-Gamma, Packard, Downers Grove, IL) or added to scintillant and counted for the tritiated radioligand.

Inositol Phosphate Assay. Approximately 24 h after the transfer to 24-well plates and following overnight incubation in complete medium containing 2 μCi/ml of myo-2-[^{3}H]inositol (Amersham biosciences, Les Ulis, France), the transfected cells were washed with Dubecco's Modified Eagle's Medium and then incubated for 30 min in 1 mL/well Dubecco's Modified Eagle's Medium containing 20 mM LiCl at 37 °C. The cells were washed with PI buffer at pH 7.45: phosphate-buffered saline containing 135 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA. The cells were then incubated for 60 min at 37 °C in 0.3 mL of PI buffer with or without ligands at various concentrations. The reaction was stopped by adding 1 mL of methanol/hydrochloric acid to each well, and the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad, Hercules, CA) for the determination of inositol phosphates. The columns were washed twice with 3 mL of distilled water and twice more with 2 mL of 5 mM sodium tetraborate/60 mM sodium formate. The content of each column was eluted by addition of 2.5 mL of 1 M ammonium formate/100 mM formic acid. Then 0.5 mL of the eluted fraction was added to scintillant and β radioactivity was counted.

■ ASSOCIATED CONTENT

Supporting Information. Elemental analysis results for compounds **1**−**11** and enantiomeric excess determinations for compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CCK, cholecystokinin; CCK_1-R, cholecystokinin receptor 1; CCK_2-R, cholecystokinin receptor 2; GI, gastrointestinal; CNS, central nervous system; SAfR, structure−affinity relationship; SAR, structure−activity relationship; ER, eudismic ratio; CSP, chiral stationary phase; HPLC, high performance liquid chromatography; RP-eHPLC, reverse phase enantioselective HPLC; CD, circular dichroism; ELSD, evaporative light scattering detector; SI, selectivity indices; MD, molecular dynamics; WT, wild type

■ REFERENCES

(1) Jensen, R. T.; Wank, S. A.; Rowley, W. H.; Sato, S.; Gardner, J. D. Interaction of CCK with pancreatic acinar cells. *Trends Pharmacol. Sci.* **1989**, *10*, 418–423.

(2) Liddle, R. A.; Gertz, B. J.; Kanayama, S.; Beccaria, L.; Coker, L. D.; Turnbull, T. A.; Morita, E. T. Effects of a novel cholecystokinin (CCK) receptor antagonist, MK-329, on gallbladder contraction and gastric emptying in humans. Implications for the physiology of CCK. *J. Clin. Invest.* **1989**, *84*, 1220–1225.

(3) Brawman-Mintzer, O.; Lydiard, R. B.; Bradwejn, J.; Villarreal, G.; Knapp, R.; Emmanuel, N.; Ware, M. R.; He, Q.; Ballenger, J. C. Effects of the cholecystokinin agonist pentagastrin in patients with generalized anxiety disorder. *Am. J. Psychiatry* **1997**, *154*, 700–702.

(4) Lofberg, C.; Agren, H.; Harro, J.; Oreland, L. Cholecystokinin in CSF from depressed patients: possible relation to severity of depression and suicidal behaviour. *Eur. Neuropsychopharmacol.* **1998**, *8*, 153–157.

(5) Dourish, C. T.; Hill, D. R. Classification and function of CCK receptors. Comments. *Trends Pharmacol. Sci.* **1987**, *8*, 207–208.

(6) Pisegna, J. R.; de Weerth, A.; Huppi, K.; Wank, S. A. Molecular cloning, functional expression, and chromosomal localization of the human cholecystokinin type A receptor. *Ann. N.Y. Acad. Sci.* **1994**, *713*, 338–342.

(7) Lee, Y. M.; Beinborn, M.; McBride, E. W.; Lu, M.; Kolakowski, L. F., Jr.; Kopin, A. S. The human brain cholecystokinin-B/gastrin receptor. Cloning and characterization. *J. Biol. Chem.* **1993**, *268*, 8164–8169.

(8) Dufresne, M.; Seva, C.; Fourmy, D. Cholecystokinin and gastrin receptors. *Physiol. Rev.* **2006**, *86*, 805–847.

(9) Makovec, F.; Bani, M.; Chistè, R.; Revel, L.; Rovati, L. C.; Rovati, L. A. Differentiation of central and peripheral cholecystokinin receptors by new glutaramic acid derivatives with cholecystokinin-antagonistic activity. *Arzneim. Forsch.: Drug Res.* **1986**, *36*, 98–102.

(10) de Tullio, P.; Delarge, J.; Pirotte, B. Recent advances in the chemistry of cholecystokinin receptor ligands (agonists and antagonists). *Curr. Med. Chem.* **1999**, *6*, 433–455.

(11) Revel, L.; Makovec, F. Update on Nonpeptide CCK-B Receptor Antagonists. *Drugs Future* **1998**, *23*, 751–766.

(12) Herranz, R. Cholecystokinin antagonists: pharmacological and therapeutic potential. *Med. Res. Rev.* **2003**, *23*, 559–605.

(13) Lassiani, L.; Varnavas, A. Twenty years of non-peptide CCK_1 receptor antagonists: All that glitters is not gold. *Expert Opin. Ther. Patents* **2006**, *16*, 1193–1213.

(14) García-López, M. T.; González-Muñiz, R.; Martín-Martínez, M.; Herranz, R. Strategies for Design of Non Peptide CCK1R Agonist/Antagonist Ligands. *Curr. Top. Med. Chem.* **2007**, *7*, 1180–1194.

(15) Kalindjian, S. B.; McDonald, I. M. Strategies for the Design of Non-peptide CCK2 Receptor Agonist and Antagonist Ligands. *Curr. Top. Med. Chem.* **2007**, *7*, 1195–1204.

(16) Varnavas, A.; Lassiani, L.; Valenta, V.; Berti, F.; Mennuni, L.; Makovec, F. Anthranilic acid derivatives: a new class of non-peptide CCK_1 receptor antagonists. *Bioorg. Med. Chem.* **2003**, *11*, 741–751.

(17) Varnavas, A.; Lassiani, L.; Valenta, V.; Berti, F.; Tontini, A.; Mennuni, L.; Makovec, F. Anthranilic acid based CCK1 antagonists: the 2-indole moiety may represent a "needle" according to the recent homonymous concept. *Eur. J. Med. Chem.* **2004**, *39*, 85–97.

(18) Varnavas, A.; Lassiani, L.; Valenta, V.; Ciogli, A.; Gasparrini, F.; Mennuni, L.; Makovec, F. N-Terminal Anthranoyl-Phenylalanine Derivatives as CCK_1 Receptor Antagonists: The Final approach. *Med. Chem.* **2005**, *1*, 501–517.

(19) Hadjipavlou-Litina, D.; Braiuca, P.; Lassiani, L.; Pavan, M. V.; Varnavas, A. 2D-QSAR and 3D-QSAR/CoMFA analyses of the N-terminal substituted anthranilic acid based CCK1 receptor antagonists: "Hic Rhodus, hic saltus". *Bioorg. Med. Chem.* **2009**, *17*, 5198–5206.

(20) Varnavas, A.; Lassiani, l.; Valenta, V.; Mennuni, L.; Makovec, F.; Hadjipavlou-Litina, D. Anthranilic Acid Based CCK_1 Receptor Antagonists: Preliminary Investigation On Their Second "Touch point". *Eur. J. Med. Chem.* **2005**, *40*, 563–581.

(21) Lassiani, L.; Pavan, M. V.; Berti, F.; Kokotos, G.; Markidis, T.; Mennuni, L.; Makovec, F.; Varnavas, A. Anthranilic acid based CCK1 receptor antagonists: blocking the receptor with the same "words" of the endogenous ligand. *Bioorg. Med. Chem.* **2009**, *17*, 2336–2350.

(22) D'Acquarica, I.; Gasparrini, F.; Misiti, D.; Villani, C.; Carrotti, A.; Cellamare, S.; Muck, S. Direct chromatographic resolution of carnitine and *O*-acylcarnitine enantiomers on a teicoplanin-bonded chiral stationary phase. *J. Chromatogr., A* **1999**, *857*, 145–155.

(23) Armstrong, D. W.; Berthod, A.; Chen, X.; Kullman, J. P.; Gasparrini, F.; D'Acquarica, I.; Villani, C.; Carotti, A. Role of the carbohydrate moieties in chiral recognition on teicoplanin-based LC stationary phases. *Anal. Chem.* **2000**, *72*, 1767–1780.

(24) Makovec, F.; Peris, W.; Revel, L.; Giovanetti, R.; Mennuni, L.; Rovati, L. C. Structure antigastrin activity relationships of new (*R*)-4-benzamido-5-oxopentanoic acid-derivatives. *J. Med. Chem.* **1992**, *35*, 28–38.

(25) Kennedy, K.; Escrieut, C.; Dufresne, M.; Clerc, P.; Vaysse, N.; Fourmy, D. Identification of a region of the N-terminal of the human CCKA receptor essential for the high affinity interaction with agonist CCK. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 845–852.

(26) Saillan-Barreau, C.; Clerc, P.; Adato, M.; Escrieut, C.; Vaysse, N.; Fourmy, D.; Dufresne, M. Transgenic CCK-B/gastrin receptor mediates murine exocrine pancreatic secretion. *Gastroenterology* **1998**, *115*, 988–996.

(27) Remko, M. The gas-phase acidities of substituted hydroxamic and silahydroxamic acids: a comparative ab initio study. *J. Phys. Chem. A* **2002**, *106*, 5005–5010.

(28) Garcia, B.; Ibeas, S.; Leal, J. M.; Secco, F.; Venturini, M.; Senen, M. L.; Niño, A.; Muñoz, C. Conformations, protonation sites, and metal complexation of benzohydroxamic acid. A theoretical and experimental study. *Inorg. Chem.* **2005**, *44*, 2908–2919.

(29) (a) Archer-Lahlou, E.; Tikhonova, I.; Escrieut, C.; Dufresne, M.; Seva, C.; Pradayrol, L.; Moroder, L.; Maigret, B.; Fourmy, D. Modeled Structure of a G-Protein-Coupled Receptor: The Cholecystokinin-1Receptor. *J. Med. Chem.* **2005**, *48*, 180–19. (b) Martín-Martínez, M.; Marty, A.; Jourdan, M.; Escrieut, C.; Archer, E.; González-Muñiz, R.; García-López, M. T.; Maigret, B.; Herranz, R.; Fourmy, D. Combination of Molecular Modeling, Site-Directed Mutagenesis, and SAR Studies To Delineate the Binding Site of Pyridopyrimidine Antagonists on the Human CCK1 Receptor. *J. Med. Chem.* **2005**, *48*, 4842–4850.

(30) Ripa, L.; Halberg, A. Aryl Radical Endo Cyclization of Enamidines. Selective Preparation of trans and cis Fused Octahydrobenzo-[*f*]quinolines. *J. Org. Chem.* **1998**, *63*, 84–91.

(31) Denninson, P. R.; Gibson, A.; Gray, A.; Patrick, G. L. Cyclisation studies of 5-arylalkyl-1,2,3,4,5,6,7,8-octahydroisoquinolines. *J. Chem. Soc., Perkin Trans 1* **1997**, *5*, 721–728.

(32) Andrako, J.; Smith, J. D.; Hartung, W. H. Substituted acetamidomalonic esters and DL-2-acetamido acids as antitumor agents. *J. Pharm. Sci.* **1961**, *50*, 337–340.

(33) Amblard, M.; Rodriguez, M.; Lignon, M. F.; Galas, M. C.; Bernard, N.; Artis-Noel, A. M.; Hauad, L.; Laur, J.; Califano, J. C.; Aumelas, A.; Martinez, J. Synthesis and biological evaluation of cholecystokinin analogs in which the Asp-Phe-NH2 moiety has been replaced by a 3-amino-7-phenylheptanoic acid or a 3-amino-6-(phenyloxy)-hexanoic acid. *J. Med. Chem.* **1993**, *36*, 3021–3028.

(34) Lednicer, D.; Emmert, P. E. Synthesis and Antifertility Activity of 4- and 5-(omega-arylalkyl) oxazolidinethiones. *J. Med. Chem.* **1968**, *11*, 1258–1262.

(35) Kametani, T.; Kigasawa, K.; Hiiragi, M.; Wakisaka, K.; Haga, S.; Sugi, H.; Tanigawa, K.; Suzuki, Y.; Fukawa, K.; Irino, O.; Saita, O.; Yamabe, S. Studies on the synthesis of chemotherapeutics. Part XI. Synthesis and antibacterial activities of phosphonopeptides. *Heterocycles* **1981**, *16*, 1205–1242.

(36) Tanaka, A.; Izumiya, N. Resolution of amino acids. I. Resolution of racemic phenylalanine and γ-phenyl-α-aminobutyric acid by leucine aminopeptidase. *Bull. Chem. Soc. Jpn.* **1958**, *31*, 529–530.

(37) Han, S. Y.; Kim, Y. A. Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron* **2004**, *60*, 2447–2467.

(38) Mac Pherson, L. J.; Bayburt, E. K.; Capparelli, M. P.; Carroll, B. J.; Goldstein, R.; Justice, M. R.; Zhu, L.; Hu, S.; Melton, R. A.; Fryer, L.; Goldberg, R. L.; Doughty, J. R.; Spirito, S.; Blancuzzi, V.; Wilson, D.; O'Byrne, E. M.; Ganu, V.; Parker, D. T. Discovery of CGS 27023A, a Non-Peptidic, Potent, and Orally Active Stromelysin Inhibitor That Blocks Cartilage Degradation in Rabbits. *J. Med. Chem.* **1997**, *40*, 2525–2532.

(39) Wang, W.; McMurray, J. S. A Selective Method for the Preparation of Primary Amides: Synthesis of Fmoc-L-4-Carboxamidophenylalanine and other Compounds. *Tetrahedron Lett.* **1999**, *40*, 2501–2504.

(40) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A.; , Jr., Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; M. A. Al-Laham, Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; , Pople, J. A. *Gaussian 03, revision C.02*; Gaussian, Inc., Wallingford, CT, 2004.

(41) Dewar, M. S. J.; Zoebish, E. J.; Healy, E. F.; Stewart, J. J. P. AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* **1985**, *107*, 3902–3909.

(42) Svensson, L.; Humbel, S.; Froese, R. D. J.; Matsubara, T.; Sieber, S.; Morokuma, K. ONIOM: A multilayered integrated MO+MM method for geometry optimizations and single point energy predictions. A test for Diels–Alder reactions and Pt(P(*t*-Bu)(3))(2)+H-2 oxidative addition. *J. Phys. Chem.* **1996**, *100*, 19357–19363.

(43) Becke, A. D. Density-functional exchange-energy approximation with correct asymptotic behavior. *Phys. Rev. A: At., Mol., Opt. Phys.* **1988**, *A38*, 3098–3100.

(44) Becke, A. D. Density-functional thermochemistry 0.3. The role of exact exchange. *J. Chem. Phys.* **1993**, *98*, 5648–5652.

(45) Yang, L. W.; Parr, R. G. Development of the Colle–Salvetti correlation-energy formula into a functional of the electron-density. *Phys. Rev. B: Condens. Matter Mater. Phys.* **1988**, *B37*, 785–789.

(46) Clark, M.; Cramer, R. D.; Van Optenbosch, N. Validation of the general-purpose tripos 5.2 force-field. *J. Comput. Chem.* **1989**, *10*, 982–1012.

(47) Sadowski, J.; Schwab, C.; Gasteiger, J. *Corina 3.1*; Molecular Networks GmbH Computerchemie: Erlangen, Germany, 2008.

(48) Bayly, C. I.; Black, W. C.; Leger, S.; Ouimet, N.; Ouellet, M.; Percival, M. D. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 307–312.

(49) Marco, E.; Foucaud, M.; Langer, I.; Escrieut, C.; Tikhonova, I. G.; Fourmy, D. *J. Biol. Chem.* **2007**, *282*, 28779–28790.

(50) De Lean, A.; Munson, P. J.; Rodbard, D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **1978**, *235*, E97–E102.

(51) Makovec, F.; Peris, W.; Revel, L.; Giovanetti, R.; Mennuni, L.; Rovati, L. C. Structure–antigastrin activity relationships of new (*R*)-4-benzamido-5-oxopentanoic acid derivatives. *J. Med. Chem.* **1992**, *35*, 28–38.

(52) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(53) Bishop, L. A.; Gerskowitch, V. P.; Hull, R. A.; Shankley, N. P.; Black, J. W. Combined dose-ratio analysis of cholecystokinin receptor antagonists, devazepide, lorglumide and loxiglumide in the guinea-pig gall bladder. *Br. J. Pharmacol.* **1992**, 61–66.

(54) Chang, R. S. L.; Lotti, V. J. Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4923–4926.

(55) Fourmy, D.; Lopez, P.; Poirot, S.; Jimenez, J.; Dufresne, M.; Moroder, L.; Powers, S. P.; Vaysse, N. A new probe for affinity labeling pancreatic cholecystokinin receptor with minor modification of its structure. *Eur. J. Biochem.* **1989**, *185*, 397–403.