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Synthesis of N-terminal substituted anthranilic acid dimer derivatives for evaluation on CCK receptors

Antonio Varnavas [a,*], Valentina Valenta [a], Federico Berti [b], Lucia Lassiani [a]

[a] *Department of Pharmaceutical Sciences, University of Trieste, P.le Europa 1, 34127 Trieste, Italy*
[b] *Department of Chemical Sciences, University of Trieste, Via Giorgieri 1, 34127 Trieste, Italy*

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Abstract

A series of new N-substituted anthranilic acid dimer derivatives having a C-terminal Phe residue was synthesized and evaluated for their affinity for CCK receptors. These compounds resulted from a blended approach based firstly on the use of an alternative substructure embedded within asperlicin and secondly on the derivatization of this template with substituents chosen considering the C-terminal primary structure of the endogenous ligand. Although these compounds exhibited a regnylogical-type organization similar to that of CCK-4, they are characterized by about 1000-fold greater affinity for CCK-A receptor than the C-terminal tetrapeptide.

Keywords: CCK receptor ligands; Anthranilic acid dimer

1. Introduction

The polypeptide hormone cholecystokinin (CCK) was discovered in the gastrointestinal tract and subsequently in the central nervous system (CNS) [1,2]. Among the different biologically active molecular forms of CCK (CCK-58, CCK-39, CCK-33, CCK-8, CCK-4), the sulfated C-terminal octapeptide (CCK-8S), Asp^{26}-$Tyr(SO_3H)^{27}$ - Met^{28} - Gly^{29} - Trp^{30} - Met^{31} - Asp^{32} - Phe^{33}-NH_2, has the same efficacy and potency as the complete tritriacontapeptide (CCK-33) [3].

CCK is structurally related to gastrin, a gastrointestinal hormone, that acts as mediator of the gastric acid secretion. These two peptides share an identical C-terminal pentapeptide (Gly-Trp-Met-Asp-Phe-NH_2; pentagastrin) which is responsible for all the biological activities of gastrin [4].

The hormone-like effects of CCK related to the gastrointestinal functions (such as stimulation of gall bladder contraction, stimulation of pancreatic enzyme secretion) as well as its CNS actions as neurotransmitter and neuromodulator are mediated by two distinct receptor types, CCK-A and CCK-B [5,6]. Both receptors are present in the periphery and in the CNS; CCK-A receptors are found mostly in the gastrointestinal area, while CCK-B receptors are the predominant type in the brain [7,8].

These receptor types can be differentiated by their affinities for CCK-4, CCK-8S, and several receptor preferring antagonists. The C-terminal tetrapeptide (CCK-4) preferentially interacts with the central receptor ($IC_{50} = 3.2 \times 10^{-8}$ and 5.0×10^{-3} M for CCK-B and CCK-A receptors, respectively) while the sulfated octapeptide (CCK-8S) binds with nanomolar affinities at both receptors [9].

Over the past decade potent and selective non-peptide antagonists for CCK-A and CCK-B receptors have been discovered and suggested to be useful for the treatment of a variety of diseases such as irritable bowel syndrome (IBS), chronic and acute pancreatitis, gastric ulcer, anxiety, panic disorder, schizophrenia, eating disorders [10–12].

The most important strategies adopted for the design of these antagonists include the discovery of natural products followed by their chemical manipulation and the peptoid design approach based on studies of CCK primary structure. Both strategies include a final step of

* Correspondence author.
E-mail address: varnavas@univ.trieste.it (A. Varnavas).

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optimization of the obtained non-peptide lead compound performed by chemical modifications.

Thus, the discovery of asperlicin (a natural compound isolated from *Aspergillus alliaceus*) and its structure simplification provided two different classes of CCK-receptor antagonists. The first one, based on the 1,4-benzodiazepine ring system of asperlicin, include the most important non-peptide CCK receptor antagonists since devazepide [3*S*-(−)-1,3-dihydro-3-(2-indolecarbonyl-amino)-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one] and L-365,260 [(3*R*-(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl)-urea] were the first examples of highly potent, selective, non-peptide antagonists for the CCK-A and CCK-B receptors, respectively [13,14]. The second one, based on quinazolinone nucleus of asperlicin, represent a class of potent and selective CCK-B receptor antagonists [15,16].

On the other hand, the peptoid derivatives resulted from a strategy based on simplifying the C-terminal tetrapeptide to the minimal structure with a significant affinity for the receptor. These studies having established that Trp and Phe residues of CCK-4 were both necessary to obtain micromolar affinity for the CCK-B receptors, the dipeptide Boc-Trp-Phe-NH_2 was the starting point for the design of the dipeptoid series. For this dipeptide (Boc-Trp-Phe-NH_2) a sycnological interaction at the receptor has been suggested, while in the case of CCK-4 the Trp-Phe-message is non-continuous (regnylogic-type organization) [17].

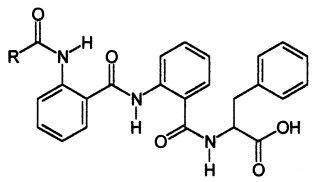
As a result of a systematic SAR of the N- and C-terminal sites of the starting dipeptide, characterized by micromolar affinity for CCK-B receptor ($K_i = 7.3 \pm 0.5 \times 10^{-5}$ M [18]), a number of dipeptoids with high affinity (nanomolar range) and selectivity for CCK-B receptor were obtained [19] and further structural modifications led to potent and selective CCK-A receptor antagonists [20].

Recently, we have proposed a new disconnection approach of asperlicin in which the rigid polycyclic system of this natural compound has been opened to give the flexible anthranilic acid dimer and Trp. In the first part of our program we demonstrated that the presence of Trp residue at the N- or C-terminus of anthranilic acid dimer led to equipotent compounds with micromolar affinity for CCK-A receptors [21]. In the subsequent step for the development of this new class of non-peptide CCK-A receptor ligands our attention was focused on the independent searching of the appropriate pharmacophoric groups for the functionalization of the N- and C-terminus of this asperlicin-derived substructure [22]. In particular, we have found that when the Trp residue is linked to the C-terminus of the anthranilic acid dimer the highest CCK-A receptor affinity was achieved with the des-amino Trp at the N-terminus of the above mentioned dimer [23].

In this paper we wish to report a novel series of N-terminal substituted anthranilic acid dimer derivatives containing, as in the case of the endogenous ligands, the Phe residue at the C-terminus of the dimer (Table 1).

Our choice was supported by the observation that compounds with similar micromolar affinity for the CCK-A receptors were obtained either in the presence at the C-terminal site of the anthranilic acid dimer, by itself inactive, of Trp — suggested by the disconnection approach of asperlicin — or Phe, a key aminoacid of the C-terminal sequence of CCK [21].

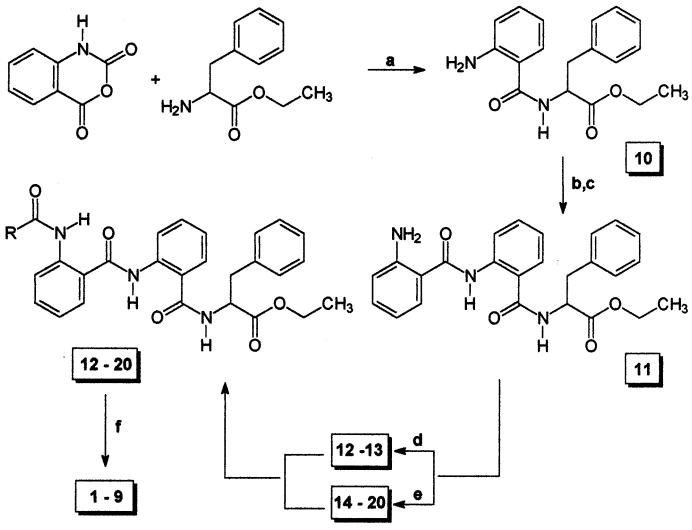
Table 1
Structure of the target compounds



Comp.	R
1	3-indolyl–$(CH_2)_2$–
2	3-indolyl–CH_2–
3	2-indolyl
4	phenyl
5	3-methylphenyl
6	benzyl
7	4-chlorobenzyl
8	phenylethenyl-
9	HOOC–$(CH_2)_2$–

2. Chemistry

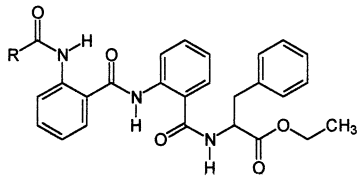
The target compounds summarized in Table 1 were synthesized by standard procedures according to the synthetic route depicted in Scheme 1. Construction of the key intermediate (compound **11**) was effected via *ortho*-amino benzoylation of the DL-phenylalanine ethyl ester with isatoic anhydride, to afford compound **10**, followed by 2-nitro benzoylation and subsequent reduction of the nitro group. Derivatives **12** and **13** were synthesized by coupling the intermediate **11** with the corresponding acid by the mixed-anhydride method with isobutyl chloroformate and triethylamine, whereas compounds **14**–**20** were obtained by condensation of compound **11** with the corresponding acyl chloride. Synthetic data of the esters **12**–**20** are listed in Tables 2–4. The free acids **1**–**9** were obtained in almost quantitative yield by base-catalyzed hydrolysis of the corresponding ethyl esters **12**–**20**; Tables 5–7 give the physical and spectroscopic properties of compounds **1**–**9**.



a) AcOEt, Et_3N; b) 2-nitrobenzoyl chloride, CH_2Cl_2, Et_3N;
c) Zn, AcOH, CH_2Cl_2; d) RCOOH, *isobutyl* chloroformate, CH_2Cl_2, Et_3N;
e) RCOCl, CH_2Cl_2, Et_3N; f) KOH, MeOH.

Scheme 1.

Table 2
Physicochemical properties of compounds **12**–**20**



Comp.	R	Molecular formula	Molecular weight	Yield (%)	Cryst. solvent [a]	R_f [b]	M.p. (°C)
12	3-indolyl–$(CH_2)_2$–	$C_{36}H_{34}N_4O_7$	602	70	A	0.51	116–117
13	3-indolyl–CH_2–	$C_{35}H_{32}N_4O_5$	588	58	MeOH	0.54	166–167
14	2-indolyl	$C_{34}H_{30}N_4O_5$	574	57	EtOH	0.69	208–210
15	phenyl	$C_{32}H_{29}N_3O_5$	535	56	MeOH	0.65	145–147
16	3-methylphenyl	$C_{33}H_{31}N_3O_5$	549	48	EtOH	0.60	123–125
17	benzyl	$C_{33}H_{31}N_3O_5$	549	68	B	0.65	86–87
18	4-chlorobenzyl	$C_{33}H_{30}ClN_3O_5$	583.5	60	A	0.80	129–130
19	phenylethenyl-	$C_{34}H_{31}N_3O_5$	561	66	EtOH	0.58	177–178
20	H_5C_2OOC–$(CH_2)_2$–	$C_{31}H_{33}N_3O_7$	559	68	MeOH	0.66	116–117

[a] Crystallizing solvents: A, AcOEt–hexane; B, EtOH 70%.
[b] AcOEt–hexane, 1:1.

3. Results and discussion

The affinities of the synthesized compounds for the CCK-A and CCK-B receptors were determined, using rat pancreatic membranes and guinea pig brain membranes, respectively, by displacement of [^{3}H]-(±)-L-364,718 and [^{3}H]-(+)-L-365,260 from their specific binding sites, as previously described [24,25].

First, the percentage of inhibition (*I*, %) was determined at the highest dose of 10 μM and when the *I* values were lower than 20%, the compounds were considered inactive. For the more active ones, the IC_{50}

values were then calculated from five-point inhibition curves by log-probit plots and the reported values are the geometric means of at least three separate experiments.

The CCK receptor binding data of N-substituted anthranilic acid dimer derivatives (compounds **1**–**9)** are summarized in Table 8.

The present results indicate that indole-containing derivatives, i.e. compounds **1**–**3**, are the most potent and selective within this series since they inhibit the [^{3}H]-(±)-L-364,718 binding to rat pancreatic membranes at micromolar concentrations and have little or no affinity for CCK-B receptor. The best result was obtained with compound **1** which has an IC_{50} of 2.3×10^{-6} M, a value about tenfold lower than that of the N-unsubstituted parent compound (compound **0**) chosen as reference.

On the contrary, all the phenyl derivatives, compounds **4**–**8**, exhibit a lower and similar affinity for both receptors thus indicating a lack of selectivity. Moreover, the structures considered — in terms of phenyl ring substitution and/or distance from the anthranilic acid dimer template — do not have a substantial effect on binding affinity.

Table 3
^{1}H NMR ($CDCl_3$) of compounds **12**–**20** [a]

Comp.	δ (ppm)
12	1.25 (t, 3H, –CH_3); 2.88 (m, 2H, –CH_2–ind); 3.25 (m, 4H, –CH_2–, >CH–CH_2–); 4.25 (q, 2H, –O–CH_2–); 5.06 (m, 1H, –CH<); 6.75 (d, 1H, –N*H*–CH<); 7.05–8.72 (m, 18H, ar); 7.96 (s, 1H, –NH–ind); 11.25 (s, 1H, –NH–); 11.85 (s, 1H, –NH–)
13	1.28 (t, 3H, –CH_3); 3.22 (m, 2H, –CH_2–CH<); 3.92 (s, 2H, –CH_2–CO–); 4.23 (q, 2H, –O–CH_2–); 5.02 (m, 1H, –CH<); 6.69 (d, 1H, –N*H*–CH<); 7.03–8.69 (m, 18H, ar); 8.26 (s, 1H, –NH–ind); 11.15 (s, 1H, –NH–); 11.65 (s, 1H, –NH–)
14	1.26 (t, 3H, –CH_3); 3.25 (m, 2H, >CH–CH_2–); 4.20 (q, 2H, –O–CH_2–); 5.05 (m, 1H, –CH<); 6.75 (d, 1H, –N*H*–CH<); 7.10–8.85 (m, 18H, ar); 9.23 (s, 1H, –NH–ind); 12.05 (s, 1H, –NH–); 12.50 (s, 1H, –NH–)
15	1.29 (t, 3H, –CH_3); 3.25 (m, 2H, >CH–CH_2–); 4.20 (q, 2H, –O–CH_2–); 5.05 (m, 1H, –CH<); 6.70 (d, 1H, –N*H*–CH<); 7.10–8.90 (m, 18H, ar); 12.00 (s, 1H, –NH–); 12.30 (s, 1H, –NH–)
16	1.27 (t, 3H, –CH_2–CH_3); 2.45 (s, 3H, –CH_3); 3.22 (m, 2H, >CH–CH_2–); 4.20 (q, 2H, –O–CH_2–); 5.05 (m, 1H, >CH–); 6.73 (d, 1H, –N*H*–CH<); 7.10–8.90 (m, 17H, ar); 11.98 (s, 1H, –NH–); 12.23 (s, 1H, –NH–)
17	1.2 (t, 3H, –CH_3); 3.2 (m, 2H, –CH_2–); 3.7 (s, 2H, –CH_2–); 4.2 (q, 2H, –O–CH_2–); 5.0 (m, 1H, >CH–); 6.7 (d, 1H, –NH–); 7.0–8.8 (m, 18H, ar); 11.25 (s, 1H, –NH–); 11.8 (s, 1H, –NH–)
18	1.28 (t, 3H, –CH_3); 3.25 (m, 2H, >CH–CH_2–); 3.72 (s, 2H, –CH_2–CO–); 4.22 (q, 2H, –O–CH_2–); 5.02 (m, 1H, >CH–); 6.70 (d, 1H, –N*H*–CH<); 7.10–8.65 (m, 17H, ar); 11.30 (s, 1H, –NH–); 11.90 (s, 1H, –NH–)
19	1.26 (t, 3H, –CH_3); 3.25 (m, 2H, –CH_2–CH<); 4.20 (q, 2H, –O–CH_2–); 5.05 (m, 1H, –CH<); 6.60 (d, 1H, –CO–CH=) (J = 15.5 Hz); 6.70 (d, 1H, –N*H*–CH<); 7.10–8.85 (m, 19H, ar and =C*H*–Ph); 11.60 (s, 1H, –NH–); 11.95 (s, 1H, –NH–)
20	1.25 (2×t, 6H, 2×–CH_3); 2.75 (s, 4H, 2×–CH_2–); 3.2 (m, 2H, –CH_2–); 4.2 (2×q, 4H, 2×–O–CH_2–); 5.0 (m, 1H, >CH–); 6.75 (d, 1H, –NH–); 7.0–8.8 (m, 13H, ar); 11.3 (s, 1H, –NH–); 11.9 (s, 1H, –NH–)

[a] Abbreviations: ar, aromatic; ind, indole; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Table 4
^{13}C NMR ($CDCl_3$) of compounds **12**–**20**

Comp.	δ (ppm)
12	8.79, 15.72, 32.56, 33.62, 48.15, 56.58, 105.66, 109.45, 113.40, 113.83, 115.03, 116.13, 116.51, 117.69, 118.16, 121.41, 121.81, 121.96, 123.26, 123.93, 127.58, 130.15, 130.99, 133.66, 134.91, 162.16, 162.98, 165.86, 166.24
13	14.19, 35.36, 37.99, 53.51, 61.95, 108.89, 111.08, 118.94, 119.82, 120.44, 121.20, 121.51, 121.87, 122.24, 123.17, 123.40, 123.93, 126.64, 126.95, 127.35, 127.53, 128.65, 129.34, 132.69, 132.79, 135.55, 139.19, 139.91, 167.09, 168.28, 170.66, 171.25
14	14.25, 38.07, 53.66, 62.03, 103.92, 111.99, 120.09, 120.54, 120.71, 121.31, 122.03, 122.45, 123.29, 123.37, 123.69, 124.77, 126.93, 127.40, 127.54, 127.99, 128.71, 129.42, 131.62, 133.12, 133.25, 135.62, 136.79, 139.35, 140.69, 160.08, 167.83, 168.53, 171.38
15	14.27, 38.05, 53.63, 62.02, 120.55, 120.65, 121.64, 121.97, 123.37, 123.67, 126.89, 127.45, 127.53, 128.71, 128.81, 129.41, 131.85, 133.06, 133.24, 135.09, 135.61, 139.35, 140.84, 165.71, 167.92, 168.43, 171.26
16	14.26, 21.60, 38.04, 53.64, 62.01, 120.52, 120.72, 121.70, 121.90, 123.34, 123.63, 124.33, 126.93, 127.40, 127.48, 128.45, 128.70, 129.40, 132.64, 133.04, 133.19, 135.07, 135.64, 138.61, 139.37, 140.80, 166.12, 167.89, 168.47, 171.11
17	14.27, 38.03, 45.79, 53.62, 62.03, 120.43, 120.79, 121.49, 121.77, 123.36, 126.91, 127.18, 127.23, 127.42, 128.71, 128.89, 129.41, 129.67, 132.95, 133.00, 134.71, 135.64, 139.37, 140.20, 167.47, 168.43, 170.04, 171.33.
18	14.26, 38.03, 45.00, 53.59, 62.03, 120.33, 120.62, 121.36, 121.73, 123.45, 123.63, 126.85, 127.17, 127.43, 128.72, 129.04, 129.40, 131.14, 133.01, 133.20, 135.59, 139.37, 140.16, 167.43, 168.42, 169.47, 171.25
19	14.24, 38.06, 53.59, 62.02, 120.34, 120.72, 121.72, 121.95, 122.32, 123.32, 123.73, 126.91, 127.38, 127.42, 128.14, 128.71, 128.87, 129.40, 129.89, 133.03, 133.20, 134.89, 135.57, 139.30, 140.77, 142.01, 164.45, 167.86, 168.38, 171.23
20	14.16, 29.44, 32.78, 38.02, 53.54, 60.64, 61.93, 120.33, 120.56, 121.50, 121.87, 123.12, 123.58, 126.78, 127.23, 127.35, 128.65, 129.35, 132.98, 135.54, 139.34, 140.35, 167.66, 168.34, 170.15, 171.17, 172.48

Compound **9**, characterized by an aliphatic side chain having a second free carboxyl group, was inactive on both receptors.

It is interesting to note that the present data, with respect to the CCK-A receptor, are closely related to those already observed for the same receptor with anthranilic acid dimer derivatives having a C-terminal Trp residue [23].

Hence, it may be inferred that the C-terminal aminoacid (Phe or Trp) do not significantly modulate the activity since almost equipotent compounds were obtained. Moreover, the indole moiety appears to impart the best CCK-A receptor binding affinity and can therefore be considered the optimum N-terminus group in these series of compounds.

Finally, we have also observed that the most active compound (compound **1**) resembles some structural features of CCK-4 having these two 'tetrapeptides' the same N- and C-terminal aminoacid side chains and differing for the presence of the anthranilic acid dimer instead of the Met-Asp dipeptide, respectively. For this reason, we hypothesize that compound **1** and CCK-4 could have a similar regnylogical-type organization although characterized by quite different affinities for the pancreatic receptor ($IC_{50} = 2.3 \times 10^{-6}$ M for compound **1** versus 5.0×10^{-3} M for CCK-4 [9]).

With an aim to assess this fact we have undertaken a molecular modeling study of compound **1** and CCK-4 structures.

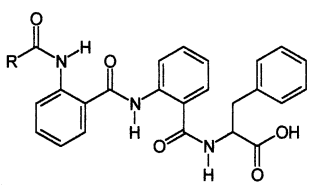
4. Molecular modeling

The geometry of compound **1** depends on the arrangement of the central anthranoyl anthranilic acid moiety: both the oxygens O1 and O2 are hydrogen bonded to the amide protons in the *ortho* position H1 and H2, as can be deduced from the interatomic distances of 1.74 and 1.72 Å, respectively (Fig. 1). The system is not planar, being the plane containing one phenyl ring of the dimer puckered by 42° with respect to the other. The phenylalanine and the indolepropionic groups lie on the same side of the structure with a distance between the indole and the phenyl rings of about 10 Å (Fig. 2).

The low-energy conformation determined for compound **1** appeared to have some common features with that described for CCK-4 [26]. Therefore, a conformational analysis was performed also on CCK-4 in order to compare the geometries of these two structures particularly with respect to the spatial arrangement of the aromatic domains of the N- and C-terminal sites previously shown to be crucial for receptor recognition.

The optimized geometry of CCK-4 obtained by our conformational search was quite similar to the absolute minimum already reported [26]. The values of the Φ and Ψ dihedral angles for the central residues of methionine and aspartate are localized in the α-helix interval and the side chains of tryptophane and phenylalanine are found on the same side of the peptide, at an average distance of 10.6 Å (Fig. 3). In this

Table 5
Physicochemical properties of compounds **1**–**9** [a]



Comp.	R	Molecular formula	Molecular weight	R_f [b]	M.p. (°C)
1	3-indolyl–$(CH_2)_2$–	$C_{34}H_{30}N_4O_7$	574	0.40	208–210
2	3-indolyl–CH_2–	$C_{33}H_{28}N_4O_5$	560	0.50	210–211
3	2-indolyl	$C_{32}H_{26}N_4O_5$	546	0.54	269–270
4	phenyl	$C_{30}H_{25}N_3O_5$	507	0.74	158–160
5	3-methylphenyl	$C_{31}H_{27}N_3O_5$	521	0.69	110–112
6	benzyl	$C_{31}H_{27}N_3O_5$	521	0.31	146–147
7	4-chlorobenzyl	$C_{31}H_{26}ClN_3O_5$	555.5	0.46	210–212
8	phenylethenyl-	$C_{32}H_{27}N_3O_5$	533	0.50	163–165
9	HOOC–$(CH_2)_2$–	$C_{27}H_{25}N_3O_7$	503	0.25	182–184

[a] Compounds **1**–**9** were crystallized from methanol.
[b] AcOEt–EtOH, 1:1.

Table 6
^{1}H NMR (DMSO-d_6) of compounds **1**–**9** [a]

Comp.	δ (ppm)
1	2.69 (t, 2H, –CH_2–ind); 2.99 (t, 2H, –CH_2–CH_2–); 3.15 (m, 2H, >CH–CH_2–); 4.64 (m, 1H, –CH<); 6.90–8.50 (m, 18H, ar); 9.05 (d, 1H, –N*H*–CH<); 10.70 (s, 1H, –NH– ind); 10.78 (s, 1H, –NH–); 11.85 (s, 1H, –NH–)
2	3.66 (m, 2H, –CH_2–CH<); 3.76 (s, 2H, –CH_2–CO–); 4.68 (m, 1H, –CH<); 6.8–8.35 (m, 18H, ar); 9.15 (d, 1H, –N*H*–CH<); 10.72 (s, 1H, –NH– ind); 11.05 (s, 1H, –NH–); 11.75 (s, 1H, –NH–)
3	3.10 (m, 2H, –CH_2–); 4.70 (m, 1H, –CH<); 7.05–8.65 (m, 18H, ar); 9.15 (d, 1H, –N*H*–CH<); 11.95 (s, 2H, –NH– and –NH– ind); 12.10 (s, 1H, –NH–)
4	3.07 (m, 2H, –CH_2–); 4.67 (m, 1H, –CH<); 7.06–8.55 (m, 18H, ar); 9.13 (d, 1H, –N*H*–CH<); 11.82 (s, 1H, –NH–); 12.05 (s, 1H, –NH–)
5	2.40 (s, 3H, –CH_3); 3.05 (m, 2H, –CH_2–); 4.68 (m, 1H, –CH<); 7.11–8.51 (m, 17H, ar); 9.14 (d, 1H, –N*H*–CH<); 11.74 (s, 1H, –NH–); 12.04 (s, 1H, –NH–)
6	3.07 (m, 2H, –CH_2–); 3.67 (s, 2H, –CH_2–); 4.68 (m, 1H, >CH–); 7.1–8.5 (m, 18H, ar); 9.11 (d, 1H, –NH–); 10.7 (s, 1H, –NH–); 11.8 (s, 1H, –NH–)
7	3.08 (m, 2H, >CH–CH_2–); 3.68 (s, 2H, –CH_2–CO–); 4.67 (m, 1H, –CH<); 7.10–8.42 (m, 17H, ar); 9.08 (d, 1H, –N*H*–CH<); 10.65 (s, 1H, –NH–); 11.79 (s, 1H, –NH–)
8	3.08 (m, 2H, –CH_2–); 4.68 (m, 1H, –CH_2–C*H*<); 6.90 (d, 1H, –CO–CH=) (J = 15.6 Hz); 7.02–8.51 (m, 19H, ar and =CH–Ph); 9.13 (d, 1H, –N*H*–CH<); 10.88 (s, 1H, –NH–); 11.87 (s, 1H, –NH–)
9	3.1 (m, 2H, –CH_2–); 3.3 (m, 4H, 2×–CH_2–); 4.7 (m, 1H, >CH–); 6.9–8.6 (m, 13H, ar); 9.0 (d, 1H, –NH–); 11.4 (s, 1H, –NH–); 12.0 (s, 1H, –NH–)

[a] Abbreviations: ar, aromatic; ind, indole; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

case, the Phe side chain is relatively close to the pyrrole ring of Trp (ca. 8 Å) whereas in compound **1** the indole ring offers its phenyl ring towards the Phe moiety.

Finally, a geometrical comparison was made by an rms overlay between compound **1** and CCK-4. This superimposition was performed matching 11 atom pairs (the five atoms of the pyrrole ring of the Trp and the six aromatic carbons of the Phe) and the rms error in the overlay resulted to be 1.07 Å (Fig. 4).

It can be seen that compound **1** exhibits an extensive overlap with CCK-4, the main difference regarding the tetrapeptide backbone as compared with the anthranilic acid dimer scaffold. In fact, the three peptide bonds of the CCK-4 sequence form a S-like bend whereas compound **1** presents a staggered U-shaped conformation. In both cases the aromatic rings of the N- and C-terminal sites approach each other, thus indicating a similar regnylogical-type organization.

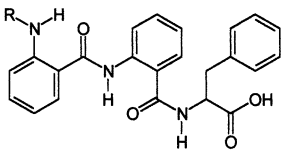
5. Conclusions

In conclusion, we have shown that compounds with micromolar affinity for CCK-A receptor could be obtained by anchoring two appropriate pharmacophoric groups on the anthranilic acid dimer template. The best results were obtained with the N- and C-terminal aromatic rings already found in the likewise spatial ori-

Table 7
^{13}C NMR (DMSO-d_6) of compounds **1**–**9**

Comp.	δ (ppm)
1	20.49, 36.09, 37.65, 53.96, 111.12, 113.12, 117.98, 118.04, 120.63, 120.70, 121.72, 122.03, 122.99, 123.36, 123.52, 126.13, 126.74, 127.38, 127.89, 128.08, 128.84, 132.03, 135.99, 137.76, 138.00, 138.41, 166.00, 168.18, 170.73, 172.51
2	34.36, 36.05, 53.86, 107.12, 111.20, 118.10, 118.37, 120.51, 120.70, 120.89, 121.02, 122.44, 123.05, 123.13, 124.58, 126.17, 126.90, 126.94, 127.90, 128.84, 132.06, 136.16, 137.70, 138.13, 138.33, 165.71, 168.22, 169.97, 172.46
3	36.05, 53.85, 102.74, 112.30, 119.97, 120.87, 120.99, 121.05, 121.39, 121.71, 123.33, 123.89, 126.16, 126.81, 127.40, 127.90, 128.19, 128.85, 131.28, 132.21, 132.70, 136.92, 137.67, 138.19, 139.03, 159.12, 166.62, 168.27, 172.44
4	36.03, 53.85, 120.76, 120.89, 121.37, 122.06, 123.27, 123.60, 126.17, 126.87, 127.41, 127.91, 128.16, 128.69, 128.84, 131.85, 132.17, 132.57, 134.29, 137.67, 138.19, 138.98, 164.53, 166.53, 168.24, 172.46
5	20.82, 36.00, 53.84, 120.75, 120.84, 121.53, 122.38, 123.27, 123.64, 123.78, 126.18, 127.42, 127.67, 127.92, 128.14, 128.55, 128.84, 132.17, 132.45, 132.59, 134.30, 137.65, 138.05, 138.18, 138.81, 164.74, 166.47, 168.24, 172.46
6	36.03, 43.68, 53.82, 120.49, 120.65, 121.76, 123.02, 123.60, 123.80, 126.18, 126.48, 127.30, 127.92, 128.09, 128.16, 128.84, 129.18, 131.92, 132.05, 135.01, 137.68, 138.37, 165.81, 168.22, 169.12, 172.46
7	36.00, 42.65, 53.82, 120.31, 120.51, 121.85, 122.94, 123.75, 124.21, 126.18, 127.37, 127.93, 128.07, 128.84, 131.16, 131.90, 132.05, 133.89, 137.41, 137.70, 138.39, 165.69, 168.22, 168.80, 172.48
8	36.02, 53.82, 120.29, 120.68, 121.89, 122.07, 123.15, 123.62, 123.78, 126.18, 127.31, 127.55, 127.77, 128.14, 128.72, 128.85, 129.35, 129.74, 132.05, 133.20, 134.29, 135.57, 138.30, 140.82, 163.47, 166.05, 168.23, 172.44
9	28.57, 31.48, 36.02, 53.81, 120.62, 120.71, 121.52, 123.07, 123.30, 126.18, 127.37, 127.92, 128.11, 128.89, 132.07, 137.68, 138.06, 138.35, 166.02, 168.21, 169.94, 172.44, 173.42

Table 8
CCK receptors binding data



Comp.	R	CCK-A (%) [a]	CCK-B (%) [b]
0	H	$(26 \times 10^{-6}$ M) [c]	IN [d]
1	1*H*-indol-3-yl–$(CH_2)_2$–CO–	$(2.3 \times 10^{-6}$ M) [c]	30
2	1*H*-indol-3-yl–CH_2–CO–	$(6.8 \times 10^{-6}$ M) [c]	28
3	1*H*-indol-2-yl–CO–	$(3.8 \times 10^{-6}$ M) [c]	25
4	C_6H_5–CO–	30	35
5	C_6H_4-*m*-CH_3–CO–	35	43
6	C_6H_5–CH_2–CO–	38	28
7	C_6H_4-*p*-Cl–CH_2–CO–	40	43
8	C_6H_5–CH=CH–CO–	43	37
9	HOOC–$(CH_2)_2$–CO–	IN [d]	IN [d]

[a] Percentage of inhibition at 10 μM of [^{3}H]-(±)-L-364,718 binding in rat pancreatic membranes.
[b] Percentage of inhibition at 10 μM of [^{3}H]-(+)-L-365,260 in guinea pig brain membranes.
[c] IC_{50} (μM) given as the mean of at least three independent determinations. The maximum standard error was always less than 20% of the geometric mean.
[d] Inactive: percentage of inhibition less than 20% at 10 μM.

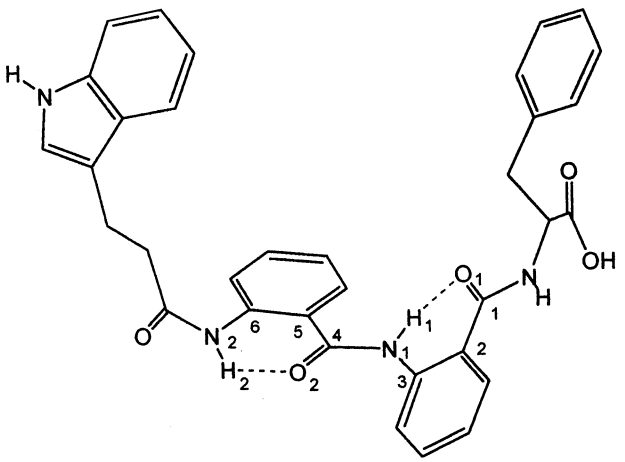


Fig. 1. Stable conformation of compound **1**.

ented sequence of CCK-4 as well as in the dipeptoid series otherwise characterized by a sycnological-type organization.

The presence of such pharmacophoric groups joined together by means of a spacer can therefore be considered as important elements for CCK receptor recognition and we are continuing our efforts to search for new templates that are able to support these groups with the correct spatial arrangement.

6. Experimental

6.1. Chemistry

All chemicals and solvents used in syntheses were reagent-grade products and were used without addi-

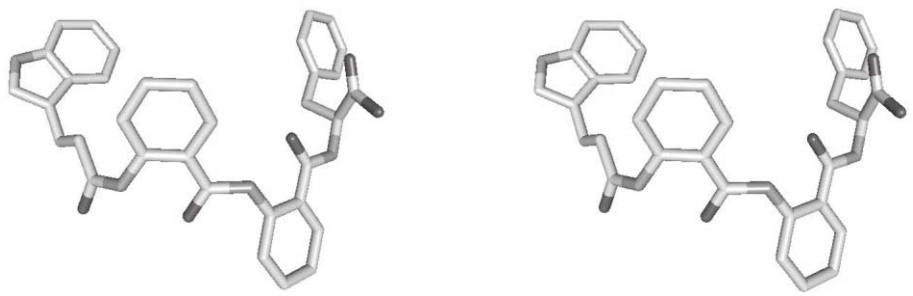


Fig. 2. Stereo view of the lowest energy conformer of compound **1**.

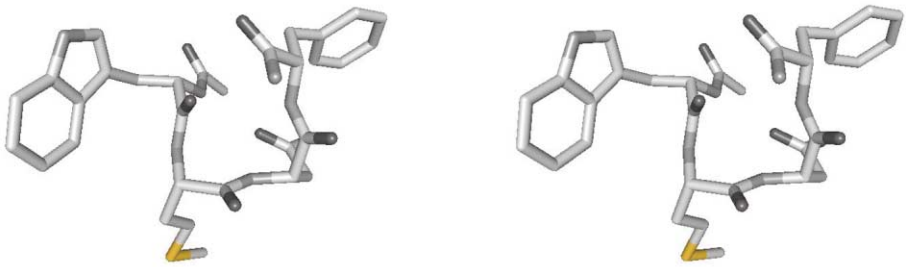


Fig. 3. Stereo view of the lowest energy conformer of Ac-CCK-4.

tional purification. Melting points were determined on a Büchi 510 melting point apparatus (Büchi, Flawil, Switzerland) and are uncorrected. Ascending thin-layer chromatography (TLC) was performed on precoated silica gel plates (60F-254 Merck) using UV light to visualize the chromatograms. Proton (^{1}H NMR, 200 MHz) and carbon (^{13}C NMR, 50 MHz) NMR spectra were recorded on a Varian-Gemini 2000 Fourier Transform spectrometer. Chemical shifts are reported in parts per million (ppm, δ units) relative to tetramethylsilane (TMS) as internal standard. Splitting patterns are designated as s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; and m, multiplet. Spectral data are consistent with the assigned structures.

*6.1.1. N-Anthranoyl-DL-phenylalanine ethyl ester (**10**)*

A suspension of DL-phenylalanine ethyl ester hydrochloride (4.59 g, 20 mmol) in 500 ml of ethyl acetate was treated with triethylamine (2.81 ml, 20 mmol) followed by isatoic anhydride (3.26 g, 20 mmol). The resulting mixture was refluxed under stirring for 2 h, cooled to room temperature (r.t.) and filtered. The organic phase was thoroughly washed with 1 M NaOH (2 × 50 ml), water (2 × 50 ml), dried over anhydrous sodium sulfate and concentrated in vacuo. Trituration with petroleum ether (40–70°C) afforded the analytically pure title compound in 79% yield.

R_f 0.69 (AcOEt–hexane, 1:1); m.p. 83–84°C; ^{1}H NMR ($CDCl_3$): δ 1.24 (t, 3H, $-CH_3$); 3.21 (m, 2H, $-CH_2-CH<$); 4.18 (q, 2H, $-CH_2-O-$); 4.97 (m, 1H, $>CH-$); 5.45 (s, 2H, $-NH_2$); 6.52 (d, 1H, –NH–); 6.61–7.28 (m, 9H, arom). ^{13}C NMR ($CDCl_3$): δ 14.19, 38.05, 53.20, 61.63, 115.41, 116.69, 117.29, 127.15, 127.43, 128.60, 129.43, 132.59, 136.04, 148.84, 168.66, 171.75.

*6.1.2. N-(N-Anthranoyl) anthranoyl-DL-phenylalanine ethyl ester (**11**)*

A solution of *N*-anthranoyl-DL-phenylalanine ethyl ester (**10**) (6.24 g, 20 mmol) in 100 ml of dry dichloromethane cooled at 0°C was treated with triethylamine (2.81 ml, 20 mmol) followed by 2-nitro-benzoyl chloride (3.70 g, 20 mmol). The resulting mixture was stirred at r.t. for 2 h. The reaction mixture was washed in succession with 1 M NaOH (2 × 30 ml) and water (2 × 30 ml). The dried (sodium sulfate) organic phase was concentrated to give the crude nitro derivative, which was used without further purification. The residue was taken up with 100 ml of dichloromethane and treated with 10 g of zinc dust. The resulting suspension was cooled at 0°C and, within 10 min, 12 ml of glacial acetic acid was added dropwise with stirring. The mixture was stirred for 1 h at r.t. and then filtered. The organic phase was washed with 1 M NaOH (2 × 50 ml), water (2 × 50 ml), dried over sodium sulfate, and evaporated. Crystallization from 90% ethanol afforded the analytically pure compound **11** in 71% yield.

R_f 0.73 (AcOEt–hexane, 1:1); m.p. 137°C; ^{1}H NMR ($CDCl_3$): δ 1.28 (t, 3H, $-CH_3$); 3.23 (m, 2H, $-CH_2-$); 4.24 (q, 2H, $-O-CH_2-$); 5.03 (m, 1H, $-CH<$); 5.75 (s, 2H, $-NH_2$); 6.68–8.67 (m, 14H, arom and –NH–); 11.62 (s, 1H, –NH–). ^{13}C NMR ($CDCl_3$): δ 14.14, 37.97, 53.48, 61.83, 115.68, 116.92, 117.39, 120.29, 121.65, 122.71, 126.68, 127.28, 127.80, 128.60, 129.33, 132.73, 135.58, 139.93, 149.71, 167.94, 168.47, 171.22.

*6.1.3. General procedure for the synthesis of N-[N-(substituted)anthranoyl] anthranoyl-DL-phenylalanine ethyl ester derivatives (**12** and **13**)*

A solution of 10 mmol of the corresponding acid in 100 ml of dry dichloromethane cooled at −10°C was treated with triethylamine (1.40 ml, 10 mmol) followed by isobutyl chloroformate (1.31 ml, 10 mmol). The resulting mixture was stirred at −10°C for 20 min and

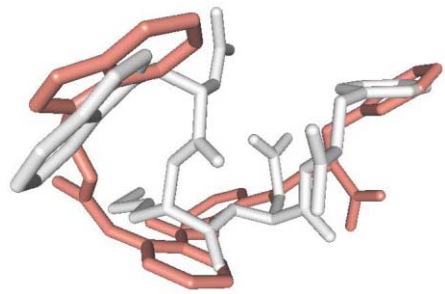


Fig. 4. Superimposition (heavy atoms only) of the preferential conformation of compound **1** (red) and Ac-CCK-4 (gray).

treated dropwise with a solution of compound **11** (4.31 g, 10 mmol) in 50 ml of dry dichloromethane. After the addition was complete, the reaction was stirred at r.t. for 1 h and then refluxed for 3 h. The solvents were evaporated, the residue was dissolved in dichloromethane; the organic layer was washed with diluted aqueous sodium hydroxide solution and with water, dried (sodium sulfate), and evaporated to dryness. The residue was purified by crystallization to yield compounds **12** and **13** (Table 2).

*6.1.4. General procedure for the synthesis of N-[N-(substituted)anthranoyl] anthranoyl-DL-phenylalanine ethyl ester derivatives (**14–20**)*

A solution of 10 mmol of the corresponding acyl chloride (the indole-2-carbonyl chloride was prepared according to the procedure described by Kermack et al. [27]) in 30 ml of dry dichloromethane was gradually added to a solution of compound **11** (4.31 g, 10 mmol) in the same solvent (50 ml). The pH of the reaction was adjusted to 9.5 with triethylamine and the solution stirred for 2 h. The reaction mixture was diluted with 100 ml of dichloromethane and washed in succession with 0.1 N NaOH, water, 0.1 N HCl and water. The dried (sodium sulfate) organic phase was rotary evaporated and the residue was purified by crystallization to yield compounds **14–20** (Table 2).

*6.1.5. General procedure for the synthesis of compounds **1–9***

A mixture of 5 mmol of the corresponding ethyl ester (compounds **12–20**) in methanol (50 ml) and in the presence of potassium hydroxide (0.56 g, 10 mmol) was gently warmed for 4 h. The solvent was removed under reduced pressure and the residue was taken up with water. After cooling, the solution was adjusted to pH 2–3 with diluted HCl to obtain the precipitation of the acid (compounds **1–9**, Table 5).

6.2. Biological evaluation

6.2.1. General

Male rats (Wistar) and male guinea pigs (Hartley) were obtained from Charles River, Calco, Como (Italy). [^{3}H]-(±)-L-364,718 and [^{3}H]-(+)-L-365,260 were purchased from NEN Research Products (Bruxelles) with specific activities of 87 and 75.3 Ci/mmol, respectively. (*R*,*S*)-L-364,718 and (*R*,*S*)-L-365,260 were synthesized in our laboratory as previously described [28]. Radioactivity was counted with 4 ml of Aquassure (NEN) high performance LSC cocktail in a Packard TRI-CARB 300 liquid scintillator.

6.2.2. Binding studies

All experiments were performed in triplicate. CCK-A receptor binding of [^{3}H]-(±)-L-364,718 was performed as previously described [24]. Briefly, samples of 0.4 ml containing 0.8 mg of wet tissue (≈7.2 μg/ml protein) were incubated for 30 min at 37°C in the presence of [^{3}H]-L-364,718 (0.2 nM final concentration) and a solution of various concentrations of the compounds to be tested. The buffer used for binding assay was 50 mM Tris–HCl (pH 7.4 at 37°C), 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mg/ml of bovine serum albumin and 0.14 mg/ml bacitracin. The samples were then filtered under reduced pressure using glass fiber GF/B (Whatman) filters and rinsed four times with 4 ml of cooled Tris buffer (50 mM, pH 7.4). Non-specific binding was determined in the presence of (*R*,*S*)-L-364,718 (0.3 μM final concentration) and was always less than 10% of the total binding.

CCK-B receptor affinities were determined by displacement of [^{3}H]-(+)-L-365,260 from guinea pig cerebral cortex membranes as previously described by Chang et al. [25]. The buffer used was 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 130 mM NaCl and 0.25 mg/ml bacitracin, pH 6.5 and the glass fiber filters were GF/C (Whatman). Briefly, displacement experiments were performed by incubation of 0.5 ml of brain membranes corresponding to 6.2 mg of wet tissue (200 μg/ml protein) for 30 min at 25°C in the presence of [^{3}H]-(+)-L-365,260 (1 nM final concentration) plus various concentrations of the compounds to be tested. Non-specific binding was determined in the presence of (*R*,*S*)-L-365,260 (2 μM final concentration). Specific binding was defined as the radioactivity after subtracting non-specific binding and was 45% of the total.

6.3. Computational procedures

All calculations were carried out on the form of the tetrapeptide (CCK-4) with the NH_2-terminal amino group blocked by an acetyl group.

The minimum energy conformations of compound **1** and CCK-4 were obtained from a conformational search carried out with a modification of the weighted random Monte Carlo search algorithm of Chang et al. [29], implemented in Conformer (Princeton Simulations).

The dihedral angles O1–C2–C3–N1, C3–N1–C4–O2, C5–C6–N2–H2, and the two α, β torsional angles at phenylalanine and indolepropionic acid were selected for the generation of the starting geometries of compound **1**, while all the Φ and Ψ angles of the peptide backbone were used for the starting geometries of CCK-4.

The initial geometries obtained from the algorithm were optimized first with the MM2 forcefield, and the searches were considered complete when either 1000 initial conformations in a row were rejected as similar to previously found conformations, or the number of consecutive minimized conformations which are proved

to be the same as the conformations previously found reach 2500. Two minimized conformations were considered different if the steric energies differ by more than 0.5 kcal/mol or a torsional angle by torsional angle comparison shows that one or more torsional angles differ by more than 0.1 rad.

The geometries obtained from the search were refined by a semiempirical calculation using the AM1 hamiltonian as implemented in MOPAC-93 [30] (RHF, SCF convergence 1×10^{-5}; the geometry optimization was carried out with the Polak–Ribiere algorithm at a convergence limit of 1×10^{-4} kcal/Å mol).

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