Replacement of Glycine with Dicarbonyl and Related Moieties in Analogues of the C-Terminal Pentapeptide of Cholecystokinin: CCK$_2$ Agonists Displaying a Novel Binding Mode

Bruno Bellier,[†,‡] Marie-Emmanuelle Million,[†,‡] Sophie DaNascimento,[‡] Hervé Meudal,[‡] Safia Kellou,[§] Bernard Maigret,[§] and Christiane Garbay*,[‡]

Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM, UMR 8600 CNRS, UFR des Sciences Pharmaceutiques et Biologiques, 4, Avenue de l'Observatoire, 75270 Paris Cedex 06, France, and Laboratoire de Chimie Théorique, S.R.S.M.C. · UMR 7565 CNRS, Université Nancy 1 - Université Henri Poincaré, B.P. 239 - Domaine Scientifique Victor Grimard, 54506 Vandoeuvre-les-nancy Cedex, France.

Recent advances in the field of cholecystokinin have indicated the possible occurrence of multiple affinity states of the CCK$_2$ receptor. Besides, numerous pharmacological experiments performed "in vitro" and "in vivo" support the eventuality of different pharmacological profiles associated to CCK$_2$ ligands. Indeed, some agonists are essentially anxiogenic and uneffective in memory tests, whereas others are not anxiogenic and appear as able to reinforce memory. The reference compound for the latter profile is the CCK-8 analogue BC 264 (Boc-Tyr(SO$_3$H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH$_2$). However, although tetrapeptide ligands based on CCK-4 (Trp-Met-Asp-Phe-NH$_2$) are known to possess sufficient structural features for CCK$_2$ recognition, none shares the properties of BC 264. Hence we have developed new short peptidic or pseudo-peptidic derivatives containing the C-terminal tetrapeptide of BC 264. Our results indicate that some compounds characterized by the presence of two carbonyl groups at the N-terminus, as in **2b** (HO$_2$C-CH$_2$-CONH-Trp-(NMe)Nle-Asp-Phe-NH$_2$), are likely to show a BC 264-like profile, bind to the CCK$_2$ receptor in a specific way, and display remarkable affinities (**2b**: 0.28 nM on guinea-pig cortex membrane preparations). This original binding mode is discussed and further enlightened by NMR and molecular modeling studies.

Introduction

The neurotransmitter and neuromodulator peptide cholecystokinin (CCK) is known to intervene in various physiological processes, in both central and peripheral nervous systems. Its biological actions are mediated by two G-protein coupled receptors designated CCK$_1$ and CCK$_2$.[1] The latter is involved in phenomenons as diverse as anxiety,[2] analgesia,[3] memory processes,[4] or psychiatric disorders.[5] The actions of CCK are predominantly due to the sulfated octapeptide CCK-8, Asp-Tyr(SO$_3$H)-Met-Gly-Trp-Met-Asp-Phe-NH$_2$, and they may result from interactions with other neurotransmitters such as dopamine[6] or enkephalins[7] and, as shown more recently, serotonine.[8]

On the basis of binding experiments using CCK$_2$ selective agonists[9a−c] or antagonists,[9d,e] several authors have suggested the occurrence of CCK$_2$ receptor subtypes or subsites. Nevertheless, the cloning of CCK$_2$ receptors has not proven the existence of structurally different entities yet, as far as proteic primary sequence is concerned.[10]

Pharmacological studies conducted in our laboratory have focused on two peptidic probes to further explore CCK$_2$ receptor potential heterogeneity: the cyclic octapeptide BC 197 (Boc-c[D-Asp-Tyr(SO$_3$H)-Nle-D-Lys]-Trp-Nle-Asp-Phe-NH$_2$)[11] and the modified heptapeptide BC 264 (Boc-Tyr(SO$_3$H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH$_2$), which is resistant to enzymatic degradation.[12] These two compounds exert somewhat different pharmacological properties, although both of them are selective CCK$_2$ agonists.

First, behavioral experiments have proved BC 197 to be anxiogenic for rats in the (+)-maze test[13] and to have no effect on the locomotor activity,[15a] whereas BC 264 does not exert any anxiogenic effects at doses as high as 10 mg/kg[13] and increases the activity of the animals in both the actimeter and the open-field tests.[15a]

Besides, in the more elaborated memory test of Dellu et al.,[14] BC 264 increases the memory of rats while BC 197 seems to be amnesia-inducing.[15b] In the Y-maze test, which measures the attention and/or the working memory of the animals, BC 264 promotes the attention, while BC 197 remains uneffective.[15a] Moreover, these compounds have also opposite actions on the grooming behavior of animals, which is enhanced by BC 264 and inhibited by BC 197.[15c] Last, "in vitro"[15d] and "in vivo" measurements of dopamine release in the anterior nucleus accumbens have once again confirmed a differential action of BC 197 and BC 264, the latter being able in particular to increase the extracellular levels of dopamine in the nucleus accumbens of awake animals while BC 197 is not.[15e] Thus, the differential effects of BC 197 and BC 264 have been associated with pharmacological profiles termed respectively "CCK-B$_1$" and

* Address correspondence to Professor Ch. Garbay. Tel: (33) 01-43-25-50-45. Fax: (33) 01-43-26-69-18. E-mail: Garbay@pharmacie.univ-paris5.fr.
† Both authors contributed equally to this work.
‡ Département de Pharmacochimie Moléculaire et Structurale.
§ Laboratoire de Chimie Théorique.

"CCK-B₂", which will now, according to the IUPHAR nomenclature, be renamed "CCK$_{2A}$" and "CCK$_{2B}$".

To support the assumption of two dissociated profiles for CCK₂ agonists, it was essential to search for new compounds belonging to other chemical series and meeting the characteristics of either profile. Since CCK-4 (Trp-Met-Asp-Phe-NH₂) is the minimal feature for CCK₂ recognition, it was then focused on tetrapeptide-like compounds, hoping they would be separable between "CCK$_{2A}$" and "CCK$_{2B}$" agonists. Thus, cyclic CCK-4 analogues RB 360 ([*N*-cycloamido)-αMe(R)-Trp-[(2*S*)-2-amino-9-(cycloamido)carbonyl)nonanoyl]]-Asp-Phe-NH₂) and RB 380 ([cyclo-*S-S*-[(5-thiopentanoyl)-αMe(R)Trp-Cys]]-Asp-Phe-NH₂)[16] were designed to mimic the preferential folded conformation of the linear tetrapeptide Boc-Trp-(NMe)Nle-Asp-Phe-NH₂. These compounds were shown to be anxiogenic and devoid of effect in the Y-maze test and on the locomotor activity. This strongly suggested that these two compounds had a BC 197-like profile, that is to say "CCK$_{2A}$". Until the beginning of the research reported here, no CCK-4 analogue had revealed a "CCK$_{2B}$" profile so far, when this profile is clearly the most interesting. The search for new "CCK$_{2B}$" compounds appeared then to be crucial, as well for a better understanding of the potential pharmacological heterogeneity of CCK₂ receptors, as for developing tools chemically simpler than BC 264.

Keeping in mind that CCK-4 analogues may furnish a convenient basis for designing new CCK₂ agonists, the C-terminal tetrapeptide of BC 264 (Trp-(NMe)Nle-Asp-Phe-NH₂) was chosen as the starting point for the quest of "CCK$_{2B}$" molecules. Unfortunately, NMR and computer-aided studies on BC 264 did not identify a preferential conformation of this compound. Therefore, the only possible rational guideline was to add step by step the N-terminal structural features of BC 264 in order to determine the key pattern for "CCK$_{2B}$" selectivity.[17] As a matter of fact, previous investigations had essentially been directed on the CCK-8 series, with a systematic analysis of the role played by each amino acid in the octapeptidic sequence. CCK-4 derivatives have also been widely studied with the aim of obtaining small and restrained CCK₂ ligands. But the influence of the N-terminus of a "CCK-5-like" structure remained to be focused on, for no particular interest had been taken in pentapeptide CCK analogues. In this paper, we describe several types of modifications (constrained or not) performed on the N-terminal part of the CCK-4 analogue Trp-(NMe)Nle-Asp-Phe-NH₂ and try to propose a differential mode of receptor recognition for these compounds in order to analyze their binding characteristics.

Results

Chemistry. The starting material TFA·Trp-(NMe)-Nle-Asp(OBzl)-Phe-NH₂ (**I**) was prepared in large amounts by standard liquid phase peptide synthesis essentially as previously described.[18] Some agents to be used to modify the N-terminus of **I** had to be prepared. Thus, malonic acid monobenzyl ester (**2**) or monomethyl ester (**3**) or methylmalonic acid monoethyl ester (**8**) were obtained by selective monosaponification of the corresponding diesters.[19a] Compound **8** was obtained as a racemic mixture, which did not need to be separated.

Subsequent amidation of **2** with benzylamine in the presence of BOP led to the corresponding amide derivative. Direct amidation of **2** with ammonia did not provide the monoamide of malonic acid. Alternatively, alcoholysis of ethyl cyanoacetate, following procedure B, yielded the desired product **6**.[19b] Last, monobenzyl succinate **4** was prepared by the nucleophilic ring opening of succinic anhydride by benzyl alcohol.[19c]

Thereafter, introduction of supplementary substituents at the N-terminus of **I** was again achieved by standard techniques: acid esters **2−4**, and **8**, or commercial sulfoacetic acid (**7**), monoethyl fumarate (**9**), monoethyl adipate (**10**), methoxyacetic acid (**11**), Z-glycine (**12**), Z-alanine (**13**), Z-D-alanine (**14**), Z-aspartic acid β-benzyl ester (**15**), Z-D-aspartic acid β-benzyl ester (**16**), Boc-β-alanine (**17**), acetoacetic acid (**18**), Boc-L or D-aspartic acid α or β benzyl ester (**19−22**), or acid amides **5** (prepared following procedure C) and **6** were coupled to **I** in the presence of BOP and DIEA to give protected pentapeptides **2a−22a**. Catalytic hydrogenation of **2a−7a** and **12a−22a** or saponification of **8a−10a** gave the final deprotected compounds **2b−22b** (Scheme 1).

All these products were purified by semipreparative HPLC. Compound **8b** (methylmalonyl-Trp-(NMe)Nle-Asp-Phe-NH₂) was the mixture of two epimers; only one could be isolated as a pure entity, while the other was obtained in a 40% diastereomeric excess. Compound **17b** (Boc-β-Ala-Trp-(NMe)Nle-Asp-Phe-NH₂) was further deprotected in a TFA/CH₂Cl₂ mixture with anisole as scavenger, to yield **17c** (Scheme 1).

Compounds **23a−26a** were synthesized by acylation of **I** with ethyl oxalyl chloride, methyl chloroformate, propionyl chloride, and isobutyryl chloride, respectively. Deprotection of the aspartate side chain by saponification (**23a**) or hydrogenolysis (**24a−26a**) led to **23b−26b**. Nucleophilic opening of maleic anhydride by **I**[19d] in a mixture of DMF and triethylamine gave **27a**, whose deprotection finally afforded **27b** (Scheme 1).
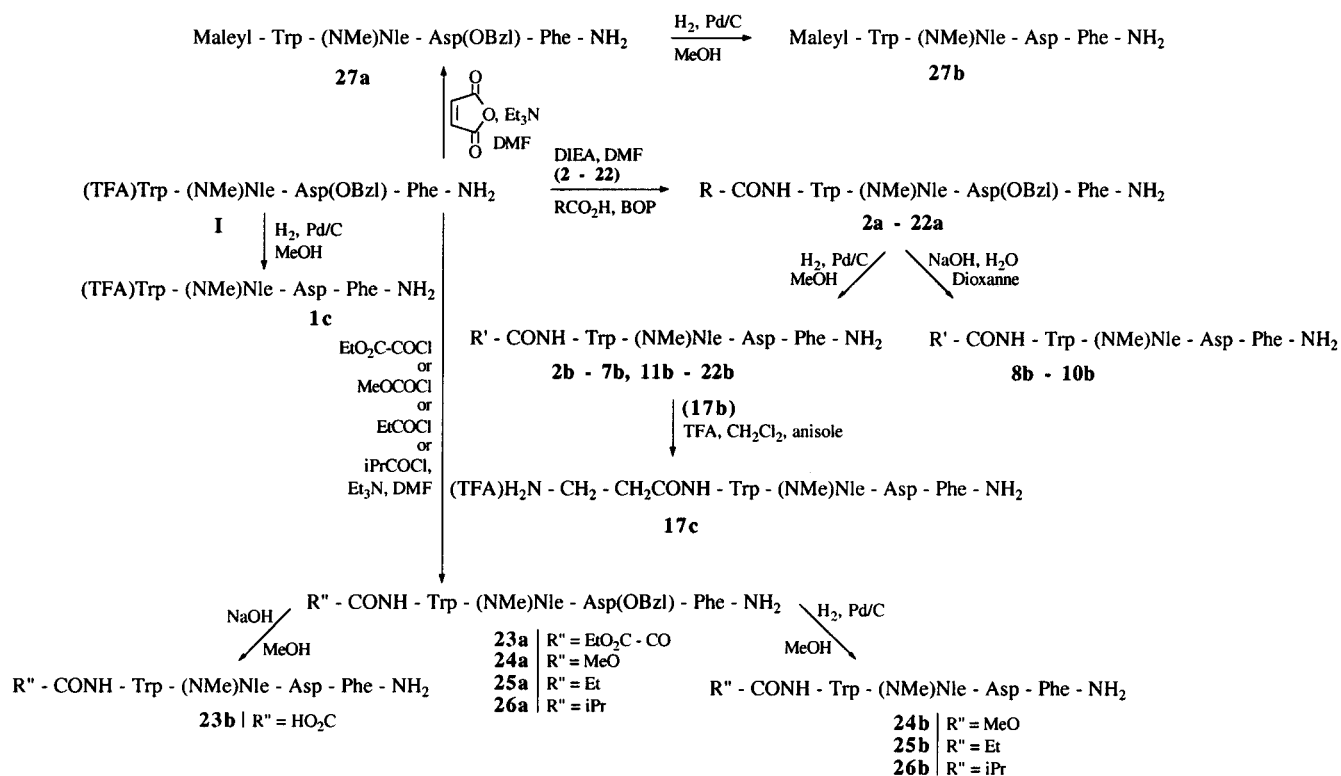
Derivatives **28b** (Ac-Trp-(NMe)Nle-Asp-Phe-NH₂) and **29b** (malonyl-(NMe)Trp-(NMe)Nle-Asp-Phe-NH₂) were obtained from the intermediate tripeptide **II** (NMe)Nle-Asp(OBzl)-Phe-NH₂, which was condensed with Ac-Trp-OH, or Boc-NMe-Trp-OH giving aspartate-protected **28a** and **29a**, respectively. Compound **29a** was deprotected with TFA, coupled to malonic acid monobenzyl ester (**2**), and the resulting pentapeptide was submitted to H₂ on Pd/C to give **29b**, while hydrogenolysis of **28a** gave **28b** (Scheme 2).

N-Acylation of **I** and **II** by either pathway and deprotection of the esters and carbamates were always carried out with good yields and did not necessitate any intermediate purification but only extractions and/or washings. It is important to note that all (NMe)Nle-containing peptides were characterized by the occurrence of two NMR signals for numerous protons, due to the equilibrium between two rotamers around the Trp-(NMe)Nle bond.

Discussion

The aim of this study was to explore the influence of the N-terminal extension of CCK-4-like CCK₂ agonists represented by the derivative Trp-(NMe)Nle-Asp-Phe-NH₂ (**1c**). The compounds synthesized were tested for
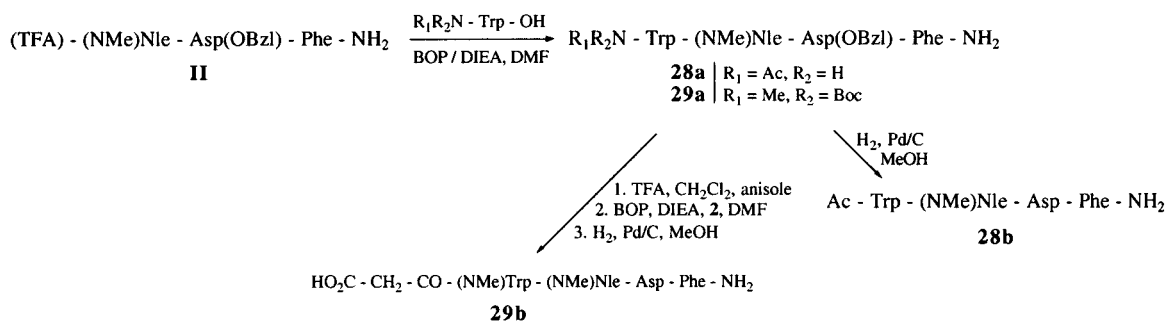
Scheme 1

Maleyl - Trp - (NMe)Nle - Asp(OBzl) - Phe - NH$_2$ $\xrightarrow[\text{MeOH}]{\text{H}_2, \text{Pd/C}}$ Maleyl - Trp - (NMe)Nle - Asp - Phe - NH$_2$

27a   **27b**

O, Et$_3$N, DMF

(TFA)Trp - (NMe)Nle - Asp(OBzl) - Phe - NH$_2$ $\xrightarrow[\text{RCO}_2\text{H, BOP}]{\text{DIEA, DMF (2 - 22)}}$ R - CONH - Trp - (NMe)Nle - Asp(OBzl) - Phe - NH$_2$

I   **2a - 22a**

H$_2$, Pd/C, MeOH

(TFA)Trp - (NMe)Nle - Asp - Phe - NH$_2$

1c

$\xleftarrow{\text{H}_2, \text{Pd/C, MeOH}}$ $\xrightarrow{\text{NaOH, H}_2\text{O, Dioxane}}$

R' - CONH - Trp - (NMe)Nle - Asp - Phe - NH$_2$ R' - CONH - Trp - (NMe)Nle - Asp - Phe - NH$_2$

2b - 7b, 11b - 22b   **8b - 10b**

EtO$_2$C-COCl or MeOCOCl or EtCOCl or iPrCOCl, Et$_3$N, DMF

(17b) TFA, CH$_2$Cl$_2$, anisole

(TFA)H$_2$N - CH$_2$ - CH$_2$CONH - Trp - (NMe)Nle - Asp - Phe - NH$_2$

17c

R" - CONH - Trp - (NMe)Nle - Asp(OBzl) - Phe - NH$_2$ $\xrightarrow{\text{H}_2, \text{Pd/C, MeOH}}$

NaOH, MeOH

R" - CONH - Trp - (NMe)Nle - Asp - Phe - NH$_2$ R" - CONH - Trp - (NMe)Nle - Asp - Phe - NH$_2$

23b | R" = HO$_2$C

23a	R" = EtO$_2$C - CO
24a	R" = MeO
25a	R" = Et
26a	R" = iPr

24b	R" = MeO
25b	R" = Et
26b	R" = iPr

	R (a series)	R' (b series)		R (a series)	R' (b series)
2	BzlO$_2$C-CH$_2$-	HO$_2$C-CH$_2$-	12	ZNH-CH$_2$-	H$_2$N-CH$_2$-
3	CH$_3$O$_2$C-CH$_2$-	CH$_3$O$_2$C-CH$_2$-	13 [c]	ZNH-CH*(CH$_3$)-	H$_2$N-CH*(CH$_3$)-
4	BzlO$_2$C-(CH$_2$)$_2$-	HO$_2$C-(CH$_2$)$_2$-	14 [d]	ZNH-CH*(CH$_3$)-	H$_2$N-CH*(CH$_3$)-
5	PhCH$_2$NHCOCH$_2$CO-	PhCH$_2$NHCOCH$_2$CO-	15 [c]	ZNH-CH*(CH$_2$CO$_2$Bzl)-	H$_2$N-CH*(CH$_2$CO$_2$H)-
6	H$_2$NCOCH$_2$CO-	H$_2$NCOCH$_2$CO-	16 [d]	ZNH-CH*(CH$_2$CO$_2$Bzl)-	H$_2$N-CH*(CH$_2$CO$_2$H)-
7	HO$_3$S-CH$_2$-	HO$_3$S-CH$_2$-	17	BocNH-CH$_2$-CH$_2$-	BocNH-CH$_2$-CH$_2$-
8 [a]	C$_2$H$_5$O$_2$C-CH(CH$_3$)-	HO$_2$C-CH(CH$_3$)-	18	CH$_3$COCH$_2$-	CH$_3$COCH$_2$-
9 [b]	C$_2$H$_5$O$_2$C-CH=CH-	HO$_2$C-CH=CH-	19 [c]	BocNH-CH*(CH$_2$CO$_2$Bzl)-	BocNH-CH*(CH$_2$CO$_2$H)-
10	C$_2$H$_5$O$_2$C-(CH$_2$)$_5$-	HO$_2$C-(CH$_2$)$_5$-	20 [d]	BocNH-CH*(CH$_2$CO$_2$Bzl)-	BocNH-CH*(CH$_2$CO$_2$H)-
11	CH$_3$OCH$_2$-	CH$_3$OCH$_2$-	21 [c]	BocNH-CH*(CO$_2$Bzl)CH$_2$	BocNH-CH*(CO$_2$H)CH$_2$
			22 [d]	BocNH-CH*(CO$_2$Bzl)CH$_2$	BocNH-CH*(CO$_2$H)CH$_2$

[a]. Racemic mixture ; [b]. The double bond is trans ; [c]. S(L)configuration at the * carbon ; [d]. R(D)configuration at the * carbon.

Scheme 2

(TFA) - (NMe)Nle - Asp(OBzl) - Phe - NH$_2$ $\xrightarrow[\text{BOP / DIEA, DMF}]{\text{R}_1\text{R}_2\text{N - Trp - OH}}$ R$_1$R$_2$N - Trp - (NMe)Nle - Asp(OBzl) - Phe - NH$_2$

II

28a	R$_1$ = Ac, R$_2$ = H
29a	R$_1$ = Me, R$_2$ = Boc

H$_2$, Pd/C, MeOH

1. TFA, CH$_2$Cl$_2$, anisole
2. BOP, DIEA, **2**, DMF
3. H$_2$, Pd/C, MeOH

Ac - Trp - (NMe)Nle - Asp - Phe - NH$_2$

28b

HO$_2$C - CH$_2$ - CO - (NMe)Trp - (NMe)Nle - Asp - Phe - NH$_2$

29b

their ability to displace [^{3}H]pCCK-8 from preparations of CHO cells stably transfected with the rat CCK$_2$ receptor and, for the most interesting ones, from guinea-pig pancreatic membranes to assay their CCK_1 affinity, and their affinities were compared to the those of the reference agonists **1b** (Boc-Trp-(NMe)Nle-Asp-Phe-NH$_2$, K_i = 2.6 nM) and BC 264 (K_i = 0.31 nM). The results are reported in Tables 2 and 3, while the affinities of standard CCK ligands are recalled in Table 1. Finally,

the compounds endowed with the best affinities were evaluated for their CCK$_2$ agonists profile by measuring their ability to stimulate inositol phosphate production in transfected CHO cells, as described previously.[16]

At first sight, the enlargement of **1c** was designed to obtain C-terminal fragments of BC 264 and thus to determine the elements responsible for the specific activity of the parent compound. This resulted in the preparation of pentapeptidic derivatives characterized

Table 1. Affinities of Standard Cholecystokinin Agonists

Compd	Formula	$K_i(CCK_2)$ [a]	$K_i(CCK_1)$ [b]	CHO cells	
				K_i [b]	EC_{50} [c]
CCK-8	Asp - Tyr(SO₃H) - Met - Gly - Trp - Met - Asp - Phe - NH₂	0.3	0.7	0.6	0.63
BC 264	Boc - Tyr(SO₃H) - gNle - mGly - Trp - NMe(Nle) - Asp - Phe - NH₂	0.15	96	0.31	3.4
BC 197	Boc - D.Asp - Tyr(SO₃H) - Nle - D.Lys - Trp - Nle - Asp - Phe - NH₂	6.4	2900	198	17
CCK-4	Trp - Met - Asp - Phe - NH₂	19.5	36133	93	1.4
Boc-CCK-4	Boc - Trp - Met - Asp - Phe - NH₂	42 [d]	11900 [d]	9.4	1
1b	Boc - Trp - (NMe)Nle - Asp - Phe - NH₂	0.8	> 5000	2.6	1.5
2b RB 400	HO₂C - CH₂CO - Trp - (NMe)Nle - Asp - Phe - NH₂	0.28	> 3000	0.75	0.40
RB 360	αMe - D.Trp - NH - *CH - CO - Asp - Phe - NH₂ [e] / NHCO - (CH₂)₇	15	2210	18	7

[a] K_i values (expressed in nM) represent the mean of three separate experiments, each performed in triplicate for determining CCK₂ and CCK₁ affinities on guinea-pig cortex and pancreatic memebranes respectively. [b] K_i values are the mean of three separate experiments, each performed in triplicate on the rat brain CCK₂ receptor expressed in CHO cells. [c] Results (expressed in nM) are the mean of three separate measurements of inositol phosphate production induced by the compounds each in triplicate. [d] From ref 24. [e] S configuration at the * carbon.
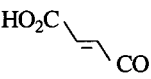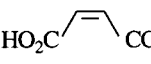
by the N-terminal feature X-CO-CH₂-CO-NH-Trp, corresponding to the retro-inverso bond characteristic of the sequence of BC 264. *In the following sections, the N-terminus of the discussed compounds (R-NH-Trp-(NMe)Nle-Asp-Phe-NH₂) will be recalled if necessary for perfect clarity, for instance* **2b** *(R = HO₂C-CH₂-CO).* The binding properties of these compounds are presented in Table 2, which brings out the remarkable subnanomolar affinities of **2b** (R = HO₂C-CH₂-CO) and **7b** (R = HO₃S-CH₂-CO) (0.75 nM and 0.54 nM, respectively). Thus, an acidic N-terminus seems favorable for receptor recognition, but it must not be compulsory, as proved by compounds **3b** (R = H₃CO₂C-CH₂-CO), **5b** (R = PhCH₂-NH-CO-CH₂-CO), and **6b** (R = H₂N-CO-CH₂-CO), which retain nanomolar affinities. These first results highlighted that a hydrophilic N-terminal capping of CCK-4 induced an increase in affinity, as did a hydrophobic capping. This indicated the occurrence of two different binding processes to the receptor followed by **2b** and **1b**, respectively. Therefore, several new derivatives were prepared in order to further characterize the new hydrophilic binding mode.

Optimization of the Length of the N-Terminal Acid Substituent. Considering the successful addition of a malonate residue, mimicking the retro-inverso glycine in BC 264, we further attempted to optimize the spatial location of the N-terminal negative charge. The distance between the two carbonyl groups was lengthened to two and five methylenes, with compounds **8b** (R = HO₂C-(CH₂)₂-CO) and **10b** (R = HO₂C-(CH₂)₅-CO), respectively. Addition of one methylene bridge to **2b** already decreases the affinity by a 4-fold factor, while a longer extension is really dramatic. On the contrary, shortening this distance by replacing the malonate by an oxalate in **23b** (R = HO₂C-CO) also considerably reduced the affinity, probably because of both an increased rigidity and an unsufficient distance to the tryptophan at this level. This suggests relatively strict

requirements for the recognition of a N-terminal carboxylate by the receptor.

Conformationally Restricted Derivatives. Another mean of studying the apparently important role of the N-terminal acidic moiety was to introduce conformational restraints at this level. The *N*-methylation of the tryptophan residue in **2b** was a way in this purpose. The corresponding di-*N*-methylated pentapeptide **29b** (HO₂C-CH₂-CO-(NMe)Trp-(NMe)Nle-Phe-NH₂) suffered a dramatic loss in affinity (K_i = 3860 nM), probably indicating either a steric hindrance or the loss of an hydrogen bond essential for receptor recognition. Ron and co-workers[20] had already studied the effects of various backbone *N*-methylations in the CCK-8 series. Single *N*-methylation of Trp did not seem to avoid binding to CCK₂ receptors, but in our case, double *N*-methylation probably results in too strong steric constraints and eventually in an increased number of inactive conformers. Replacement of the malonate by either a fumarate (**9b**) or a maleate (**21b**) constrained the carboxylate moiety with only a little increase in the distance to the tryptophan amide nitrogen. Curiously, this N-acylation of **I** resulted in both cases in two separable products, each of these being itself a couple of N-cis/N-trans rotamers around the Trp-(NMe)Nle bond as revealed by the presence of two NMR signals for the *N*-methyl group. NMR and mass spectra of these compounds strongly suggested that they also be rotamers of a single molecule, presumably because of a slow isomerization of the conjugated N-terminal system. Actually, the CH-CO bonds are here partial double bonds and may present sufficiently high rotation barriers to give rise to separable products. However, none of these showed a good affinity for the CCK₂ receptor, but conversely, all had micromolar potencies. This probably indicated the blockade of the acid moiety in an unappropriate position, or electronic and/or steric unfavorable effects of the N-terminal unsaturated chain.

**Table 2.** Affinities of N-Terminal Modified RNH-Trp-(NMe)Nle-Asp-Phe-NH$_2$ Derivatives of CCK-4

Compd	R	Ki(CCK$_1$) [a]	Ki(CCK$_2$) [b]	EC$_{50}$ (CHO) [c]
1b	Boc	> 5000	2.6	1.5
Malonate derivatives				
2b	HO$_2$C - CH$_2$ - CO	> 3000	0.75	0.40
3b	H$_3$CO$_2$C - CH$_2$ - CO	n.d.	3.61	2
5b	PhCH$_2$NHOC - CH$_2$ - CO	n.d.	1.81	3.30
6b	H$_2$NOC - CH$_2$ - CO	22800	2.05	1.64
7b	HO$_3$S - CH$_2$ - CO	n.d.	0.54	0.56
18b	CH$_3$CO - CH$_2$ - CO	n.d.	3.84	n.d.
Influence of the chain length				
2b	HO$_2$C - CH$_2$ - CO	> 3000	0.75	0.40
8b	HO$_2$C - (CH$_2$)$_2$ - CO	> 3000	3.02	1.5
11b	HO$_2$C - (CH$_2$)$_5$ - CO	n.d.	992	n.d.
23b	HO$_2$C - CO	n.d.	1460	n.d.
Constrained derivatives				
9b [d]	HO$_2$C (cis) CO	n.d.	{2490 / 1280}	n.d.
21b [d]	HO$_2$C (trans) CO	n.d.	{10200 / 317}	n.d.
Amine derivatives				
1c	H	> 3000	2.6	n.d.
12b	H$_2$N - CH$_2$ - CO	> 3000	18	n.d.
17c	H$_2$N - CH$_2$ - CH$_2$ - CO	n.d.	95	n.d.
17b	BocHN - CH$_2$ - CH$_2$ - CO	4620	1.35	n.d.
Influence of the chain length				
1b	Boc	> 5000	2.6	1.5
11b	CH$_3$O - CH$_2$ - CO	n.d.	2.52	n.d.
24b	CH$_3$O - CO	n.d.	23.8	n.d.
25b	CH$_3$ - CH$_2$ - CO	> 6000	4.35	n.d.
28b	CH$_3$ - CO	n.d.	228	n.d.

Compd	R	Ki(CCK$_1$) [a]	Ki(CCK$_2$) [b]	Remarks
Aspartate derivatives				
15b	HO$_2$C - CH$_2$ - CH*(NH$_2$)CO	n.d.	4.8	L configuration at the * carbon
16b	HO$_2$C - CH$_2$ - CH*(NH$_2$)CO	n.d.	11.9	D configuration at the * carbon
19b	HO$_2$C - CH$_2$ - CH*(NHBoc)CO	n.d.	7.31	L configuration at the * carbon
20b	HO$_2$C - CH$_2$ - CH*(NHBoc)CO	n.d.	7.76	D configuration at the * carbon
21b	HO$_2$C - CH*(NHBoc)CH$_2$CO	n.d.	4.07	L configuration at the * carbon
22b	HO$_2$C - CH*(NHBoc)CH$_2$CO	n.d.	2.7	D configuration at the * carbon

[a]−[c] See footnotes a−c in Table 1. [d] Each value corresponds to a putative rotamer (see text for details). n.d.: not determined.

Table 3. Influence of a a-substitution of the N-terminal Residue in CCK-5 Derivatives RNH-Trp-(NMe)Nle-Asp-Phe-NH$_2$

Compd	R	Ki (CCK$_1$) [a]	Ki((CCK$_2$) [b]	Remarks
1b	Boc	> 5000	2.6	
2b	HO$_2$C-CH$_2$-CO	> 3000	0.75	
4b	HO$_2$C-CH(CH$_3$)-CO [c]	n.d.	3340	Pure 1st diastereoisomer
			224	2nd diastereoisomer in a 40% diastereomeric excess
12b	H$_2$N-CH$_2$-CO	> 3000	18	
13b	H$_2$N-CH*(CH$_3$)-CO	> 3000	20	L configuration at the * carbon
14b	H$_2$N-CH*(CH$_3$)-CO	> 3000	15	D configuration at the * carbon
25b	CH$_3$-CH$_2$-CO	> 6000	4.35	
26b	CH$_3$-CH(CH$_3$)-CO	n.d.	1.37	

^{a,b} See footnotes a and b in Table 1. ^{c} Diastereoisomers could not be unambiguously characterized. n.d.: not determined.

Scheme 3
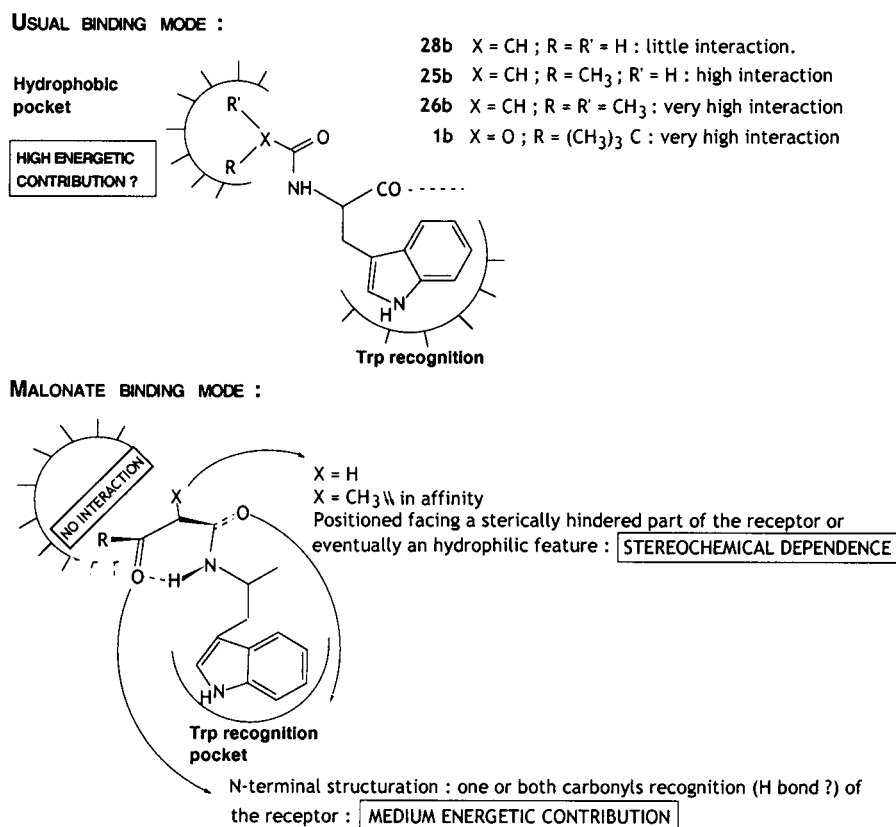
Alkylated N-Terminal Derivatives. To fully understand the influence of a carbonyl functionality at a γ position from the Trp NH (Scheme 3), a second series of derivatives of **I** was prepared, with alkyl or amine N-terminal substituents. In the alkyl series, compound **28b** (R = H$_3$C-CO) curiously exerted only a weak affinity (228 nM) whereas Ac-CCK-4 is a potent CCK$_2$ agonist. This is all the more surprising as the substitution of

Met by (NMe)Nle in the CCK-4 series generally increases the affinity for the CCK$_2$ receptor, as exemplified by the comparison of **1b** (K_i = 2.6 nM) with Boc-CCK-4 (K_i = 9.4 nM). However, increasing the alkyl chain length improves the affinity, as proved by the sequence **28b** (R = acetyl, K_i = 228 nM); **25b** (R = propionyl, K_i = 4.35 nM); **1b** (R = *tert*-butoxycarbonyl, K_i = 2.6 nM). Conversely, the affinity of positively charged derivatives

of **I** decreases when the distance from the NH Trp to the N-terminal charge is increased: this is shown by the sequence **1c** (R = H, K_i = 2.6 nM); **12b** (R = Gly, K_i = 18 nM); **17c** (R = βAla, K_i = 95 nM). Compound **12b** (Gly-Trp-(NMe)Nle-Asp-Phe-NH$_2$) had already been described with an affinity for rat cortex of 3 nM. Surprising as it may be, the 6-fold difference found here in CHO cells is not fully inconsistent, since variations between biological models have often been observed, as exemplified by the 4-fold difference in affinity of the reference antagonist L-365,260 for rat cortex (3.9 nM) and CHO cells (15 nM).

These two series of results strongly tend to indicate the existence of an interaction of N-terminal hydrophobic moieties with a hydrophobic pocket at this level of the receptor. Thus, the conformational restraint induced by (NMe)Nle would prevent recognition of this pocket by the acetyl moiety, while propionyl and Boc are long enough to counteract this effect. This would explain why only **28b** (R = CH$_3$CO) is far less efficient than its Met-containing analogue. On the contrary, a positively charged N-terminus is only tolerated when it does not produce too much unfavorable hydrophilic/hydrophobic repulsion, that is to say when the amine moiety may be far enough from this putative hydrophobic pocket. However, this interpretation may seem inconsistent with the ability of hydrophilic, acid-N-terminal derivatives such as **2b** (R = HO$_2$C−CH$_2$CO) to bind with the highest affinities to the receptor, unless these compounds have a specific binding mode. Such an hypothesis necessitated further inquiries on the role of the γ-carbonyl or eventually of the sequence CO-CH$_2$-CO, which is specific of BC 264 in the octapeptide series and hence a major point of interest.

N-Terminal Branched Analogues. Therefore, compound **4b** (R = HO$_2$C-CH(CH$_3$)CO) was prepared in order to study the influence of a substitution at the β position (toward the NH Trp). Astonishingly, the introduction of a methyl substituent produced a considerable (more than 200-fold for the better diastereomer, 4000-fold for the other) loss of affinity as compared to **2b**. This was all the more surprising as several potent CCK$_2$ ligands are substituted at this position, which is the case for BC 197. In particular, substitution of Gly[29] by Ala or D-Ala in the CCK-8 series did not induce such a decrease in affinity, but only a 2- and 5-fold loss, respectively.[22a,b] Consequently, the compounds **13b** and **14b** were prepared, with Ala and D-Ala replacing the N-terminal methylmalonate. Both pentapeptides retained a good affinity (20 and 15 nM, respectively) for CCK$_2$ receptors, and they did not show any significant difference with their glycine-containing analogue **12b** (see Table 3). Taken together, these results again indicate a very original behavior of malonate-containing derivatives of **1b**: in fact, the β position is crucial only for the latter whereas it seems of little importance for fully peptidic derivatives.

Thus, two hypothesis may be inferred here about the role of the N-terminal carbonyl group. On one hand, it could participate in the structuration of the ligand in a particular way; on the other hand, it could recognize a particular feature of the receptor. In the first case, substituting the β position by a methyl could prevent the convenient folding and thus any recognition of the

receptor. In the second case, such a substitution could lead to steric repulsion between the methyl and a neighboring group in the receptor. The 20-fold (at least) difference in affinity between the two isomers of **4b** speaks rather for the second hypothesis, since the spatial position of the features of the ligand is generally crucial for receptor recognition. The structural hypothesis presented in these sections are schematized in Scheme 3.

Aspartate Derivatives. Finally, an attempt was made to combine the favorable hydrophobic features found in **17b** (R = Boc-βAla, K_i = 1.35 nM) to the hydrophilic malonates, with the introduction of an aspartyl residue at the N-terminus. The amide functionality was left unprotected in **15b** (R = L-Asp, K_i = 4.8 nM) and **16b** (R = D-Asp, K_i = 11.9 nM) (which can be considered as hybrids of **8b** (R = HO$_2$C-(CH$_2$)$_2$-CO, K_i = 3 nM) and **12b** (R = H$_2$N-CH$_2$-CO, K_i = 18 nM)) whose affinities were little different from those of the related compounds.

Protection of the amine with a Boc group was hoped to significantly improve the affinity of these pentapeptides, as in **17b** (R = Boc-βAla, K_i = 1.35 nM) versus **17c** (R = βAla, K_i = 95 nM). Unfortunately, the four Boc-aspartate-containing compounds **19b**−**22b** had all comparable affinities (Table 3), suggesting that the favorable effects of a N-terminal hydrophobic group and of a second carbonyl at the same level cannot be added, presumably because the binding of either feature to the corresponding sites of the receptor furthers the other off its own binding pocket. The negligible effect of the configuration of the chiral carbons on the affinity further supports this hypothesis, since it proves that neither compound is able to simultaneously recognize two (or more) essential regions of the receptor.

Additional Implications for the Receptor Binding Requirements. The properties of the aspartate derivatives compared to that of, on one hand, the alkyl analogues (**1b** (R = Boc)-**28b** (R = CH$_3$CO)-**25b** (R = CH$_3$CH$_2$CO)) and, on the other hand, the malonate ones (**2b** (R = HO$_2$C-CH$_2$CO), **8b** (R = HO$_2$C-(CH$_2$)$_2$-CO), and **4b** (R = HO$_2$C−CH(CH$_3$)CO), in particular) deserve a more accurate discussion. It seems surprising that aspartate derivatives, which can be seen also as branched succinates, are tolerated by the receptor while the substitution of the β position by a methyl (compound **8b**) was forbidden. This must mean that the binding feature crucial for the high affinity of aspartate derivatives **19b**−**22b** is the Boc group and that the resulting spatial arrangement does not lead to the same orientation of the carboxylate as in **2b** and thus avoids the steric hindrance at the β position seen in **4b**. Schematically, as outlined in Scheme 4, since it appears as impossible to recognize both features of the receptor, the ligand makes the choice of the hydrophobic pocket as a priority over the feature binding of the malonate (or more generally hydrogen bond-accepting) derivatives. In fact, the thermodynamic contribution of the hydrophobic interactions at this level of the receptor might override that of the malonate recognition, but the steric constraints involved in the hydrophilic recognition may also force the ligand to adopt another fit.

This suggests that the "default" binding mode of the ligands to the CCK$_2$ receptor is that described for
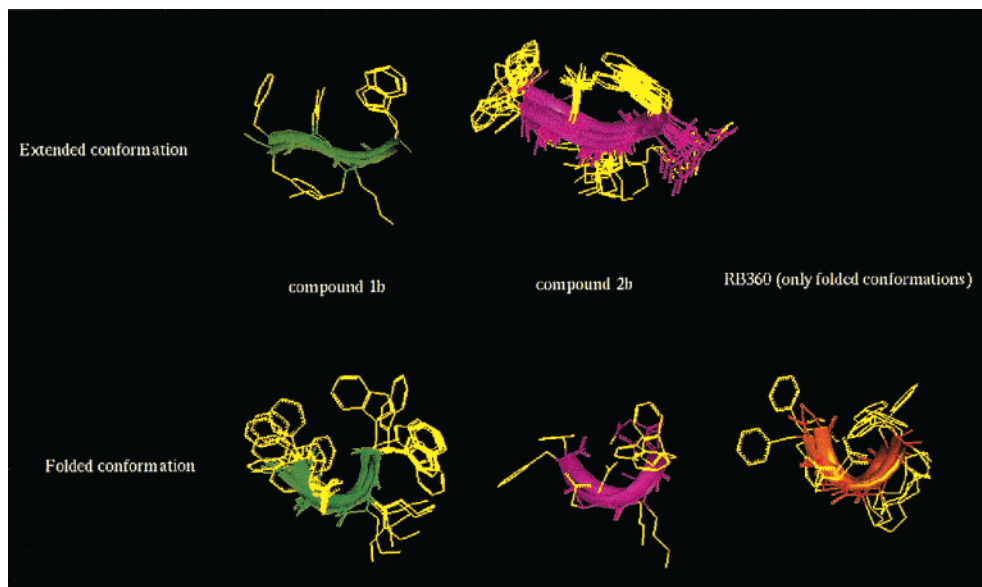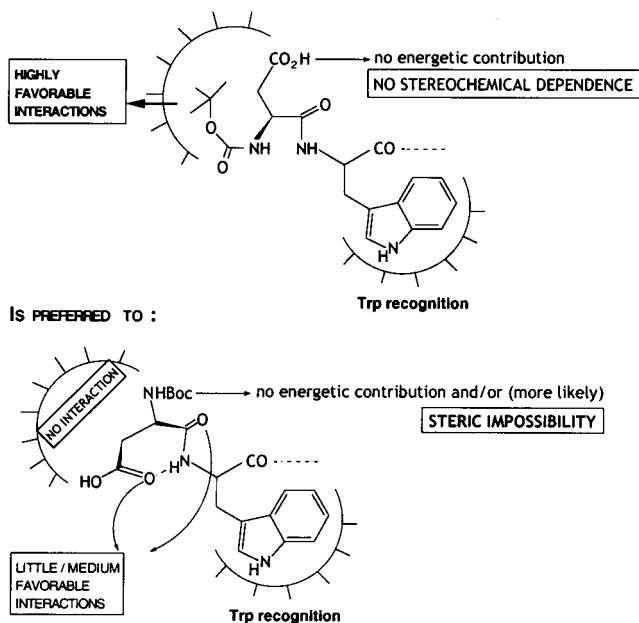
Figure 1. Conformational analysis of compounds **1b**, **2b**, and RB 360 (cyclic analogue of **1b**). It clearly appears that extended conformations are more important in the case of **2b** and folded ones in the case of **1b**. Only folded conformations are found in the case of the cyclic RB 360.

Scheme 4

compound **1b**, which could explain that BC 264 had remained an exception for such a long time.

Structural Studies. Following the structure−affinity results suggesting a differential binding mode for the malonate derivatives derived from **1b** (Boc-Trp-(NMe)Nle-Asp-Phe-NH₂), structural investigations were conducted on **2b** (HO₂CCH₂CONH-Trp-(NMe)Nle-Asp-Phe-NH₂) to further characterize a putative specificity of this lead compound. Unfortunately, 2D NMR experiments, performed under the conditions that had given NOE data for **1b**,[23] did not give any precise information about the position of the carboxylate which seems to be crucial in the affinity of **2b** for CCK₂ receptors. However, our results again support the hypothesis of a differential behavior for **1b** and **2b** recognition: indeed, the absence in the NMR spectra of **2b** of NOE cross-peaks, which

characterized the folded structure of **1b**, proves that **2b** adopts a different, presumably unfolded conformation. Very interestingly, this is also the case for BC 264, whose dominant conformations are likely to be unfolded. Computer-aided molecular studies were also performed on **1b** and **2b**, involving energetic minimization followed by conformational analysis. Interestingly, the super-position of the backbones of the resulting conformers suggested the occurrence of two families of conformations, extended and folded (Figure 1). The folded conformations seemed to be predominant in **1b** while the extended ones would be more populated in **2b**, in accordance with NMR data. As expected, the analysis of the cyclic derivative of **1b**, RB 360 (whose formula and properties are recalled in Table 1), showed only the folded structures, which confirms the validity of our approach.

Conclusion

The search for new CCK₂ agonists possessing the desirable pharmacological properties of the well-described compound BC 264 has led us to an extensive structure−affinity study of the role of the N-terminal residue in CCK-derived pentapeptides. Introduction of derivatives of malonic acid has led to highly selective CCK₂ agonists, eventually endowed with subnanomolar affinities. Most interestingly, some of the new compounds have been shown to bind to the receptor in an unusual way, which can be thought to be that of BC 264. This resemblance is supported by NMR and computer-aided structural studies, confirming a similar behavior of malonate-containing pentapeptides and BC 264. The results presented above support the proposal of two binding modes to CCK₂ receptors: one chosen by CCK-4-derived ligands possessing an hydrophilic N-terminus, the other being that of hydrophobic analogues. Among the two binding modes characterized, hydrophobic interactions seem to be dominant in the recognition of all formerly known ligands and should have priority on hydrophilic recognition. This hydrophilic mode probably characterizes BC 264 and mal-

onate-containing derivatives such as **2b**. Structural hypotheses are described, which suggest how this can occur. In particular, NMR and computer-aided analysis support the assumption of a greater flexibility of **2b** and its analogues, maybe authorizing a particular adaptation process to the agonist binding pocket of the CCK_2 receptor. Taken together, this yields highly valuable information about the CCK ligand−receptor interactions.

Thus, the new compounds with dicarbonyl moieties on the N-terminal of Trp-(NMe)Nle-Asp-Phe-NH$_2$, presented here, are greatly expected to exert the same favorable pharmacological effects as BC 264. Their pharmacological profile is now under study; preliminary results indicated that compound **2b** is devoid of anxiogenic effects in the (+)-maze and able to increase the spontaneous alternation of rats in the Y-maze at a dose of 0.3 mg/kg. These results, analogous to those obtained with BC 264, suggest that **2b** exerts a "CCK_{2B}" pharmacology. Further studies on this compound and its analogues are now requested, together with extended structural investigation, to fully understand this unusual behavior.

Experimental Section

Chemistry. Amino acids and derivatives were purchased from Bachem (Switzerland), BOP was from Novabiochem, and other reagents from Aldrich (France) or Acros (Belgium). Solvents were from SDS (France).

Commercial chemicals were used without further purification. Anhydrous solvents were used when necessary after drying over 4 Å molecular sieves. TLC were performed on Merck plates precoated with F254 silica gel, 0.2 mm. Plates were developed with UV, iodine vapor, TFA/ninhydrin, and Ehrlich's reagent. Column chromatography was performed using Merck silica gel (230−400 mesh). Final compounds were purified by semipreparative HPLC on a Waters apparatus (column Vydac C$_{18}$, 300 Å, 10 × 250 mm), using the following solvent system {A: H$_2$O + 0.1% TFA, B: CH$_3$CN/H$_2$O/TFA 70/30/0.09 v/v/v}, flow rate: 2 mL/min, detection 210 nm.

The structures of all compounds were confirmed by ^{1}H NMR spectroscopy in DMSO-d_6, with concentrations ranging 3−5 mM. Chemical shifts were measured in ppm with TMS as internal standard. The purity of the final compounds was checked by HPLC (Shimadzu apparatus, Vydac C$_{18}$ column, 300 Å, 4.6 × 150 mm) with the same solvent system as that for semipreparative purifications, flow rate 1 mL/min, UV detection 214 nM. Compounds **I** and **II** were prepared as previously described.[18]

Mass spectra were performed by Quad Service (Poissy, France) by electrospray techniques using acetonitrile/water/acetic acid (50/50/0.1, v/v/v) as solvent.

Abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxy-tris(dimethylamino) phosphonium-hexafluorophosphate; CCK, cholecystokinin; CHO cells, Chinese hamster ovary cells; DIEA, diisopropyl ethylamine; NOE, nuclear Overhauser effect; pCCK-8, propionyl-CCK-8, TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

Procedure A. Preparation of the Monoesters of Malonic Acid. 1. Malonic Acid, Monobenzyl Ester.[19a] A solution of 5 g of dibenzylmalonate (17.59 mmol) in 50 mL of benzyl alcohol cooled to 0 °C were treated with 1.01 g of potassium hydroxide (18.03 mmol, 1.025 equiv) dissolved in 200 mL of benzyl alcohol under vigorous stirring. The reaction was complete after 2 days, with formation of a white precipitate. This precipitate was filtered off, washed with Et$_2$O, and redissolved in 100 mL of water. The aqueous solution was acidified to pH 2 and extracted with 2 × 70 mL of ether. The resulting organic phases were washed with water and brine,

dried over Na$_2$SO$_4$, and evaporated to dryness, yielding 2.66 g (78%) of a yellow oil, crystallizing to a white solid. ^{1}H NMR (DMSO + TFA) δ 3.43 (2 H, s, COCH$_2$CO), 5.12 (2 H, s, CH$_2$-Ph), 7.37 (5 H, m, Ar).

2. Malonic acid monomethyl ester and methylmalonic acid monoethyl ester were prepared in the same manner from dimethyl malonate and diethyl methylmalonate using methanol and ethanol as solvents, respectively, instead of benzyl alcohol.

Procedure B. Malonic Acid Monoamide, Sodium Salt.[19b] A total of 7.5 mL (70.5 mmol) of ethyl cyanoacetate was dissolved in 4.125 mL of ethanol (70.5 mmol, 1 equiv). The mixture was put under a slow stream of hydrogen chloride during 16 h, with formation of a white precipitate. This precipitate was filtered off, washed with ethanol and dried in a vacuum to yield 4.583 g (33%) of a solid, which was pyrolyzed at 115 °C in an oil bath. A total of 500 mg of the resulting product was redissolved in acetone, filtered, and evaporated to dryness to yield an oil, which crystallized quickly to a beige solid (284 mg, 85%), identified as malonic acid monoethyl ester, monoamide. The latter product was saponified with sodium hydroxide to yield quantitatively the desired malonic acid monoamide, sodium salt.

Procedure C. Malonic Acid, Substituted Monoamide. A solution of malonic acid monobenzyl ester (370 mg, 1.905 mmol), in 5 mL of DMF, was treated at 0 °C with benzylamine (210 μL, 1.905 mmol, 1 equiv), BOP (1.011 g, 2.286 mmol, 1.2 equiv), and diisopropyl ethylamine (1.17 mL, 6.671 mmol, 3.5 equiv), and the mixture was allowed to stand overnight at room temperature. After evaporation of the solvents, the residue was redissolved in ethyl acetate, washed with aqueous citric acid 10%, water, sodium hydrogenocarbonate 10%, water, brine. The resulting organic phase was dried over sodium sulfate and evaporated to dryness giving 450 mg (90%) of malonic acid benzyl ester, benzylamide. The latter was dissolved in 5 mL of methanol, which was added to a suspension of 100 mg of Pd 10% on charcoal in 5 mL of methanol. The mixture was stirred overnight under hydrogen, then filtrated on Celite and evaporated to dryness, giving 210 mg of the expected substituted monoamide of malonic acid (76%).

General Procedure for the Preparation of Pentapeptides from I: Malonyl-Trp-(NMe)Nle-Asp-Phe-NH$_2$ (2b). A total of 750 mg (0.94 mmol) of TFA·H-Trp-(NMe)Nle-Asp-(OBzl)Phe-NH$_2$ (**I**) was dissolved in 15 mL of anhydrous DMF with 182 mg (0.94 mmol, 1 equiv) of malonic acid, monobenzyl ester, prepared according to procedure A. To this cooled (0 °C) stirred solution, were added 457 mg (1.03 mmol, 1.1 equiv) of BOP and 654 μL (3.76 mmol, 4 equiv) of DIEA.

The mixture was allowed to stand overnight at room temperature, then concentrated. The residue was redissolved in 50 mL of ethyl acetate and washed successively with water (50 mL), 10% aqueous citric acid (3 × 50 mL), water (50 mL), 10% aqueous sodium hydrogenocarbonate (3 × 50 mL), water (2 × 50 mL), and brine. The resulting organic phase was dried over sodium sulfate and evaporated to dryness, yielding 811 mg (99%) of the intermediate malonyl-Trp-(NMe)Nle-Asp-(OBzl)-Phe-NH$_2$.

A solution of 800 mg of the latter (0.92 mmol) in 10 mL of ethanol was added to an activated suspension of Pd/C (55 mg, 60 mg/mmol) in 5 mL of methanol. The mixture was stirred under an H$_2$ atmosphere during 24 h, then filtrated on Celite and evaporated. The residue was purified by HPLC and lyophilized to a white powder.

Biological Assays. Binding experiments and inositol phosphate assays were performed under the same conditions as described previously.[16]

Molecular Design. The calculations were performed on Silicon Graphics workstations using the Insight/Discover software package (BIOSYM Technologies Inc., San Diego). The initial structures were built using standard parameters for amino acids supplied with the software and were submitted to a first energy minimization cycle (200 steps using the steepest descents algorithm followed by 1000 steps using the conjugate gradient algorithm).

The conformational analysis was then performed using high temperature (1000 K) simulated annealing. The structures obtained thereafter were refined by using the minimization protocol described initially. This was repeated 100 times, and the 100 minimized structures were clustered using the RMS superposition method, according to conformational similarities.

NMR Studies. NMR experiments were conducted under the same conditions and with the same solvent systems as described in ref 23.

Supporting Information Available: Analytical data of the final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Reviewed in (a) Noble, F.; Wank, S. A.; Crawley, J. N.; Bradwejn, J.; Seroogy, K. B.; Hamon, M.; Roques, B. P. Structure, distribution and functions of CCK receptors using the rational nomenclature defined by the International Union of Pharmacology. *Pharmacol. Rev.* **1999**, *51* (4), 745−781. (b) Crawley, J. N.; Corwin, R. L. Biological actions of cholecystokinin. *Peptides* **1994**, *15*, 731−755.
(2) Reviewed in Van Megen, H.; Westenberg, H.; den Boer, J.; Kahn, R. Cholecystokinin in anxiety. *Eur. Neuropsychopharmacol.* **1996**, *6*, 263−280.
(3) Faris, P. L.; Komisaruk, B. R.; Watkins, L. R.; Mayer, D. J. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science* **1983**, *219*, 310−312.
(4) Itoh, S.; Lal, H. Influences of cholecystokinin and analogues on memory processes. *Drug Dev. Res.* **1996**, *21*, 257−276.
(5) Reviewed in Daugé, V.; Roques, B. P. Reward versus anxiety: The opposite pharmacology of opioids and CCK. *In cholecystokinin and Anxiety from Neuron to Behaviour*, Molecular Biology Intelligence Unit; Bradwejn, J., Vasar, E., Eds.; Springer Press R. G. Landes Co.: Austin, TX, 1995; Chapter 8, pp 151−171.
(6) Crawley, J. N. Subtype-selective cholecystokinin receptor antagonists block cholecystokinin modulation of dopamine-mediated behaviours in the rat mesolimbic pathway. *J. Neurosci.* **1992**, *12*, 3380−3391.
(7) Maldonado, R.; Derrien, M.; Noble, F.; Roques, B. P. Association of the peptidase inhibitor RB 101 and a selective CCK-B antagonist strongly enhances antinociceptive responses. *Neurol. Rep.* **1993**, *4*, 947−950.
(8) Rex, A.; Fink, H. Effects of cholecystokinin-receptor agonists on cortical 5-HT release in guinea pigs on the X-maze. *Peptides* **1998**, *19* (3), 519−526.
(9) (a) Durieux, C.; Coppey, M.; Zajac, J. M.; Roques, B. P. Occurrence of two cholecystokinin binding sites in guinea-pig brain cortex. *Biochem. Biophys. Res. Commun.* **1986**, *137*, 1167−1173. (b) Knapp, R. J.; Vaughn, L. H.; Fang, S.; Bogert, C. L.; Yamamura, M. S.; Hruby, V. J.; Yamamura, H. I. A new highly selective CCK-B receptor radioligand ([³H][*N*-methyl-Nle²⁸,³¹]-CCK₂₆₋₃₃): Evidence for CCK-B receptor heterogeneity. *J. Pharmacol. Exp. Ther.* **1990**, *255*, 1278−1286. (c) Huang, S.; Fortune, K. P.; Wank, S. A.; Kopin, A. S.; Gardner, J. D. Multiple affinity states of different cholecystokinin receptors. *J. Biol. Chem.* **1994**, *269*, 26121−26126. (d) Harper, E. A.; Roberts, S. P.; Shankley, N. R.; Black, J. W. Analysis of variation in L-365,260 competition curves in radioligand binding assays. *Br. J. Pharmacol.* **1996**, *118*, 1717−1726. (e) Bellier, B.; McCort-Tranchepain, I.; Ducos, B.; Danascimento, S.; Meudal, H.; Noble, F.; Garbay, C.; Roques, B. P. Synthesis and biological properties of new constrained CCK-B antagonists: discrimination of two affinity states of the CCK-B receptor on transfected CHO cells. *J. Med. Chem.* **1997**, *40*, 3947−3956.
(10) Leinre, S.; Schoneberg, T.; Meyer, R.; Martin, H.; Kaufmann, R. Pharmacological characterization of CCK-B receptors in human brain: No evidence for receptor heterogeneity. *Neurosci. Lett.* **1996**, *217*, 45−49.
(11) Charpentier, B.; Dor, A.; Roy, P.; England, P.; Pham, I.; Durieux, C.; Roques, B. P. Synthesis and binding affinities of cyclic and related linear analogues of CCK₈ selective for central receptors. *J. Med. Chem.* **1989**, *32*, 1184−1190.

(12) Charpentier, B.; Durieux, C.; Pélaprat, D.; Dor, A.; Reibaud, M.; Blanchard, J. C.; Roques, B. P. Enzyme-resistant CCK analogues with high affinities for central receptors. *Peptides* **1988**, *9*, 835−841.
(13) Derrien, M.; McCort-Tranchepain, I.; Ducos, B.; Roques, B. P.; Durieux, C. Heterogeneity of CCK−B receptors involved in animal models of anxiety. *Pharmacol. Biochem. Behav.* **1994**, *49*, 133−141.
(14) Dellu, F.; Mayo, W.; Cherkaoui, J.; LeMoal, M.; Simon, H. A two trial memory task with automated recording: study in yound and aged rats. *Brain Res.* **1992**, *588*, 132−139.
(15) (a) Ladurelle, N.; Keller, G.; Blommaert, A.; Roques, B. P.; Daugé, V. The CCK−B agonist, BC 264, increases dopamine in the nucleus accumbens and facilitates motivation and attention after intraperitoneal injection in rats. *Eur. J. Neurosci.* **1997**, *9*, 1804−1814. (b) Léna, I.; Simon, H.; Roques, B. P.; Daugé, V. Opposing effects of two CCK-B agonists on the retrieval phase of a two-trial memory task after systemic injection in the rat. *Neuropharmacology* **1999**, *38* (4), 543−553. (c) Ladurelle, N.; Sebret, A.; Garbay, C.; Roques, B. P.; Daugé, V. Opposite effects of CCK-B agonists in grooming behaviour in rats: further evidence for two CCK-B subsites. *Br. J. Pharmacol.* **1998**, *124*, 1091−1098. (d) Léna, I.; Roques, B. P.; Durieux, C. Dual modulation of dopamine release from anterior nucleus accumbens through cholecystokinin-B receptor subsites. *J. Neurochem.* **1997**, *68*, 162−168. (e) Ladurelle, N.; Keller, G.; Roques, B. P.; Daugé, V. Effects of CCK-8 and of CCK-B selective agonist BC 264 on extracellular dopamine content in the anterior and posterior nucleus accumbens: A microdialysis study in freely moving rats. *Brain Res.* **1993**, *628*, 254−262.
(16) Blommaert, A.; Dhotel, H.; Ducos, B.; Durieux, C.; Goudreau, N.; Bado, A.; Garbay, C.; Roques, B. P. Structure-based design of new constrained cyclic agonists of the cholecystokinin CCK-B receptor. *J. Med. Chem.* **1997**, *40*, 647−658.
(17) Million, M.-E.; Léna, I.; DaNascimento, S.; Noble, F.; Daugé, V.; Garbay, C.; Roques, B. P. Development of new potent agonists able to interact with two postulated subsites of the cholecystokinin CCK-B receptor. *Lett. Pept. Sci.* **1997**, *4*, 407−410.
(18) Corringer, P. J.; Weng, J. H.; Ducos, B.; Durieux, C.; Boudeau, P.; Böhme, A.; Roques, B. P. CCK-B agonist or antagonist activities of structurally hindered and peptidase-resistant Boc-CCK₄ derivatives. *J. Med. Chem.* **1993**, *36*, 166−172.
(19) (a) Curtius, T.; Sieber, W. Umwandlung von alkylierten Malonsäuren in α-Amino-saüren, II. Mitteilung. *Ber. Dtsch. Chem. Ges.* **1922**, *55*, 1543. (b) Pinner, A. Ueber Imidoäther. *Ber. Dtsch. Chem. Ges.* **1895**, *28*, 473−488. (c) Flavin, M. Preparation of O-succinyl-L-homoserine. *Methods Enzymol.* **1971**, *17B*, 418. (d) Liwschitz, Y.; Zilkha, A. Syntheses of β-DL-aspartyl peptides with maleic anhydride. *J. Am. Chem. Soc.* **1955**, *77*, 1265−1266.
(20) Ron, D.; Gilon, C.; Hanani, M.; Vromen, A.; Selinger, Z.; Chorev, M. N-methylated analogues of Ac[Nle²⁸,³¹]CCK₍₂₆₋₃₃₎: Synthesis, activity and receptor selectivity. *J. Med. Chem.* **1992**, *35*, 2806−2811.
(21) Hruby, V. J.; Fang, S.; Knapp, R.; Kazmierski, W.; Lui, G. K.; Yamamura, H. I. Cholecystokinin analogues with high affinity and selectivity for brain membrane receptors. *Int. J. Pept. Protein Res.* **1990**, *35*, 566−573.
(22) (a) Knight, M.; Barone, P.; Tamminga, C. A.; Steardo, L.; Chase, T. N. Cholecystokinin octapeptide analogues stable to brain proteolysis. *Peptides* **1985**, *6*, 631−634. (b) Holladay, M. W.; Bennett, M. J.; Tufano, M. D.; Lin, C. W.; Asin, K. E.; Witte, D. G.; Miller, T. R.; Bianchi, B. R.; Nikkel, A. L.; Bednarz, L.; Nadzan, A. M. Synthesis and biological activity of CCK heptapeptide analogues. Effects of conformational constraints and standard modifications on receptor subtype selectivity, functional activity in vitro and appetite suppression in vivo. *J. Med. Chem.* **1992**, *35*, 2919−2928.
(23) Goudreau, N.; Weng, J. H.; Roques, B. P. Conformational analysis of CCK−B agonists using ¹H NMR studies and restrained molecular dynamics: Comparison of biologically active Boc-Trp-(NMe)Nle-Asp-Phe-NH₂ and inactive Boc-Trp-(NMe)-Phe-Asp-Phe-NH₂. *Biopolymers* **1994**, *34*, 155−169.
(24) Harhammer, R.; Schäfer, U.; Henklein, P.; Ott, T.; Repke, H. CCK₈-related C-terminal tetrapeptides: Affinities for central CCK-B and peripheral CCK-A receptors. *Eur. J. Pharmacol.* **1991**, *209*, 263−266.