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PSEUDOPEPTIDE CCK-4 ANALOGUES INCORPORATING THE Ψ[CH(CN)NH] PEPTIDE BOND SURROGATE

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Abstract: The synthesis, binding to CCK receptors, and *in vitro* functional activity of pseudopeptide CCK-4 analogues incorporating the (*R*) or (*S*) Ψ[CH(CN)NH] peptide bond surrogate at the Nle^{31}-Asp^{32} or Trp^{30}-Nle^{31} bonds are described. Z-TrpΨ[(*S*)CH(CN)NH]Nle-Asp-Phe-NH_2 retained the high CCK-B receptor binding affinity of Boc-[Nle^{31}]-CCK-4, and was a potent and selective CCK-B antagonist in the isolated guinea pig ileum.

Isosteric peptide bond replacements in biologically active peptides have been widely used to increase their metabolic stability and as a step towards enzyme inhibitors and peptidomimetics.[1] On the basis of semiempirical quantum mechanic calculations, we suggested that the [CH(CN)NH] group could be a good peptide bond surrogate,[2] and, consequently, we developed a general method for the synthesis of cyanomethyleneamino pseudopeptides.[3] Biological data of neurotensin analogues incorporating this surrogate supported our hypothesis.[4] In order to further investigate the utility of this peptide bond replacement, we have now explored the extension of this approach to cholecystokinin (CCK). CCK represents a family of related peptides found in the periphery and in the central nervous system as a hormone and as a neurotransmitter/neuromodulator.[5] There are at least two subtypes of receptors for CCK, namely CCK-A, found predominantly in peripheral tissues, and CCK-B, localised in the central nervous system.[6]

We herein describe the synthesis, binding affinity for CCK receptors, and *in vitro* functional activity in the isolated guinea pig ileum of the *N*-protected pseudotetrapeptides Boc-Trp-NleΨ[CH(CN)NH]Asp-Phe-NH_2 [(***R***)- and (***S***)-**7**, scheme 1] and Z-TrpΨ[CH(CN)NH]Nle-Asp-Phe-NH_2 [(***R***)- and (***S***)-**11a**, scheme 2], analogues of the CCK *C*-terminal tetrapeptide (CCK-4, H-Trp^{30}-Met^{31}-Asp^{32}-Phe^{33}-NH_2), which is the minimal sequence with high affinity for CCK-B receptors. Moreover, as the Trp and Phe residues are considered essential structural requirements for CCK-4 recognition,[5,7] we have also prepared the shorter analogues (***R***)- and (***S***)-**10c**, and (***R***)- and (***S***)-**11b** (scheme 2). It has been shown that the replacement of Met^{31} by Nle or Leu has not significant influence on the biological activity of CCK-4 analogues.[5] Therefore, in order to avoid the Met instability, this residue has been replaced by Nle. Since *N*-acylation (Boc, Z, and Ac) in CCK analogues increases resistance to enzymatic hydrolysis, and, usually, leads to compounds of enhanced potency,[5] Boc- and Z-protected pseudopeptides have been prepared as final compounds .

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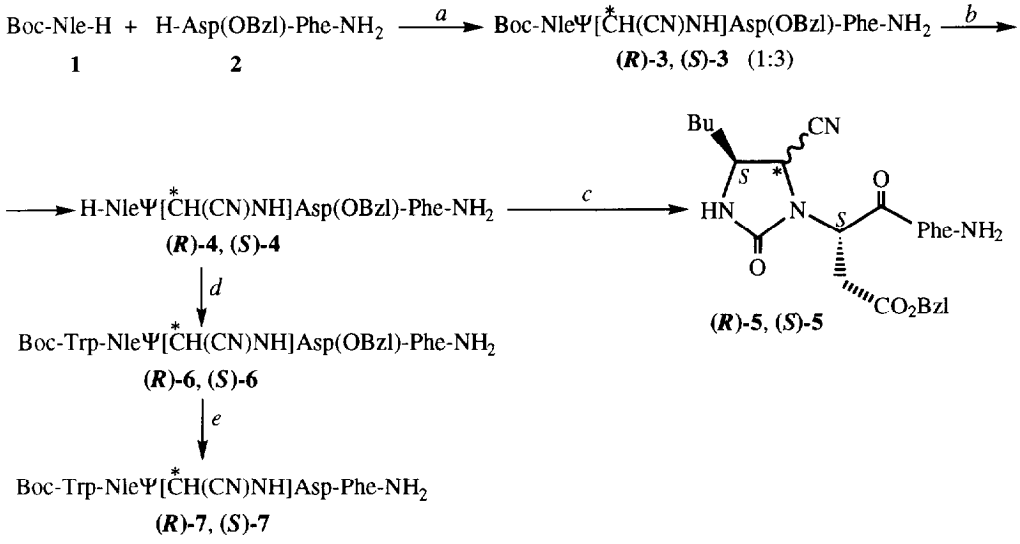
Chemistry

As indicated in scheme 1, pseudotetrapeptides (***R***)- and (***S***)-**7**, epimers at the Ψ[CH(CN)NH] stereogenic centre, were synthesised in solution, using Boc and Bzl protecting groups for the α-amino groups and the Asp side chain, respectively, and standard methods for peptide bond couplings. The peptide bond surrogate was introduced into pseudotripeptides (***R***)- and (***S***)-**3**, which were obtained in a (1:3) ratio in 65% overall yield, using our previously reported method.[3] This involved the $ZnCl_2$ catalysed addition of TMSCN to the *in situ* formed imine from Boc-Nle aldehyde (**1**) and dipeptide **2**.

Scheme 1



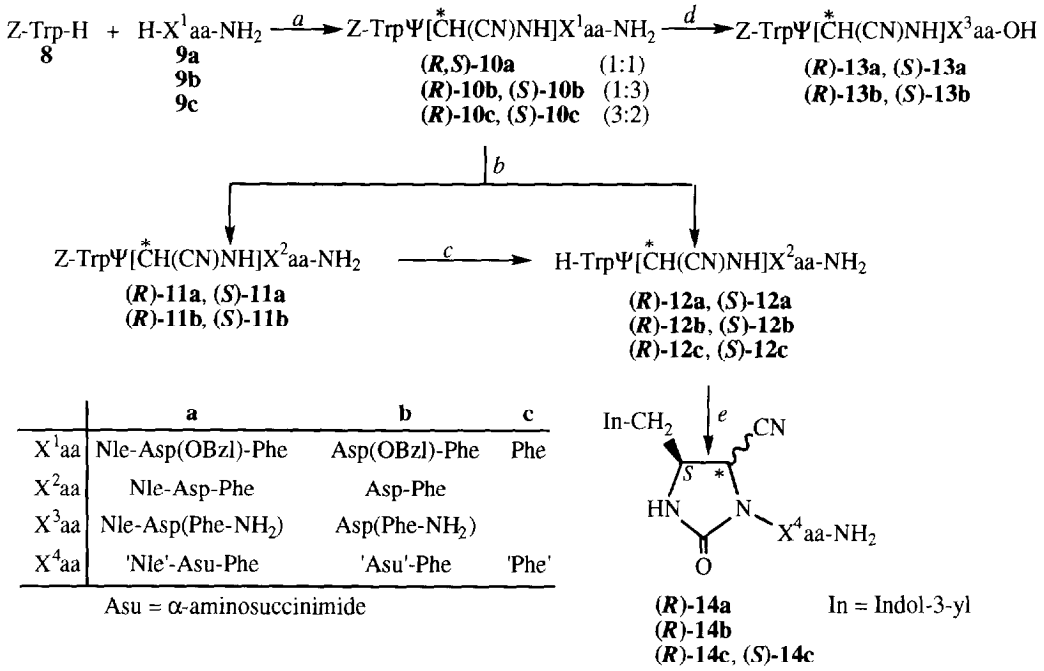
Reagents: (*a*) $ZnCl_2$, TMSCN (70%); (*b*) 3M HCl , EtOAc (92-100%); (*c*) $(Cl_3CO)_2CO$, TEA (50-80%); (*d*) Boc-Trp-OH, BOP, TEA (75-80%); (*e*) 10% Pd(C), H_2 (51-58%).

The configuration assignment at Ψ[CH(CN)NH] in the epimeric pseudotripeptides (***R***)- and (***S***)-**3**, and therefore in (***R***)- and (***S***)-**7**, was established by the imidazolidin-2-one ring H_4,H_5 coupling constant and NOE effects observed in the ^{1}H-NMR spectra of their respective derivatives (***R***)- and (***S***)-**5**. These derivatives were obtained by reaction of the *N*-deprotected pseudotripeptides **4** with bis(trichloromethyl)carbonate and triethylamine.[3] Boc deprotections were carried out with HCl in EtOAc to avoid *N*-terminus trifluoroacetylation[8] of (***R***)- and (***S***)-**4** , which took place when TFA was used.

Initially, the synthesis of pseudopeptides **10c**, **11a** and **11b** was also planned using Boc/Bzl protecting groups. However, due to the extreme susceptibility of the Trp residue of these CCK-4 analogues to oxidative degradation in acid media, it was not possible to carry out the final *N*-Boc deprotection, not even by using different described conditions to minimise this problem.[9] Therefore, since the Ψ[CH(CN)NH] configuration assignment requires *N*-deprotection for the subsequent formation of the corresponding imidazolidin-2-one derivative, the Boc group was replaced by the Z protection. The synthesis and configuration assignment for compounds **10c**, **11a** and **11b** is shown in scheme 2. Thus, TMSCN addition to the imine formed by *in situ*

reaction of Z-triptophanal (**8**) with tripeptide **9a** led to a (1:1) epimeric mixture of pseudotetrapeptides (***R,S*)-10a** in 90% yield, which could not be resolved. Since Z and Bzl are not orthogonal protections, the mild 10% Pd(C) catalysed hydrogenolysis (1 atm, room temperature) of (***R,S*)-10a** gave a mixture of the corresponding debenzylated and fully deprotected compounds (***R,S*)-11a** (50%) and (***R,S*)-12a** (25%), respectively, which after column chromatography and RPHPLC[10] was resolved into the four components. Z Removal in (***R*)-** and (***S*)-11a** yielded the respective deprotected pseudotetrapeptides (***R*)-** and (***S*)-12a**. Although the rapid treatment (30 min) of (***R,S*)-10a** with an equivalent of NaOH in (1:1) dioxane/H_2O removed the Asp side chain protection selectively, isomerization occurred quantitatively to provide the β-Asp containing pseudopeptides (***R*)-** and (***S*)-13a**. These compounds were identical to those obtained from (***R*)-** and (***S*)-11a**, respectively, after the same treatment with NaOH. In the reaction of the *N*-deprotected pseudotetrapeptides (***R*)-** and (***S*)-12a** with bis(trichloromethyl)carbonate, only the (*R*)-epimer gave the corresponding imidazolidin-2-one derivative (***R*)-14a**. Aspartimide formation at the Asp residue also took place in this compound. The assignment of (*R*) configuration to this epimer was based on the imidazolidin-2-one ring H_4,H_5 coupling constant (3 Hz).

Scheme 2



	a	b	c
X^1aa	Nle-Asp(OBzl)-Phe	Asp(OBzl)-Phe	Phe
X^2aa	Nle-Asp-Phe	Asp-Phe	
X^3aa	Nle-Asp(Phe-NH_2)	Asp(Phe-NH_2)	
X^4aa	'Nle'-Asu-Phe	'Asu'-Phe	'Phe'

Asu = α-aminosuccinimide

Reagents: (*a*) $ZnCl_2$, TMSCN (60-90%); (*b*) 10% Pd(C), H_2 (**11a,b**: 50%, **12a,b**: 25%); (*c*) 10% Pd(C), H_2 (100%); (*d*) NaOH (100%); (*e*) $(Cl_3CO)_2CO$, TEA (30-40%).

The shorter pseudopeptides (***R*)-, (*S*)-10c** and (***R*)-, (*S*)-11b** were obtained following a similar synthetic scheme. As in the case of pseudotetrapeptides **10a,** the Bzl removal in pseudotripeptides (***R*)-** and (***S*)-10b** by NaOH treatment also led to the complete isomerization of the α-Asp residue to β-Asp. All β-peptides (**13a,b**)

showed lower t_R in RPHPLC analysis[11] than their corresponding α-isomers (**11a,b**),[12] and a slight shielding of 0.07-0.24 ppm for the β-Asp 2-H in their ^{1}HNMR spectra.[13] The FABMS analysis of both α and β isomeric peptides produced the same $(M+H)^+$ ion.

Biological Activity

The target pseudopeptides (***R*)-** and (***S*)-7, -11a,b, -10c**, and those obtained containing β-Asp (***R*)-** and (***S*)-13a,b** were evaluated for their potency in displacing the binding of [^{3}H]propionyl-CCK-8 to CCK-A and CCK-B receptors, using rat pancreatic and cerebral cortex homogenates[14], respectively (Table 1). For comparative purposes CCK-8 and the dipeptoid CCK-B antagonist PD-135,158[15] were also included in the assay. The reported IC_{50} values for Boc-CCK-4 and Boc-[Nle^{31}]CCK-4 at guinea pig pancreatic (CCK-A) and cortical (CCK-B) receptors[16] are also shown in table 1.

Table 1.-Inhibition of specific [^{3}H]propionyl-CCK-8 binding to rat pancreas (CCK-A) and rat cerebral cortex membranes (CCK-B) by Ψ[CH(CN)NH] pseudopeptide CCK-4 analogues

Compd.	Structure	IC_{50}(nM)[a] CCK-A	CCK-B	A/B
CCK-8		1.08	6	0.18
Boc-CCK-4[b]		1800	25	72
Boc-[Nle^{31}]CCK-4[b]		4000	65	62
PD-135,158		1426	13	110
(*R*)-7	Boc-Trp-NleΨ[(*R*)CH(CN)NH]Asp-Phe-NH_2	10000	180	56
(*S*)-7	Boc-Trp-NleΨ[(*S*)CH(CN)NH]Asp-Phe-NH_2	10000	920	11
(*R*)-11a	Z-TrpΨ[(*R*)CH(CN)NH]Nle-Asp-Phe-NH_2	3846	953	4
(*S*)-11a	Z-TrpΨ[(*S*)CH(CN)NH]Nle-Asp-Phe-NH_2	1714	14.9	115
(*R*)-13a	Z-TrpΨ[(*R*)CH(CN)NH]Nle-Asp(Phe-NH_2)OH	483	45.3	11
(*S*)-13a	Z-TrpΨ[(*S*)CH(CN)NH]Nle-Asp(Phe-NH_2)OH	189	938	0.2
(*R*)-11b	Z-TrpΨ[(*R*)CH(CN)NH]Asp-Phe-NH_2	719	>10000	<0.07
(*S*)-11b	Z-TrpΨ[(*S*)CH(CN)NH]Asp-Phe-NH_2	344	>10000	<0.03
(*R*)-13b	Z-TrpΨ[(*R*)CH(CN)NH]Asp(Phe-NH_2)OH	272	8630	0.03
(*S*)-13b	Z-TrpΨ[(*S*)CH(CN)NH]Asp(Phe-NH_2)OH	305	>10000	<0.03
(*R*)-10c	Z-TrpΨ[(*R*)CH(CN)NH]Phe-NH_2	5220	>10000	<0.5
(*S*)-10c	Z-TrpΨ[(*S*)CH(CN)NH]Phe-NH_2	>10000	>10000	--

[a] Values are the mean of at least three experiments performed in triplicate (Standard errors within ± 10-15% of the mean).[b] Reported IC_{50} values at guinea pig cortical (CCK-B) and pancreatic (CCK-A) receptors.[16]

The replacement of the Boc-[Ne^{31}]-CCK-4 central peptide bond with a (*R*)- or (*S*)-Ψ[CH(CN)NH] surrogate led to a 3- and 14-fold decrease in the binding affinity of pseudopeptides **(*R*)-** and **(*S*)-7,** respectively, for CCK-B receptors. Also a 14-fold reduction in affinity for brain receptors was observed when the surrogate with (*R*) configuration was introduced at the Trp-Nle peptide bond in **(*R*)-11a**. In contrast, its epimer at the backbone modification **(S)-11a** displayed similar CCK-B binding potency (14.9 nM) to that of the dipeptoid PD-135,158 (13 nM), and in the same range to that reported for Boc-[Nle^{31}]-CCC-4 (65 nM).[16] Moreover, the

CCK-B selectivity of **(*S*)-11a** and PD-135,158 were almost twice that of Boc-[Nle^{31}]-CCK-4. These results suggest a higher susceptibility of the Boc-CCK-4 binding properties to backbone modifications at the central peptide bond that at the Trp-Nle bond.

In the case of β-pseudotetrapeptides **(*R*)-** and **(*S*)-13a**, the former retained the Boc-[Nle^{31}]-CCK-4 affinity for CCK-B receptors, but with a significant 5-fold decrease in CCK-B selectivity, while the epimer **(*S*)-13a** changed its preference to CCK-A receptors, displaying a modest affinity for these receptors (189 nM). The α- and β-pseudotripeptides **(*R*)-**, **(*S*)-11b** and **(*R*)-**, **(*S*)-13b** also showed modest CCK-A affinity and selectivity. Neither the surrogate configuration nor the presence of α- or β-Asp had significant influence in the binding properties of these compounds. Pseudodipeptides **(*R*)-** and **(*S*)-10c** did not bind to CCK receptors at concentrations below 10^{-5}M.

The CCK-4 analogues were tested for their antagonism to the contractions elicted by CCK-8 and CCK-4 in the isolated longitudinal muscle myenteric plexus preparations from guinea pig ileum.[17] In this assay CCK-8 produces a contractile effect mainly by stimulation of CCK-A and CCK-B receptors, whereas CCK-4 stimulates only the CCK-B receptor subtype. The dipeptoid PD-135,158 was also included for comparative purposes. Pseudopeptides which antagonised the CCK-8 or CCK-4 effect are shown in table 2. In agreement with the binding data, pseudotetrapeptides **(*S*)-11a** and **(*R*)-13a** were the most potent antagonists, and inhibited the CCK-4 induced contractions with pA_2 values of 8.0 and 7.1, respectively. Like PD-135,158, **(*S*)-11a** was a more selective CCK-B receptor antagonist by approximately three orders of magnitude. **(*S*)-11a** showed also an intrinsic contractile effect in the ileum preparation that was completely prevented by the CCK-B antagonist L-265,260 (10^{-6} M). Other CCK-4 analogues[18] as well as some dipeptoids such as PD-135,158[19] also behave as partial CCK-B agonists.

Table 2.- Antagonism to the contractions induced by CCK-8 and CCK-4 in guinea pig ileum longitudinal muscle

	CCK-8		CCK-4	
Compd.	Ant, %[a]	pA_2 (CL)[b]	Ant, %[a]	pA_2 (CL)[b]
(*R*)-7	20	--	48	--
(*S*)-7	23	--	52	--
(*R*)-11a	48	--	100	5.2 (4.5-5.4)
(*S*)-11a	78	5.1 (4.7-5.3)	100	8.0 (7.5-8.3)
(*R*)-13a	83	6.0 (5.5-6.3)	93	7.1 (6.5-7.4)
(*S*)-13a	70	--	56	--
PD-135,158	66	5.3 (4.9-5.6)	85	8.1 (7.9-8.3)

[a]Compounds initially tested at a fixed 10^{-5} M concentration for their antagonism to the contraction induced by CCK-8 (10^{-8} M) or CCK-4 (10^{-6} M.) Values are the mean of at least three experiments performed in triplicate (Standard errors within ± 10-15% of the mean).[b]confidence limits (95%) for pA_2 values.

In conclusion, pseudotetrapeptide Z-TrpΨ[(*S*)CH(CN)NH]Nle-Asp-Phe-NH_2 retains the Boc-[Nle^{31}]-CCK-4 receptor binding affinity, and appears to be a potent and selective CCK-B antagonist. This compound

could therefore be used to analyse, at the molecular level, the agonist and antagonist states of the CCK-B receptor, and could be of importance to investigate the occurrence and physiological relevance of CCK-B receptor subsites. Besides these findings, the results here reported show the utility of the Ψ[CH(CN)NH] group as an appropriate peptide bond surrogate.

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10. Preparative RPHPLC was performed on Delta-Pak C_{18} (25x100 mm, 15 μm, 300 Å) cartridges, with a 6.5 ml/min flow rate. Solution A was 0.05% TFA in H_2O, and solution B was CH_3CN.
11. Analytical RPHPLC was performed on a Nova-Pak C_{18} (3.9x150 mm, 4 μM, 60Å) column, with a 1ml/min flow rate. Solution A was 0.05% TFA in H_2O, and solution B was CH_3CN.
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