Synthesis and Biological Activities of Pseudopeptide Analogues of the C-Terminal Heptapeptide of Cholecystokinin. On the Importance of the Peptide Bonds

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A series of pseudopeptide analogues of the C-terminal heptapeptide of cholecystokinin in which each peptide bond, one at a time, has been replaced by a CH_2NH bond were synthesized: Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (1), Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (2), Z-Tyr(SO_3^-)-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (3), Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (4), Z-Tyr(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (5), Z-Tyr(SO_3^-)-Met-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (6), Z-Tyr(SO_3^-)-Met-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (7), Z-Tyr(SO_3^-)-Met-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (8). These derivatives were studied for their ability to stimulate amylase release from rat pancreatic acini and to inhibit the binding of labeled CCK-9 to rat pancreatic acini and to guinea pig brain membrane CCK receptors. They were compared to the potent CCK-8 analogue Boc-Asp-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2. All of these pseudopeptides were able to stimulate amylase secretion with the same efficacy as CCK-8 but with varying potencies. These compounds were also potent in inhibiting the binding of labeled CCK-9 to CCK receptors from rat pancreatic acini and from guinea pig brain membranes.

Cholecystokinin is a 33 amino acid peptide first isolated from hog intestine,[1] where it stimulates gastrointestinal motility and induces both gall bladder contraction and pancreatic amylase release.[2,3] Structure-activity relationship studies[4] on cholecystokinin have shown that the full spectrum of CCK-like biological activities can be reproduced by the C-terminal heptapeptide of CCK of structure Tyr(SO_3^-)-Met-Gly-Trp-Met-Asp-Phe-NH_2. In addition, CCK-8 was found in the brain,[5] where it functions as a neurotransmitter and neuromodulator.[6,7]

We recently showed the importance of the peptide bonds in the C-terminal tetrapeptide of gastrin for exhibiting biological activity on acid secretion and for binding to gastric mucosal cells.[8] Replacing a peptide bond by a CH_2NH bond in the C-terminal tetrapeptide of gastrin led to compounds having a high apparent affinity for the gastrin receptor but behaving either as gastrin agonists or antagonists on acid secretion, depending on which peptide bond was replaced. Gastrin and cholecystokinin have the same C-terminal pentapeptide amide fragment (-Gly-Trp-Met-Asp-Phe-NH_2), and they overlap in their biological activities but are modulated in their potency by different N-terminal extensions.[9,10] In order to evaluate the importance of the peptide bonds in the active fragment of CCK (e.g., the C-terminal heptapeptide, CCK-7), we synthesized five pseudopeptide analogues of CCK-7 in which each peptide bond, one at a time, has been replaced by a CH_2NH bond, "reduced peptide bond",[11] e.g., Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (1), Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (2), Z-Tyr(SO_3^-)-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (3), Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (4), and Z-Tyr(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (5). In these pseudopeptide analogues of the C-terminal heptapeptide of CCK, for easier syntheses and better stability of the ensuing compounds, the two methionine residues (Met^{28} and Met^{31}) have been replaced by two norleucines. This change has been shown in many cases not to influence the biological activities of the analogues.[12,13] As supplementary examples, we synthesized compounds Z-Tyr(SO_3^-)-Met-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (6), Z-Tyr(SO_3^-)-Met-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (7), and Z-Tyr(SO_3^-)-Met-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (8), in which only Met in position 31 has been replaced by Nle. The pseudopeptides we have synthesized were protected on their N-terminus by a benzyloxycarbonyl group (Z) because it had already been demonstrated that analogues of the C-terminal heptapeptide of CCK bearing an N^α protecting group were somewhat more potent and more stable than the heptapeptide analogues with a free α-amino group.[14] The replacement of a peptide bond by a CH_2NH bond does not really affect the conformation of the pseudopeptide as compared with that of the parent peptide.[26]

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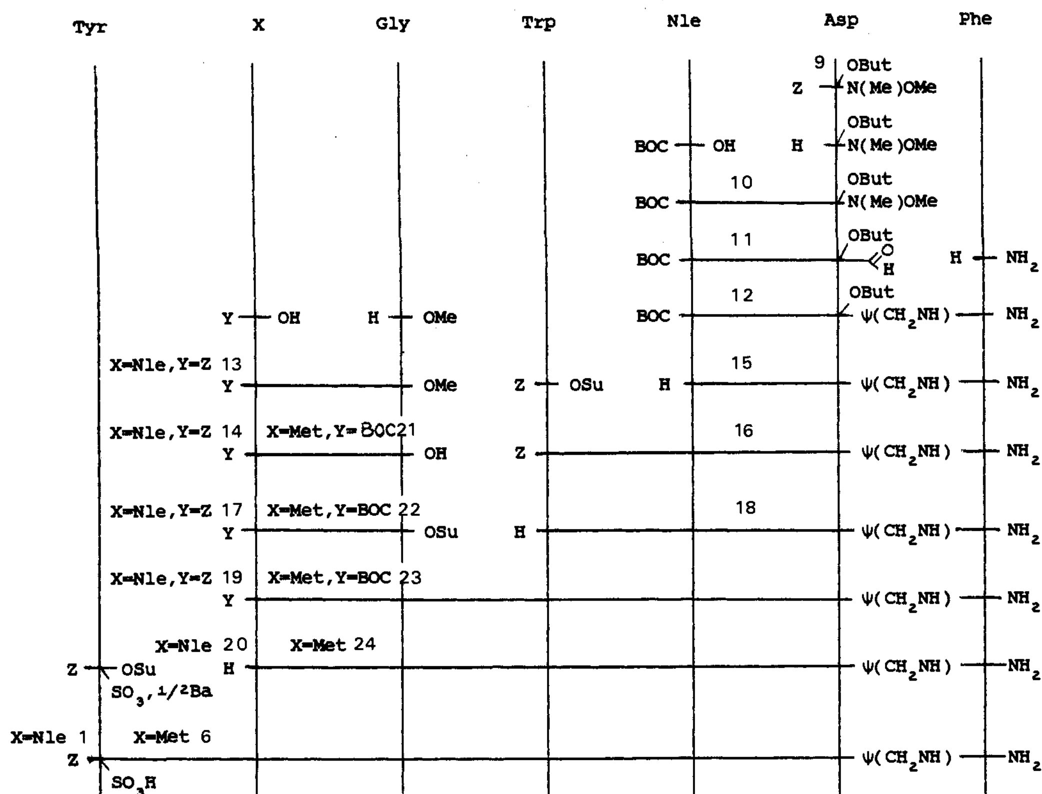
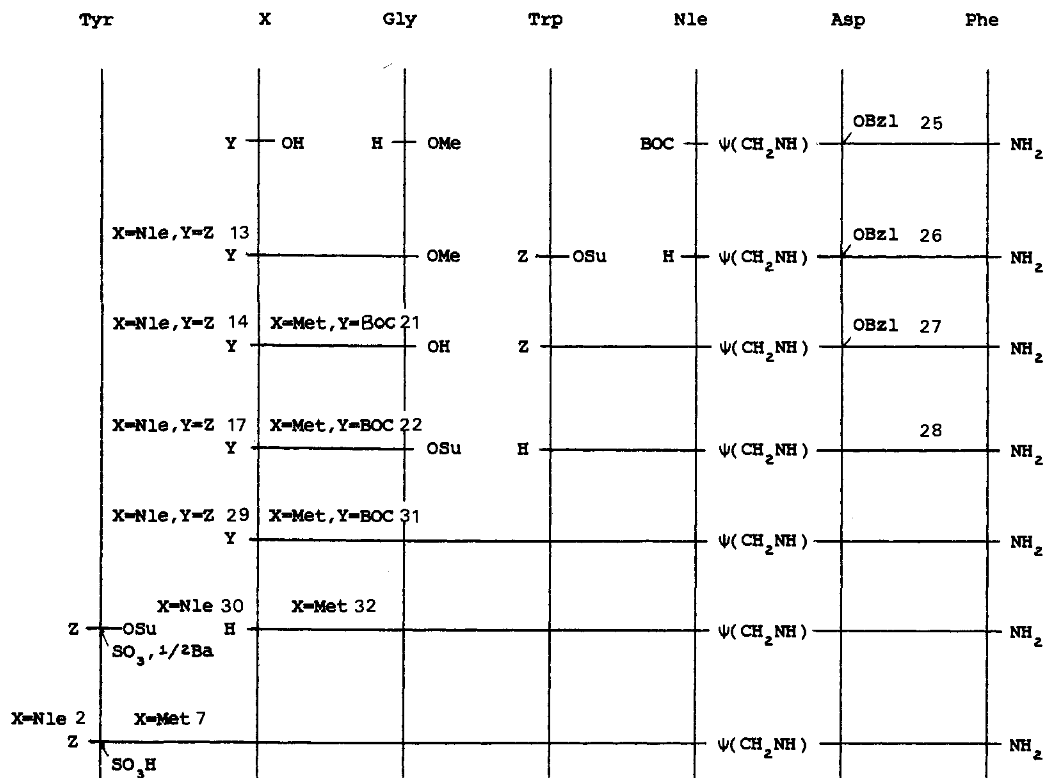
Scheme I



Scheme II



However, as a peptide bond induces some rigidity to the molecule, its replacement by a CH_2NH bond induces some motional freedom by the possibility of rotation around this "reduced bond". On the other hand, this modification

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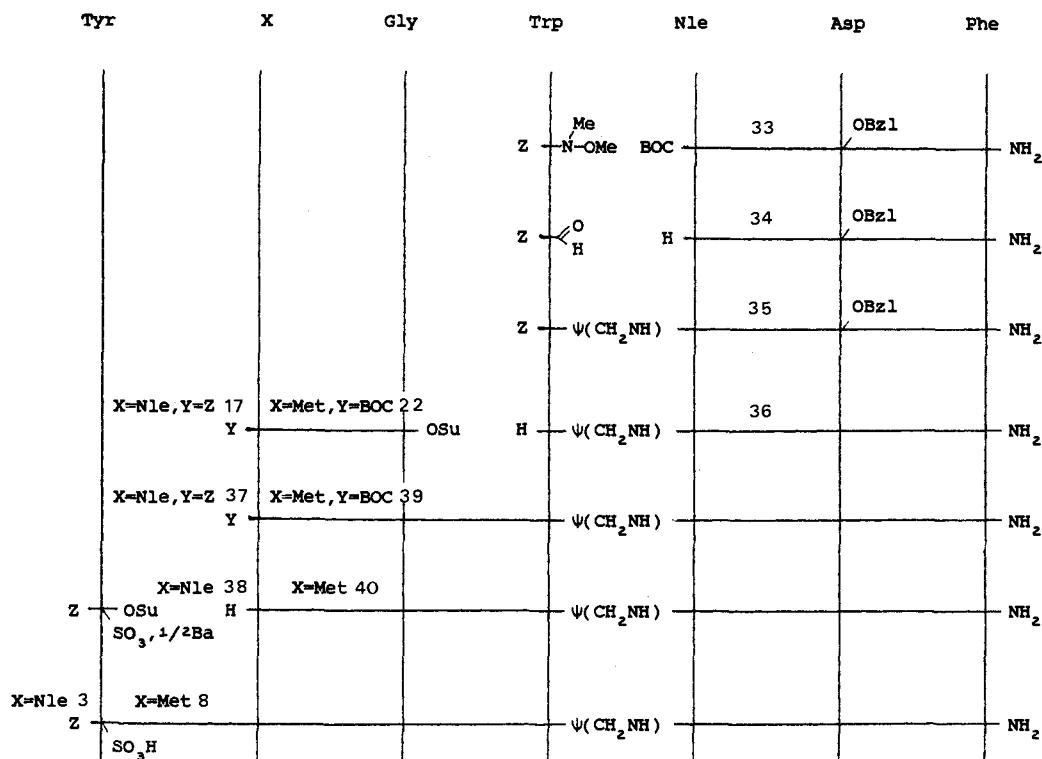
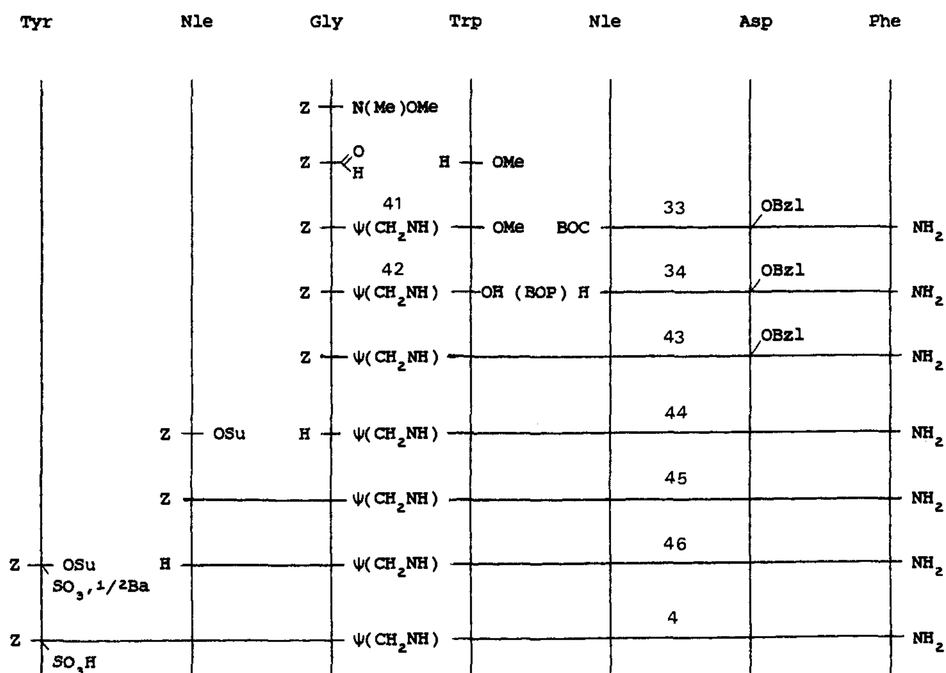
Scheme III



Scheme IV



introduces into the molecule a new possible charge that might be of some importance for the interactions with the receptor.

Chemistry

The syntheses of the pseudopeptides prepared in this work are summarized in Schemes I–V. The aldehyde of the N-protected amino acid obtained from the corresponding *N*,*O*-dimethylhydroxamate according to Ferhentz and Castro[16] was allowed to react with the α-amino function of the protected amino acid or peptide, in the presence of $NaBH_3CN$ as reducing reagent in a methanol/acetic acid mixture,[17] to yield the corresponding pseudopeptide with a CH_2NH bond.[8] Elongation of the peptide chains was performed stepwise or by fragment condensation using active ester derivatives as acylating agents and isobutyl chloroformate or BOP as coupling reagent.[18] During the activation of pseudodipeptide derivatives (e.g., **42**) for coupling or during condensation of dipeptide aldehydes (e.g., **11**), no racemization can occur by the oxazolone mechanism. *N*-(Benzyloxycarbonyl)-L-

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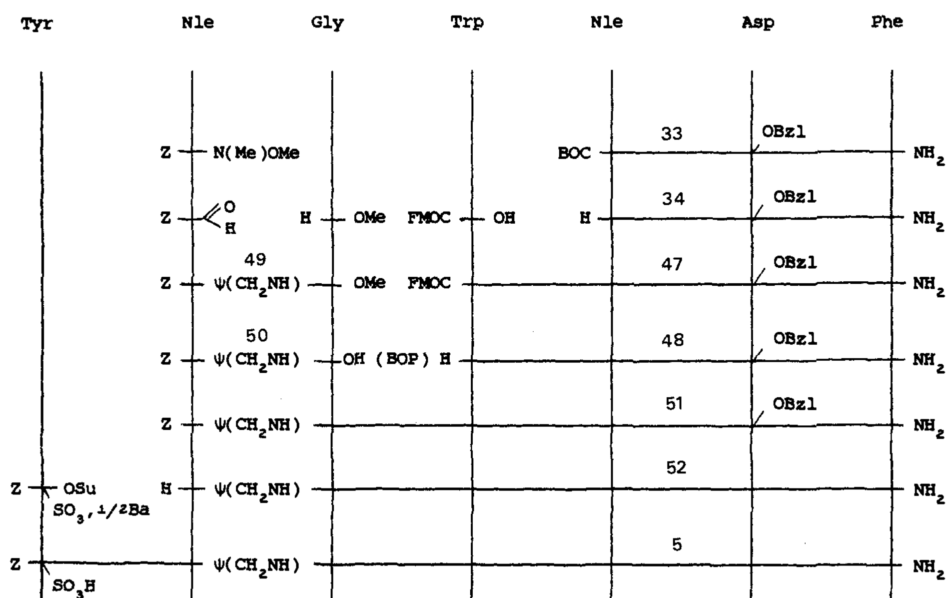
Scheme V



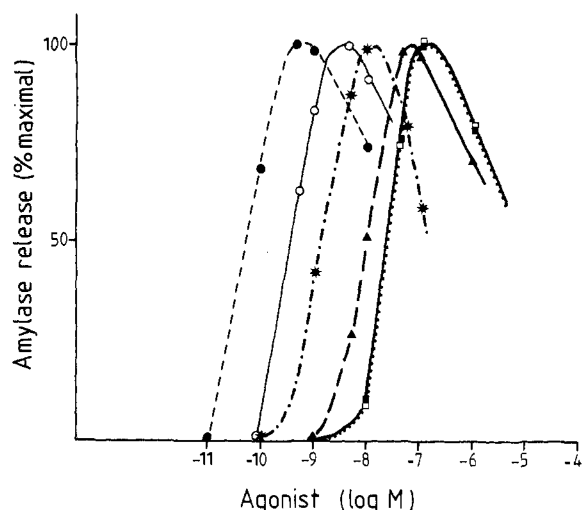


Figure 1. The effects of Boc-[Nle[28],Nle[31]]-CCK-8 (●) and of pseudopeptides Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)-Phe-NH_2 (1) (■), Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (2) (*), Z-Tyr(SO_3^-)-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (3) (□), Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (4) (▲), and Z-Tyr(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (5) (○) on amylase release from rat pancreatic acini. Dispersed pancreatic acini from rat pancreas were prepared and amylase release was measured according to previously described procedures.[20] Amylase activity was determined by the method of Ceska et al.[28] using the Phadebas reagent. Amylase release was measured as the difference of amylase activity at the end of incubation that was released into the extracellular medium, with and without secretagogue, and expressed as percent of maximal stimulation. In each experiment, each value was determined in duplicate, and the results given are the means from at least three separate experiments.

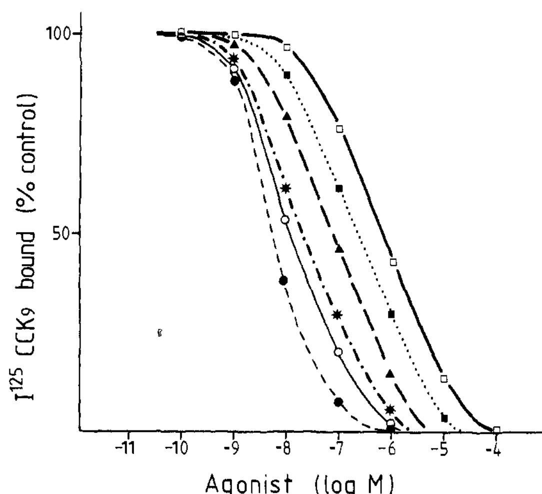


Figure 2. Ability of compounds 1–8 to inhibit binding of labeled ^{125}I-BH-CCK-9 to rat pancreatic acini. Binding of Bolton-Hunter CCK-9 (that was identical to that of BH-CCK-8 from Amersham) was performed as described by Jensen et al.[20] Acini were incubated for 30 min at 37 °C with various concentrations of Boc-[Nle[28],Nle[31]]-CCK-9 (●) or of pseudopeptides Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (1) (■), Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (2) (*), Z-Tyr(SO_3^-)-Nl-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (3), (□), Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (4), (▲), and Z-Tyr-(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (5), (○), plus 50 pM labeled CCK-9. Values are expressed as the percentage of the value obtained with labeled CCK-9 alone. In each experiment, each value was determined in duplicate, and the results given are the means from at least three separate experiments.

tyrosine *O*-sulfate (prepared according to the procedure of Moroder et al.[19]) was introduced in the form of its *N*-hydroxysuccinimide active ester obtained in situ. Pseudopeptides 1–8 were purified by HPLC on a Beckman Ultrasphere ODS (5 μm) column using a mixture of 0.01 M ammonium acetate (pH 6.5)/methanol (Table I). Their 360-MHz ^1H NMR spectra showed the influence of the reduced peptide bond on the $C^{\alpha}H$ of the neighbor amino acid residues. As we already observed, the $C^{\alpha}H$ of the amino acid bearing the CH_2 is less affected than the $C^{\alpha}H$ bearing the NH.[26]

All peptide and pseudopeptide intermediates were purified by silica gel chromatography until they showed a single spot on TLC in various solvents and were identified by ^1H NMR spectroscopy and elemental analysis. Physical and analytical data are reported in Table II.

Biological Results

The pseudopeptides 1–8 were evaluated for their ability to stimulate in vitro amylase release from rat pancreatic acini[4] (Figure 1) and to inhibit the binding of ^{125}I-BH-CCK-9 to isolated rat pancreatic acini[20] (Figure 2) and to guinea pig brain membranes[21] (Figure 3). (^{125}I-BH-CCK-9 is the Bolton-Hunter derivative of the C-terminal nona-

Table I. Physical and Analytical Data of the Pseudopeptide Analogues of the C-Terminal Heptapeptide of Cholecystokinin Used in the Biological Tests

pseudopeptides	mp, °C	$[\alpha]_D$, deg (*c* 1, DMF)	t_R,[a] min	R_f[b] D	R_f[b] E
Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**1**)	180 dec	-10.5	6.7 (A)	0.27	0.47
Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**2**)	190 dec	-16.8	13.9 (A)	0.16	0.36
Z-Tyr(SO_3^-)-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**3**)	180 dec	-16.1	10.9 (A)	0.20	0.41
Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (**4**)	205 dec	-22.6	7.2 (A)	0.22	0.42
Z-Tyr(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (**5**)	195 dec	-19.6	7.7 (A)	0.25	0.50
Z-Tyr(SO_3^-)-Met-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**6**)	175 dec	-14.7	11.6 (B)	0.26	0.44
Z-Tyr(SO_3^-)-Met-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**7**)	180-185	-18.1	15.0 (A)	0.16	0.33
Z-Tyr(SO_3^-)-Met-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**8**)	175-180	-20.0	11.4 (B)	0.20	0.36

[a] Solvent proportions used in HPLC, (A) 32:68 and (B) 34:66 ratios of a mixture of ammonium acetate 0.01 M, pH 6.5/methanol, at a flow rate of 3 mL/min. We used a Beckman Ultraspere ODS 0.05 column (0.5 μm) and a Hitachi instrument, with a UV detection at 279 nm. [b] Solvents: D, ethyl acetate/pyridine/acetic acid/water, 80:20:5:10, and E, ethyl acetate/pyridine/acetic acid/water, 60:20:5:10.

Table II. Analytical and Physical Data of Synthetic Peptide and Pseudopeptide Derivatives

compounds	mp, °C	$[\alpha]_D$, deg (*c* 1, DMF)	R_f[a]	anal. C, H, N
Z-Asp(But)-*N*,*O*-dimethylhydroxamate (**9**) (ref 8)	75-76	-12	(B) 0.65	
Boc-Nle-Asp(But)-*N*,*O*-dimethylhydroxamate (**10**)	oil		(B) 0.68	
Boc-Nle-Asp(But)ψ(CH_2NH)Phe-NH_2 (**12**) (ref 25)	115-117	-13.5	(A) 0.55	
Z-Nle-Gly-OMe (**13**)	110-112	-5	(A) 0.84, (B) 0.73	$C_{17}H_{24}N_2O_5$
Z-Nle-Gly-OH (**14**)	135-137	-4.3	(C)0.39, (F) 0.63	$C_{16}H_{22}N_2O_5$
Boc-Met-Gly-OH (**21**) (ref 29)	125-126	-11.8		
Z-Nle-Gly-OSu (**17**)	175-178	-12.2	(A) 0.62, (B) 0.46	$C_{20}H_{25}N_3O_7$
Boc-Met-Gly-OSu (**22**)	126-128	-19.1	(B) 0.50, (B) 0.33	$C_{16}H_{25}N_3O_7S$
Z-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**16**)	115 dec	-11.6	(C) 0.10, (D) 0.52	$C_{38}H_{46}N_6O_7$
Z-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**19**)	145 dec	-17	(D 0.40, (F) 0.25	$C_{46}H_{60}N_8O_9$
Boc-Met-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**23**)	125-135	-15.8	(D) 0.41, (E) 0.82	$C_{42}H_{60}N_8O_9S$
Boc-Nleψ(CH_2NH)Asp(OBzl)-Phe-NH_2 (**25**) (ref 25)	177-179	-14.3	(B) 0.37	$C_{31}H_{44}N_4O_6$
Z-Trp-Nleψ(CH_2NH)Asp(OBzl)-Phe-NH_2 (**27**)	190-195	-12.3	(A) 0.39, (B) 0.10	$C_{45}H_{52}N_6O_7$
Z-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**29**)	115 dec	-13.8	(D) 0.32, (F) 0.15	$C_{46}H_{60}N_8O_9$
Boc-Met-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**31**)	120-125	-18.7	(D) 0.34, (E) 0.51	$C_{42}H_{60}N_8O_9S$
Z-Trp-*N*,*O*-dimethylhydroxamate	122-125	-39.3	(A) 0.63, (B) 0.43	$C_{21}H_{23}N_3O_4$
Boc-Nle-Asp(OBzl)-Phe-NH_2 (**33**) (ref 25)	135-137	-78.6	(A) 0.59	
Z-Trpψ(CH_2NH)Nle-Asp(OBzl)-Phe-NH_2 (**35**)	110 dec	-21.2	(A) 0.34, (B) 0.06	$C_{45}H_{52}N_6O_7$
Z-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**37**)	140 dec	-11.7	(D) 0.36, (F) 0.23	$C_{46}H_{60}N_8O_9$
Boc-Met-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**39**)	105-120	-17.5	(D) 0.34, (E) 0.55	$C_{42}H_{60}N_8O_9S$
Z-Gly-*N*,*O*-dimethylhydroxamate	74-76		(A) 0.59, (B) 0.46	$C_{12}H_{16}N_2O_4$
Z-Glyψ(CH_2NH)Trp-OMe (**41**)	oil		(C) 0.37	
Z-Glyψ(CH_2NH)Trp-OH (**42**)	195-198	+14.7[b]	(C) 0.10	$C_{21}H_{23}N_3O_4$
Z-Glyψ(CH_2NH)Trp-Nle-Asp(OBzl)-Phe-NH_2 (**43**)	225 dec	-24.9	(C) 0.34, (F) 0.81	$C_{47}H_{55}N_7O_8$
Z-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (**45**)	230 dec	-24.8	(D) 0.48, (F) 0.28	$C_{46}H_{60}N_8O_9$
Z-Nle-*N*,*O*-dimethylhydroxamate	oil		(B) 0.66	
FMOC-Trp-Nle-Asp(OBzl)-Phe-NH_2 (**47**)	215-220	-27.4	(A) 0.35	$C_{52}H_{54}N_6O_8$
Z-Nleψ(CH_2NH)Gly-OMe (**49**)	oil		(B) 0.41	
Z-Nleψ(CH_2NH)Gly-OH (**50**)	220-223	-21.7[b]	(A) 0.25	
Z-Nleψ(CH_2NH)Gly-Trp-Nle-Asp(OBzl)-Phe-NH_2 (**51**)	204-207	-21.3	(D) 0.80, (F) 0.75	$C_{53}H_{66}N_8O_9$

[a] Solvents: A, AcOEt; B, ethyl acetate/hexane, 7:3; C, chloroform/methanol/acetic acid, 85:10:5; D, ethyl acetate/pyridine/acetic acid/water, 80:20:5:10; E, ethyl acetate/pyridine/acetic acid/water, 60:20:5:10; F, ethyl acetate/pyridine/acetic acid/water, 80:20:3:3. [b] Measured in 0.1 N NaOH as solvent.

peptide of cholecystokinin.) They were compared in the same experiments with the potent CCK-8 analogue[15] Boc-[Nle^{28},Nle^{31}]-CCK-8, Boc-Asp-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2. Each pseudopeptide tested had, on stimulation of amylase release from rat pancreatic acini, a biphasic dose-response curve similar to that of Boc-[Nle^{28},Nle^{31}]-CCK-8 in that, as the pseudopeptide concentrations increased, amylase secretion increased, became maximal, and then decreased by approximately 50% at supramaximal concentrations (Figure 1). The most potent was compound **5**, having a reduced peptide bond between Nle^{28} and Gly^{29}, which exhibited a maximal stimulation at 3 nM. It was also very potent in inhibiting the binding of ^{125}I-BH-CCK-9 to its receptors on pancreatic acini, giving half-maximal inhibition at 10 nM. Compounds **2** and **4**, having a CH_2NH bond between Nle^{31} and Asp^{32} and between Gly^{29} and Trp^{30}, respectively, were still very potent on the stimulation of amylase secretion. Compound **2** was 30 times less potent than the CCK-8 analogue (maximal response was observed at 10 nM), and compound **4** was 150 times less potent than the CCK-8 analogue (maximal response at 50 nM). Both compounds were able to recognize the CCK receptor with a good apparent affinity (IC_{50} = 30 nM for **2** and 70 nM for **4**). The pseudopeptides having a reduced peptide bond between Trp^{30} and Nle^{31} and between Asp^{32} and Phe^{33}, e.g., compounds **3** and **1**, were less potent in stimulation of amylase release and on inhibition of binding of ^{125}I-BH-CCK-9 to pancreatic CCK receptors (Table III). On the inhibition of binding of ^{125}I-BH-CCK-9 to guinea pig brain membranes, compound **5** (having a CH_2NH bond between Nle^{28} and Gly^{29}) was again the most potent (half-maximal inhibition at 0.5 nM) whereas the pseudopeptides **4** (with a CH_2NH bond between Gly^{29} and Trp^{30}) and **3** (with a CH_2NH bond between Trp^{30} and Nle^{31}) were quite potent (half-maximal inhibition at 2 nM and 30 nM, approximately 3 and 50 times less potent than CCK-8, respectively). Compounds **1** and **2**, however, were less potent (half-maximal inhibition at 200 and 600 nM, repsectively) (Table III). Compounds **6**, **7**, and **8**, analogues of **1**, **2**, and **3**, respectively, but with a methionine instead of a norleucine in position 28, behaved almost similarly as **1**, **2**, and **3** both on rat pancreatic

Table III. Biological Activities of Pseudopeptide Analogues of the C-Terminal Heptapeptide of Cholecystokinin on the Binding to the Pancreatic and Brain CCK Receptors and on the Activity on Amylase Release from Rat Pancreatic Acini

	rat pancreatic acini		
compounds	amylase stim (max.)	binding (IC_{50}, M)	guinea pig brain membranes: binding (IC_{50}, M)
Boc-Asp-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2	3×10^{-10}	5×10^{-9}	6×10^{-10}
Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**1**)	1.5×10^{-7}	3×10^{-7}	2×10^{-7}
Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**2**)	10^{-8}	3×10^{-8}	6×10^{-7}
Z-Tyr(SO_3^-)-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**3**)	1.5×10^{-7}	8×10^{-7}	3×10^{-8}
Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (**4**)	5×10^{-8}	7×10^{-8}	2×10^{-9}
Z-Tyr(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (**5**)	3×10^{-9}	10^{-8}	5×10^{-10}
Z-Tyr(SO_3^-)-Met-Gly-Trp-Nle-Aspψ(CH_2NH)Phe-NH_2 (**6**)	3×10^{-7}	2×10^{-7}	7×10^{-6}
Z-Tyr(SO_3^-)-Met-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**7**)	5×10^{-9}	3×10^{-8}	10^{-6}
Z-Tyr(SO_3^-)-Met-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**8**)	3×10^{-7}	3×10^{-7}	6×10^{-6}

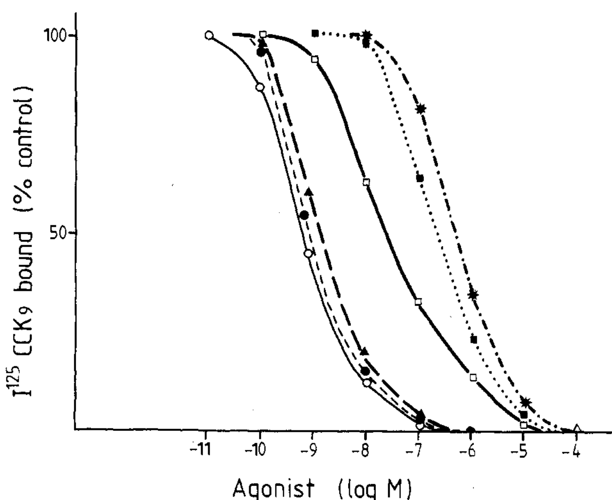


Figure 3. Abilities of Boc-[Nle[28],Nle[31]]-CCK-8 (●) and of pseudopeptides Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Aspψ(CH_2NH)-Phe-NH_2 (**1**) (■), Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nleψ(CH_2NH)Asp-Phe-NH_2 (**2**) (★), Z-Tyr(SO_3^-)-Nle-Gly-Trpψ(CH_2NH)Nle-Asp-Phe-NH_2 (**3**), (□), Z-Tyr(SO_3^-)-Nle-Glyψ(CH_2NH)Trp-Nle-Asp-Phe-NH_2 (**4**) (▲), and Z-Tyr(SO_3^-)-Nleψ(CH_2NH)Gly-Trp-Nle-Asp-Phe-NH_2 (**5**) (O) to inhibit binding of labeled CCK on guinea pig brain membranes. Guinea pig brain membranes were obtained by the method of Pelaprat et al.[21] Binding of labeled CCK-9 (identical with that of Bolton-Hunter CCK-8 from Amersham) was performed as described by Pelaprat et al.[21] Brain membranes were incubated for 40 min at 25 °C with various concentrations of Boc-[Nle[28],Nle[31]]-CCK-8 (●) or **1**,**2**,**3**,**4**, or **5** plus 15 pM labeled CCK-9. Values are expressed as the percentage of the value obtained with labeled CCK-9 alone. Nonspecific binding was determined in th e presence of 1 μM CCK-8. In each experiment, each value was determined in duplicate, and the results given are the means form four separate experiments.

acini and on guinea pig brain membranes.

Discussion

Comparison of the results on amylase release and on binding to rat pancreatic acini were in pretty good agreement (Table III). However, the apparent affinity of the various pseudopeptide derivatives differed somewhat for CCK receptors on guinea pig brain membranes. The pseudopeptide **2**, which is quite potent on pancreatic CCK receptors, displayed a weaker affinity on central CCK receptors, indicating that the replacement of the peptide bond between Nle[31] and Asp[32] influences much more the binding of the compound to the central CCK receptor (IC_{50} = 600 nM) than to the pancreatic CCK receptor (IC_{50} = 30 nM). On the contrary, the pseudopeptide **3**, in which the peptide bond between Trp[29] and Nle[30] has been replaced by a CH_2NH bond, and the pseudopeptide **4**, in which the peptide bond between Gly[28] and Trp[29] was replaced by a CH_2NH bond, were more potent in inhibiting the binding of ^{125}I-BH-CCK-9 to the central CCK receptor (IC_{50} = 30 and 2 nM, respectively) than to the pancreatic CCK receptor (IC_{50} = 800 and 70 nM, respectively).

It clearly appeared from these results that the peptide bonds of the C-terminal heptapeptide of cholecystokinin did not have the same significance as that we found in gastrin.[8] The replacement of each peptide bond (one at a time) by a CH_2NH bond led to compounds exhibiting the same biological activity as CCK-8 on amylase release from rat pancreatic acini, with the same efficacy but with varying potencies. The results obtained on the inhibition of binding of ^{125}I-BH-CCK-9 to CCK pancreatic receptor were in good agreement. The order of potency was somewhat different in the inhibition of binding of ^{125}I-BH-CCK-9 to central CCK receptor, indicating that the structural requirements for binding to peripheral and central CCK receptors might not be identical.

We recently showed that replacement of LTrp by a DTrp in modified analogues of the C-terminal heptapeptide of cholecystokinin (e.g., Boc-Tyr(SO_3^-)-Met-Gly-DTrp-Nle-Asp α-2-phenylethyl ester) led to CCK receptor antagonists.[22] This analogue had a good apparent affinity for the pancreatic CCK receptor but exhibited weaker potency on the central CCK receptor, whereas the corresponding LTrp analogue (e.g., Boc-Tyr(SO_3^-)-Met-Gly-Trp-Nle-Asp α-2-phenylethyl ester) was almost as potent as CCK-8, on both central[24] and peripheral[15] CCK receptors. These results indicated that the orientation of the indole ring of tryptophan was more significant for binding to brain than to pancreatic CCK receptors. The present work showed that the replacement of the peptide bond between Nle[31] and Asp[32] by a CH_2NH bond led also to compounds exhibiting a higher apparent affinity for the pancreatic than for the brain CCK receptor (IC_{50} = 30 and 600 nM, respectively). It appeared that along with the tryptophan position, the peptide bond between Nle[31] and Asp[32] in CCK C-terminal heptapeptide analogues was more important for binding to the brain than to the pancreatic CCK receptor. It also clearly appeared from this work that the replacement of the peptide bond between Nle[28] and Gly[29] by a CH_2NH bond affected neither the activity on amylase secretion from rat pancreatic acini nor the binding to the pancreatic or to the brain CCK receptors.

In summary, the present results showed that the replacement of the peptide bonds by "reduced peptide bonds" in the C-terminal heptapeptide analogue of CCK led to compounds exhibiting the same activity as CCK-8. On stimulation of amylase release from rat pancreatic acini, these compounds exhibited the same activity as CCK-8, with the same efficacy but with weaker potencies, in the order CCK-8 > **5** > **2** > **4** > **3** ~ **1**. The results on the inhibition of binding of ^{125}I-BH-CCK-9 to pancreatic acini were in agreement with those of stimulation of amylase

secretion. The order of potency was somewhat disturbed in inhibiting the binding of ^{125}I-BH-CCK-9 to guinea pig brain membrane CCK receptors: CCK-8 $\simeq$ **5** > **4** > **3** > **1** > **2**. Apart from this tissue difference indicating probably different CCK receptors in brain and pancreas as it has already been reported,[23] it appears from this work that the peptide bonds of the C-terminal heptapeptide of CCK are crucial neither for the pancreozymin activity nor for the binding to pancreatic or brain CCK receptors.

Experimental Section

Melting points were taken on a Büchi apparatus in open capillary tubes. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. Elemental analyses were performed by le Service de microanalyses de l'ENSCM (Montpellier, France). Ascending TLC was performed on precoated plates of silica gel 60 F_{254} (Merck) using the following solvent systems (by volume): A, ethyl acetate; B, ethyl acetate/hexane, 7:3; C, chloroform/methanol/acetic acid, 85:10:15; D, AcOEt/pyridine/AcOH/H_2O, 80:20:5:10; E, AcOEt/pyridine/AcOH/H_2O, 60:20:5:10; F, AcOEt/pyridine/AcOH/H_2O, 80:20:3:3. Peptide derivatives were located with charring reagent or ninhydrin. Column chromatographies were performed with silica gel 60, 60–229 mesh, ASTM, (Merck). L-Amino acids and derivatives were from Bachem. All reagents and solvents were of analytical grade. The following abbreviations were used: DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; DIEA, *N,N*-diisopropylethylamine; DCC, *N,N'*-dicyclohexylcarbodiimide; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate. Other abbreviations used were those recommended by the IUPAC–IUB Commission (*Eur. J. Biochem.* **1984**, *138*, 9–37).

Syntheses. The peptide derivatives described in this work were synthesized according to the general procedures detailed in this section for the preparation of compound **5**. All compounds used for biological tests were filtered on Millipore (0.45 μm) and lyophilized after HPLC purification. The purity of the lyophilized material was checked again by HPLC.

***N*-(Benzyloxycarbonyl)-L-norleucylψ(CH_2NH)glycine Methyl Ester (49).** *N*-(Benzyloxycarbonyl)-L-norleucinal (6.5 mmol) obtained from *N*-(benzyloxycarbonyl)-L-norleucine *N,O*-dimethylhydroxamate[16] was dissolved in a mixture of methanol/acetic acid, 99:1 (30 mL), containing the hydrochloride salt of glycine methyl ester (0.82 g, 6.5 mmol). Sodium cyanoborohydride (1.23 g, 19.5 mmol) was added portionwise during 45 min. After 1 h, no more glycine methyl ester could be detected by TLC. The reaction mixture was cooled in an ice/water bath, and a saturated sodium bicarbonate solution (100 mL) was added with stirring, followed by ethyl acetate (150 mL). The organic layer was collected, washed with water (1 × 20 mL), dried over sodium sulfate, and concentrated in vacuo (t < 40 °C). The residue was purified by chromatography on silica gel to yield a pure oily compound, (0.71 g), R_f (B) 0.41.

***N*-(Benzyloxycarbonyl)-L-norleucylψ(CH_2NH)glycine (50).** *N*-(Benzyloxycarbonyl)-L-norleucylψ(CH_2NH)glycine methyl ester (**49**) (0.71 g, 2.2 mmol) was saponified in methanol (3 mL) containing 0.1 N NaOH (3 mL), at 0 °C. After the reaction mixture was stirred for 30 min at room temperature, it was diluted with water (50 mL) and extracted with ether (3 × 50 mL). The aqueous solution was acidified with $KHSO_4$ (pH 6–7). The product which precipitated was collected by filtration, washed with water, and dried in vacuo: yield 0.58 g; R_f (A) 0.25; mp 220–223 °C; $[\alpha]_D$ –21.7 (c 1, 0.1 N NaOH). Anal. ($C_{16}H_{24}N_2O_4$) C, H, N.

***N*-(((Fluorenylmethyl)oxy)carbonyl)-L-tryptophyl-L-norleucyl-L-(β-benzyl)-L-aspartylphenylalanine Amide (47).** Boc-Nle-Asp-Phe-NH_2 (**33**) (1.92 g, 3 mmol)[25] was partially deprotected by TFA (5 mL) for 30 min. Addition of ether (150 mL) yielded a white solid, which was collected by filtration, washed several times with ether, and dried in vacuo over KOH. It was dissolved in DMF (5 mL) and added to a solution containing the mixed anhydride of FMOC-Trp prepared from FMOC-L-Trp (1.28 g, 3 mmol), *N*-methylmorpholine (0.34 mL, 3 mmol), and isobutyl chloroformate (0.41 mL, 3 mmol), in DMF (5 mL) (5 min at –20 °C). After 1 h at room temperature, a 0.1 M citric acid solution was added (100 mL) and the precipitate that formed was collected by filtration, washed with water, a saturated bicarbonate solution, water, and ether, and dried in vacuo: yield 2.5 g (94%); R_f (A) 0.35; mp 215–220 °C; $[\alpha]_D$ –27.4 (c 1, DMF). Anal. ($C_{52}H_{54}N_6O_8$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-norleucylψ(CH_2NH)glycyl-L-tryptophyl-L-norleucyl-(β-benzyl)-L-aspartyl-L-phenylalanine Amide (51).** Compound **47** (1.34 g, 1.60 mmol) was treated with a mixture of DMF/diethylamine, 9:1 (60 mL), for 30 min at room temperature. The solvents were concentrated in vacuo at t < 40 °C, and the residue was treated with ether. The precipitate was washed several times with ether and dissolved in DMF (10 mL) containing Z-Nleψ(CH_2NH)Gly-OH (0.5 g, 1.62 mmol), BOP (0.75 g, 1.70 mmol), and DIEA (0.28 mL, 1.62 mmol). After standing for 30 min at 0 °C, the reaction mixture was allowed to stand at room temperature for 4 h. After addition of $KHSO_4$ (0.5 N, 100 mL), a precipitate formed. It was collected by filtration, washed several times with water, with a saturated sodium bicarbonate solution, and again with water, and dried in vacuo over P_2O_5: yield 1.18 g (82%); R_f (D) 0.80; R_f (F) 0.75; mp 204–207 °C; $[\alpha]_D$ –21.3 (c 1, DMF). Anal. ($C_{53}H_{66}N_8O_9$) C, H, N.

***N*-(Benzyloxycarbonyl)-*O*-(sulfate)-L-tyrosyl-L-norleucylψ(CH_2NH)glycyl-L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine Amide (5).** Compound **51** (0.5 g, 0.52 mmol) was dissolved in a mixture of DMF/acetic acid/water, 25:3:3 (30 mL), and hydrogenated at room temperature in the presence of a 10% Pd/C as catalyst. The catalyst was removed by filtration and rinsed several times with DMF, and the solvents were concentrated in vacuo at t < 40 °C. The residue was washed several times with ether and dried in vacuo over KOH to yield compound **52**. Z-Tyr($SO_3{}^1/_2Ba$)$O^1/_2Ba\cdot 3H_2O$ (0.26 g, 0.45 mmol) (prepared according to Moroder et al.[19]) was dissolved in cold DMF (2 mL) in the presence of *N*-hydroxysuccinimide (0.16 g, 1.35 mmol) and *N,N'*-dicyclohexylcarbodiimide (0.93 g, 0.45 mmol). After overnight stirring at room temperature, a solution in DMF (2 mL) containing compound **52** and DIEA (77 μL, 0.45 mmol) was added. After 2 h of stirring at room temperature, a solution of 5% citric acid (50 mL) was added. The resulting precipitate was collected by filtration and washed several times with water and with ether, yield 0.25 g (40%). It was purified by silica gel chromatography using a mixture of ethyl acetate/pyridine/acetic acid/water, 80:20:5:10, as solvent. Pure fractions were collected and yielded compound **5** (0.15 g, 24%). It was purified by HPLC as indicated (Table I) and lyophilized before used in biological tests. Physical and analytical data are reported in Tables I and II.

Biological Tests. Materials. Male guinea pigs (280–300 g) were obtained from le Centre d'Elevage d'Animaux de Laboratoire. Ardenay, France; male Wistar rats (180–200 g) from Effa-Credo, Saint Germain l'Arbresle, France; Hepes from Boehringer Mannheim; purified collagenase from Serva; carbamylcholine (carbachol), soybean trypsin inhibitor from Sigma; Eagle's basal amino acid medium (100 times concentrated) from GIBCO; essential vitamin mixture (100 times concentrated) from Microbiological Associates, Bethesda, MD; bovine plasma albumin (fraction V) from Miles Laboratory, Elkhart, IN; Phadebas amylase test from Pharmacia Diagnostics, Piscataway, NJ; ^{125}I-labeled *N*-succinimidyl-3-(4-hydroxyphenyl)propionyl-CCK-9 was a gift of L. Pradayrol and D. Fourmy, Toulouse, France. Unless otherwise stated, the standard incubation solution contained 24.5 mM Hepes (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mg/mL bacitracin, 0.2% (w/v) albumin, 0.03% (w/v) soybean trypsin inhibitor, 1% (v/v) essential amino acid mixture, and 1% (v/v) essential vitamin mixture. The incubation solution was equilibrated with 95% O_2/5% CO_2 as the gas phase.

Tissue Preparations. Dispersed acini from guinea pig and rat pancreas were prepared by using the previously described modifications[4] of the method of Peikin et al.[27] Guinea pig brain membranes were prepared by following the procedure described by Pelaprat et al.[21]

Amylase Release. Amylase release was measured by using the procedure already described.[4] Acini were resuspended in the

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standard incubation solution complemented with 1% bovine serum albumin, 1 mM calcium, and 5 mM theophylline, containing about 1 mg of protein/mL, and samples (1 mL) were incubated at 37 °C for 30 min. Amylase activity was determined by the method of Ceska et al.[28] using the Phadebas reagent. Amylase release was measured as the difference of amylase activity with and without secretagogues at the end of the incubation. Results were expressed as percent of maximal stimulation obtained with CCK-8 (which represents ca. 35–40% of the total amylase contained in the acini).

Binding Studies. Binding of ^{125}I-BH-CCK-9 to Pancreatic Acini. Binding of ^{125}I-BH-CCK-9 on pancreatic acini was performed as described by Jensen et al.[20] The specific activities of the various preparations used in our experiments were 1000–1300 Ci/mmol. Acini from three pancreata were suspended in 100 mL of standard incubation solution complemented with 1% bovine serum albumin. Samples (1 mL containing about 1 mg of protein) were incubated with the appropriate peptide concentrations plus 50 pM ^{125}I-BH-CCK-9 for 30 min at 37 °C. After centrifugation and washings, the radioactivity associated with the acinar pellet was measured. Nonsaturable binding of ^{125}I-BH-CCK-9 was determined as the amount of radioactivity associated with the acini when the incubation contained 1 μM CCK-8. All values reported in this paper are for saturable binding, i.e., total binding minus nonsaturable binding. Nonsaturable binding has always been less than 15% of the total binding.

Binding of ^{125}I-BH-CCK-9 to Guinea Pig Brain Membranes. Binding of ^{125}I-BH-CCK-9 to brain membranes was performed according to Pelaprat et al.[21] The buffer used was 50 mM Tris·HCl, 5 mM $MgCl_2$, 0.1 mg/mL bacitracin, pH 7.4 (Tris/$MgCl_2$/bacitracin buffer). Displacement experiments were performed by incubation of 1 mL of guinea pig brain membranes (about 0.5 mg of protein) in the presence of 15 pM ^{125}I-BH-CCK-9 (or ^{125}I-BH-CCK-8) during 40 min at 25 °C. In the presence of various concentrations of the analogue to be tested, in a total volume of 1 mL. Nonspecific binding was determined in the presence of 1 μM CCK-8.

Supplementary Material Available: Tables containing ^{1}H NMR data of the pseudopeptide derivatives of the C-terminal heptapeptide of cholecystokinin (3 pages). Ordering information is given on any current masthead page.

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β-Substituted Cysteines as Sequestering Agents for Ethanol-Derived Acetaldehyde in Vivo

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A series of β-mono- and β,β-disubstituted cysteines were evaluated in rats as sequestering agents for metabolically generated acetaldehyde (AcH) during the oxidation of ethanol in vivo and compared against D-(−)-penicillamine. Both *threo*- (**5**) and *erythro*-β-phenyl-DL-cysteine (**6**) reduced ethanol-derived blood AcH by ca. 40% and 60%, respectively, whereas the corresponding β-methyl-DL-cysteines (**3** and **4**) and the α-substituted α-methyl-DL-cysteine (**8**) had no effect. β,β-Tetramethylene-DL-cysteine (**7**), however, was as effective as D-(−)-penicillamine in sequestering AcH in vivo, reducing blood AcH after ethanol to 20% of maximal values. Thus, bulky β-substitution or, better, β,β-disubstitution on cysteine is required for such activity. ^{14}C-Labeled 2(*RS*),5,5-trimethylthiazolidine-4(*S*)-carboxylic acid (**1**) prepared by the condensation of D-(−)-penicillamine with [1,2-^{14}C]acetaldehyde was found to be relatively stable in vivo, giving rise to <6% $^{14}CO_2$ excretion in the expired air and the recovery of 65.5% of the administered dose in the urine as unchanged **1**.

It has been reported that chronic alcoholics exhibit elevated levels of blood acetaldehyde (AcH) after consuming alcohol compared to normal individuals,[1] while male offsprings of chronic alcoholic parents also show a tendency to high ethanol-derived blood AcH levels.[2] The "vicious cycle" of acetaldehydemia and hepatotoxicity has been etiologically linked to AcH,[3] the first product of ethanol metabolism. Much discussion has centered on the possibilities for AcH-mediated mechanisms in alcohol dependence,[4] although the data are yet inconclusive. It is known that AcH triggers the release of catecholamines from adrenergic neurons[5] and inhibits hepatic[6] and myocardial[7] protein biosynthesis. AcH also interferes with mitochondrial function in the liver[8] and the heart,[9] and circulating AcH can combine with biogenic amines in vivo to form tetrahydroisoquinolines[10] and can interfere with biogenic amine metabolism to give rise to the formation of tetrahydropapaverolines,[11] some of which show alcohol-addicting liabilities when instilled directly into the

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