

Synthesis and Binding Affinities of Cyclic and Related Linear Analogues of CCK₈ Selective for Central Receptors

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To investigate the role of the sulfate group and the influence of cyclization on the biological properties of conformationally constrained CCK₈ analogues, three series of compounds were synthesized: Boc-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (1), Boc-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (2), and Boc-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (3) (series A); Boc-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (4), Boc-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (5), Boc-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (6), and Boc-D-Glu-Tyr(SO₃H)-Nle-D-Nle-Trp-Asp-Phe-NH₂ (7) (series B); and Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (8), Boc-γ-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (9), and Boc-γ-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (10) (series C). The selectivity of these peptides was studied by measuring their ability to displace [³H]propionyl-CCK₈ from guinea pig brain and pancreatic membranes. All the peptides displayed low affinities (K_1 values around 10⁻⁶ M) for the pancreatic receptors (A type). In contrast, both sulfated and nonsulfated cyclic analogues displayed high affinities for central-type binding sites (B type), especially compounds belonging to series C [K_1 (8) = 4.7 nM and K_1 (9) = 0.56 nM]. In all series the linear analogues had relatively poor affinities (K_1 ~ 300 nM) for B-type receptors. Compound 9 was the most potent (K_1 = 0.56 nM) and selective [K_1 (pancreas)/ K_1 (brain) = 4464] for central-type CCK receptors of guinea pig. The cyclization of the N-terminal region of CCK₈ permits one therefore to obtain probes for central receptors, and small changes directed toward the cyclic part modulate the affinity for these receptors.

The C-terminal peptide of cholecystokinin CCK₂₆₋₃₃ [Asp-Tyr(SO₃H)-Met-Gly-Trp-Met-Asp-Phe-NH₂] (CCK₈), originally found in the gut,¹ also exists in high concentrations in mammalian central nervous system.² In the brain, CCK has been shown to act primarily as modulator of dopamine, which has stimulated numerous studies aimed at developing CCK analogues as possible new antipsychotic agents.^{3,4} Moreover, recent studies have shown that CCK₈ is also able to modulate many physiological responses such as pain perception,⁵ sedation,⁶ feeding,⁷ and memory processing.^{8,9} Extensive biochemical studies have shown the existence of at least two classes of CCK receptors,^{10,11} i.e., "central" receptors (B type) discretely distributed in the brain¹²⁻¹⁴ and "peripheral" receptors (A type) primarily encountered in peripheral organs (pancreas, gall bladder, intestine) but also present in a few regions of the brain.^{12,15} Recently, nonpeptidic CCK antagonists selective for A-type receptors have been reported.¹⁶ At this time, the role of each class of brain receptor in the various CCK-induced pharmacological responses remains to be elucidated,¹⁷ and for this purpose centrally selective enzyme-resistant CCK analogues (agonists or antagonists) able to cross the blood-brain barrier are essential.

Since each type of CCK₈ receptor interacts with a distinct biologically active conformation of the native peptide, it was of interest to introduce constraints into CCK₈ with the aim of favoring the recognition of a single class of binding site. Taking as a model the N-terminal folding of CCK₈ deduced from conformational studies,¹⁸ two centrally selective cyclic CCK analogues were synthesized through internal amide bond formation between the side chains of the amino acids in positions 26 and 29 (Table I).^{19,20} In order to assess the structural requirements responsible for this selectivity, nonsulfated cyclic CCK analogues, and linear analogues structurally related to the sulfated cyclic CCK analogues, were prepared. The synthesis and the apparent affinities on guinea pig brain and

pancreatic membranes of these new compounds are reported in this paper.

- (1) Mutt, V.; Jorpes, E. *Biochem. J.* **1971**, *125*, 57.
- (2) Vanderhaeghen, J. J.; Signeau, J. C.; Gepts, W. *Nature (London)* **1975**, *257*, 604.
- (3) Hökfelt, T.; Rehfeld, J. F.; Skirboll, I.; Ivemark, B.; Goldstein, M.; Markey, K. *Nature* **1980**, *285*, 476.
- (4) Crawley, J. N.; Stivers, J. A.; Blumstein, L. K.; Paul, S. M. *J. Neurosci.* **1985**, *5*, 1972.
- (5) Barbaz, B. S.; Autry, W. L.; Ambrose, F. G.; Hall, N. R.; Liebman, J. M. *Neuropharmacology* **1986**, *25*, 823.
- (6) Crawley, J. N.; Hays, S. E.; Paul, S. M.; Goodwin, F. K. *Physiol. Behav.* **1981**, *27*, 407.
- (7) Gibbs, J.; Smith, G. *Am. J. Clin. Nutr.* **1977**, *30*, 758.
- (8) Katsuura, G.; Itoh, S. *Peptides* **1986**, *7*, 105.
- (9) Fekete, M.; Lengyel, A.; Hegedus, B.; Penke, B.; Zarandy, M.; Toth, G. K.; Telegdy, G. *Eur. J. Pharmacol.* **1984**, *98*, 79.
- (10) Innis, R. B.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 6917.
- (11) Sakamoto, C.; William, J. A.; Goldfine, I. D. *Biochem. Biophys. Res. Commun.* **1984**, *124*, 497.
- (12) Moran, T. H.; Robinson, R.; Goldrich, M. S.; McHugh, P. *Brain Res.* **1986**, *362*, 175.
- (13) Van Dijk, A.; Richards, J. G.; Trzeciak, A.; Gillessen, D.; Mohler, H. *J. Neurosci.* **1984**, *4*, 1021.
- (14) Pélaprat, D.; Broer, Y.; Studler, J. M.; Peschanski, M.; Tassin, J. P.; Glowinski, J.; Rostène, W.; Roques, B. P. *Neurochem. Int.* **1987**, *10*, 495.
- (15) Hill, D. R.; Campbell, N. J.; Shaw, T. M.; Woodruff, G. N. *J. Neurosci.* **1987**, *7*, 2967.
- (16) Evans, B. E.; Bock, M. G.; Rittle, K. E.; Di Pardo, R. M.; Whitter, W. L.; Veber, D. F.; Anderson, P. S.; Freidinger, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4918.
- (17) Crawley, J. N. *Peptides* **1985**, *6* (Suppl. 2), 129.
- (18) Fournié-Zaluski, M. C.; Belleney, J.; Lux, B.; Durieux, C.; Gérard, D.; Gacel, G.; Maigret, B.; Roques, B. P. *Biochemistry* **1986**, *25*, 3778.
- (19) Charpentier, B.; Pélaprat, D.; Durieux, C.; Dor, A.; Reibaud, M.; Blanchard, J. C.; Roques, B. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1968.
- (20) Charpentier, B.; Roques, B. P. European Patent No. 88400459.9.

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Table I. Apparent Affinities (K_i) of CCK₈ Analogues on the Binding of [³H]pCCK₈ to Brain and Pancreatic Membranes of Guinea Pig

compd	peptide sequence	K_i , nM		$K_i(\text{pancreas}) / K_i(\text{brain})$
		brain ^a	pancreas ^a	
CCK ₈	Asp-Tyr(SO ₃ H)-Met-Gly-Trp-Met-Asp-Phe-NH ₂	0.28 ± 0.01	0.64 ± 0.04	2.3
Boc[Nle ^{28,31}]CCK ₂₇₋₃₃	Boc-Tyr(SO ₃ H)-Nle-Gly-Trp-Nle-Asp-Phe-NH ₂	0.23 ± 0.05	0.93 ± 0.08	4.0
	Boc-Asp-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	120 ± 11.0 ^b	1500 ± 700 ^b	12.5
	Boc-D-Asp-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	6.43 ± 0.79	2900 ± 600	451.0
series A				
1	Boc-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	47.30 ± 7.60	1997 ± 250	42.2
2	Boc-Glu-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	29.20 ± 2.70	3850 ± 580	131.8
3	Boc-Glu-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	334 ± 50	1996 ± 580	6.0
series B				
4	Boc-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	27.50 ± 0.5	1250 ± 65	45.4
5	Boc-D-Glu-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	9.11 ± 1.23	3100 ± 100	340.3
6	Boc-D-Glu-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	313 ± 21	1550 ± 110	5.0
7	Boc-D-Glu-Tyr(SO ₃ H)-Nle-D-Nle-Trp-Nle-Asp-Phe-NH ₂	54.0 ± 1.2	1600 ± 100	29.6
series C				
8	Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	4.70 ± 0.4	1890 ± 280	402.1
9	Boc-γ-D-Glu-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	0.56 ± 0.05	2500 ± 640	4464.3
10	Boc-γ-D-Glu-Tyr(SO ₃ H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH ₂	301 ± 3	1390 ± 280	4.6

^a The tritiated ligands were used at 0.2 nM in brain and 0.1 nM in pancreas. ^b These values were obtained by using [³H]Boc[Nle^{28,31}]CCK₂₇₋₃₃ as radioligand at its K_D concentration, i.e., 0.19 nM and 4.4 nM for mouse brain and rat pancreas, respectively. The K_i values are the mean ± SEM of three independent determinations, each in triplicate. Each K_i value was obtained from computer analysis of Hill plots with 10 concentrations of inhibitors. All the peptides were used in Na salt form.

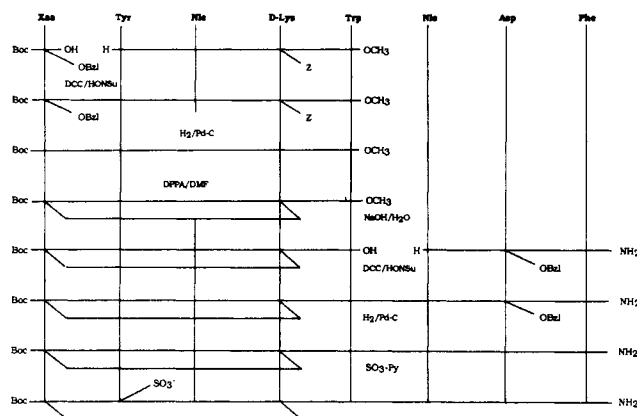


Figure 1. General scheme for the synthesis of the compounds Boc-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (2), Boc-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (5), and Boc-γ-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (9). As shown in this figure, the corresponding unsulfated analogues are obtained at the penultimate step.

Chemistry

Both cyclic and linear CCK₈ analogues were synthesized in liquid phase following a general procedure shown in Figures 1 and 2.²¹ Elongation of the peptide chain was performed stepwise or by fragment condensation. For the synthesis of cyclic peptides, the N-terminal pentapeptide was cyclized with diphenyl phosphorazidate (DPPA)²²⁻²⁴ after removal of the protecting groups from the D-Lys²⁹ side chain and from the α- or γ-carboxyl group of the Glu²⁶ residue by catalytic hydrogenation. The final cyclic pep-

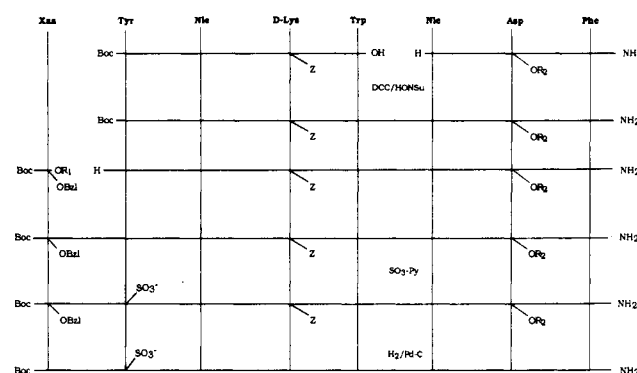


Figure 2. General scheme for the synthesis of the compounds Boc-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (3), Boc-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (6), and Boc-γ-D-Glu-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (10). For the synthesis of 3 and 6, R₁ = ONp and R₂ = H. For the synthesis of 10, R₁ = H and R₂ = Bzl.

tides were obtained by condensation of the cyclic pentapeptide with the tripeptide H-Nle-Asp(OBzl)-Phe-NH₂ using the DCC/HONSu method. Finally, after removal of the Asp³² benzyl ester by catalytic hydrogenation, the tyrosine moiety of compounds 1, 4, and 8 was sulfated, by using a pyridine-SO₃ complex,²⁵ to yield the peptides 2, 5, and 9. The linear CCK₈ analogues 3, 6, and 10 were obtained by using the intermediate H-Tyr-Nle-D-Lys-(Z)-Trp-Nle-Asp(OR)-Phe-NH₂ (R = H or benzyl), which was acylated with the appropriate glutamic acid derivative to give the octapeptides 30, 32, and 34. Sulfation of 30, 32, and 34 with a pyridine-SO₃ complex, followed by catalytic hydrogenation with Pd/C, yielded compounds 3, 6, and 10. Analogue 7 was synthesized stepwise starting from the tripeptide H-Nle-Asp(OBzl)-Phe-NH₂-TFA,²¹ with the sulfation procedure as for the last step. Given their structural similarities, the final peptides were unambiguously characterized according to two-dimensional (2D) correlated ¹H NMR spectroscopy. Proton chemical

- (21) Charpentier, B.; Durieux, C.; Menant, I.; Roques, B. P. *J. Med. Chem.* 1987, 30, 962.
- (22) Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* 1972, 94, 6203.
- (23) Kopple, K. D.; Schramper, T. J.; Go, A. *J. Am. Chem. Soc.* 1974, 96, 2597.
- (24) Ondetti, M. A.; Pluscec, J.; Sabo, F. E.; Sheehan, J. T.; Williams, N. *J. Am. Chem. Soc.* 1970, 92, 195.

- (25) Ruiz-Gayo, M.; Dauge, V.; Menant, I.; Bégué, D.; Gacel, G.; Roques, B. P. *Peptides* 1985, 6, 415.

Table II. Chemical Shift of the NH, H α , and H $\beta\beta'$ Protons for Compounds 1–10^a

residue		1	2	3	4	5	6	7	8	9	10
Glu	NH	6.67	6.70	6.90	7.08	7.24	7.17	6.76	7.04	7.42	6.23
	α	3.73	3.72	3.91	3.68	3.65	3.77	3.85	3.82	3.80	3.60
	β/β'	1.66	1.70	1.73	1.75/1.68	1.84/1.63	1.85/1.74	1.65/1.50	1.76/1.63	1.64/1.60	1.90/1.65
Tyr	NH	8.62	8.88	8.15	8.57	9.06	8.21	7.97	?	9.76	8.25
	α	4.14	4.34	4.37	4.26	4.40	4.31	4.40	4.22	4.40	4.16
	β/β'	2.93/2.71	3.03/2.80	2.99/2.73	2.81	2.84/2.81	2.92/2.85	2.89/2.61	2.94/2.73	2.95/2.82	3.01/2.65
Nle	NH	9.13	9.14	8.41	9.18	9.87	8.90	8.00	?	10.04	8.70
	α	4.00	3.92	4.04	4.10	4.17	4.00	4.16	4.13	4.08	3.98
	β/β'	1.45	1.60/1.47	1.56/1.47	1.43	1.63/1.40	1.55	1.51/1.47	1.57/1.44	1.64/1.40	1.69/1.46
Lys	NH	7.46	7.76	8.41	7.84	8.10	8.21	7.88	7.84	7.92	8.08
	α	4.06	4.10	4.02	4.15	4.11	3.99	4.16	4.19	4.12	3.89
	β/β'	1.23	1.36/1.23	1.45	1.65/1.37	1.49	1.60/1.50	1.55/1.28	1.25	1.45	1.51/1.40
Trp	NH	8.13	8.27	8.37	8.35	8.48	8.77	8.11	8.33	8.42	8.72
	α	4.35	4.40	4.38	4.40	4.42	4.36	4.53	4.49	4.30	4.32
	β/β'	3.05/2.81	3.10/2.90	3.08/2.84	3.12/2.94	3.18/2.94	3.09/2.92	3.10/2.87	3.10/2.84	3.05/2.94	3.05/2.94
Nle	NH	7.75	7.70	8.08	7.69	7.58	8.27	8.05	7.82	7.47	8.05
	α	3.91	3.96	4.00	3.91	3.82	3.93	4.13	3.94	3.84	4.09
	β/β'	1.53/1.37	1.55/1.40	1.51/1.45	1.58/1.42	1.62/1.40	1.55/1.47	1.53/1.46	1.52/1.43	1.60/1.37	1.60/1.54
Asp	NH	7.90	7.94	7.97	8.10	8.30	7.82	8.13	8.06	8.00	7.94
	α	4.19	4.22	4.29	4.26	4.16	4.22	4.44	4.28	4.13	4.35
	β/β'	2.31	2.30/2.26	2.29	2.48/2.39	2.54/2.22	2.48/2.39	2.56/2.42	2.50/2.22	2.40/2.24	2.33
Phe	NH	7.70	7.92	7.82	7.53	7.42	7.82	7.80	7.60	7.52	7.90
	α	4.21	4.14	4.20	4.13	4.13	4.13	4.29	4.22	4.16	4.22
	β/β'	2.87/2.67	3.03/2.78	3.04/2.77	2.99/2.78	2.98/2.70	2.99/2.78	2.98/2.78	2.99/2.79	2.94/2.80	3.05/2.77

^a The ¹H NMR spectra were performed in Me₂SO-*d*₆; concentration of peptide (*c* = 2–4 mM). pH of lyophilization = 6.5–7.0; internal reference = HMDS.

shifts are reported in Table II.

Biological Results and Discussion

The structure–activity study reported here was carried out with Boc[Nle^{28,31}]CCK_{27–33} as the parent compound, since we have previously found that the structural modifications introduced into this analogue increased its chemical and enzymatic stability as compared to that of CCK₈.²⁶ Moreover, this molecule retains the full biological properties of CCK₈. Cyclic CCK analogues were synthesized by the introduction of an aspartic or glutamic residue in position 26 and a D-lysine in position 29. The D configuration of this latter amino acid was selected to stabilize the β -turn around the sequence Asp²⁶-Tyr²⁷-Met²⁸-Gly²⁹ deduced from conformational analysis of CCK₈.¹⁸ The resulting peptides were cyclized by amidification between the free carboxyl group of the amino acid in position 26 and the amino group of the D-Lys²⁹ residue.

The CCK analogues synthesized were investigated for their ability to displace the specific binding of [³H]pCCK₈ from guinea pig brain and pancreatic membranes. Inhibition constants are reported in Table I. The apparent affinities of compounds Boc-D-Asp-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ and **9** are similar to those previously reported with [³H]BDNL and [¹²⁵I]BH-CCK₈ as radioligands.¹⁹

Compound Boc-Asp-Tyr(SO₃H)-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ was shown to be weakly selective for central versus peripheral receptors [*K*_I(pancreas)/*K*_I(brain) = 12.5].²¹ However, the replacement of L-Asp²⁶ by its enantiomer D-Asp²⁶ led to a 36-fold improvement in the selectivity for central receptors [*K*_I(pancreas)/*K*_I(brain) = 451] without large change in the affinity for pancreatic binding sites. Therefore, we tried to enhance the affinity for CNS binding sites by increasing the size of the cyclic

moiety. Changing the cyclic moiety from a 17- to an 18-membered ring by replacement of L-Asp by L-Glu did not significantly increase the affinity and selectivity for central receptors (Table I). As could be expected from the result obtained with the cyclic compound containing a D-Asp²⁶, the replacement of this residue by a D-Glu²⁶ residue led to a peptide **5** that exhibited a similar high affinity and selectivity for central binding sites [*K*_I(pancreas)/*K*_I(brain) = 340]. In order to further investigate the role of the cyclic moiety for preferential interaction with central receptors, the D-Glu²⁶ residue was introduced with its γ -carboxyl group linked to the amino group of Tyr²⁷ (referred as γ -D-Glu in the text). This modification, leading to compounds of series C, significantly increased the affinity for central binding sites without any modification at the level of pancreatic receptors. Thus compound **9** behaves as the most potent and selective CCK analogue for central receptors described so far.^{19,20} This could be due to an increased flexibility of the cyclic moiety allowing a better conformational adaptation to the central binding sites. Interestingly, desulfation of the cyclic analogues induces the weak decrease in affinity for central binding sites already observed with CCK₈¹⁰ but does not significantly affect pancreatic receptor recognition whereas in the case of CCK₈ this modification leads to an about 1000-fold loss of affinity.¹⁰ This suggests that, sulfated or nonsulfated, the tyrosine moiety is probably unable to fit its binding subsite in “peripheral” receptors, implying that cyclization destabilizes the biologically active conformation, at least in the region of the Tyr residue.

In order to investigate whether the constrained conformations of these CCK analogues were responsible for their selectivity for CNS receptors, the binding affinities of the structurally related linear analogues **3**, **6**, **7**, and **10** were measured. Opening of the cycle led to a decrease in the affinity for central receptors without any significant change for pancreatic binding sites. As compared to CCK₈, all CCK analogues show a similar loss of affinity for a A-type receptors (Table I). Compound **7** was synthesized in order to assess the role of the charged lysine side-chain

(26) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* 1951, 193, 265.

amino group. This derivative, while showing a similar affinity for A-type receptors as analogues 3, 6, and 10, displayed an enhanced affinity for B-type receptor. Thus the side-chain free amino group of the Lys²⁹ residue seems to induce a negative interaction with central receptors either directly or through conformational change of compound 7. An additional recognition improvement for B-type CCK receptors is observed with compound 5, showing that introduction of conformational constraints through cyclization reinforces the affinity and selectivity of these CCK analogues for brain receptors. Interestingly, small changes directed toward the cyclic portion of these CCK analogues (compounds 2, 5, and 9) are able to selectively modulate the affinity for central receptors without affecting the weak interaction with peripheral receptors. All these results show that the folded structure observed for CCK₈ in solution¹⁸ is probably not relevant for interaction with pancreatic receptors whereas, in agreement with results of conformational analysis, the high affinities of cyclic analogues for CCK central receptors could be mainly related to the folding of the N-terminal constrained part and/or structural change induced on the C-terminal part by the bulky cyclized N-terminal part. Extensive, conformational analysis of the corresponding flexible and rigid analogues is expected to provide further insight into the requirements for specific interaction with A-type or B-type CCK₈ receptors.

In conclusion, introduction of conformational constraints in the N-terminal region of CCK₈ is a powerful means to design centrally selective CCK analogues. The peptides synthesized in this work would be useful as selective drugs to investigate the physiological role of CCK in the central nervous system.

Experimental Section

Chemistry. All protected amino acids were from Bachem AG. Solvents (Prolabo) were of analytical grade. Melting points were determined with a Kofler apparatus and were uncorrected. Chromatography was carried out with Merck silica gel 60 (230–400 mesh). Thin-layer chromatography was performed on precoated plates of silica gel 60 F₂₅₄ (Merck) using the following solvent systems: (A) CHCl₃–MeOH (95:5); (B) CHCl₃–MeOH (9:1); (C) CHCl₃–MeOH (8:2); (D) CHCl₃–MeOH (7:3); (E) 1-butanol–AcOH–H₂O (4:1:1); (F) 1-butanol–AcOH–H₂O (8:1:1); (G) EtOAc–pyridine–AcOH–H₂O (60:20:6:11); (I) EtOAc–pyridine–AcOH–H₂O (80:20:6:11); (J) EtOAc–pyridine–AcOH–H₂O (100:20:6:11); (K) EtOAc–pyridine–AcOH–H₂O (140:20:6:11). Plates were developed with UV, iodine vapor, ninhydrin, or Ehrlich's reagent. The structures of the compounds and of all the intermediates were confirmed by ¹H NMR spectroscopy (Bruker WH, 270 MHz). HPLC was carried out on a Waters apparatus either on an analytical 250 × 4.6 mm S.F.C.C. (France) 5-μm column or on a preparative 300 × 7 mm S.F.C.C. (France) 5-μm column. The purity of the final peptides was checked with Et₃N–H₃PO₄ buffer (TEAP, 0.025 M, pH 6.5)/CH₃CN as eluent at a flow rate of 1.2 mL/min with UV (210-nm) detection. Final peptides were purified on HPLC before use in bioassays using Et₃N–HCOOH buffer (TEAF, 0.25 M, pH 6.5)/CH₃CN and a flow rate of 3 mL/min with UV (280-nm) detection. The CH₃CN/TEAF ratio (v:v) was CH₃CN–TEAF (43:57) for nonsulfated cyclic analogues, CH₃CN–TEAF (38:62) for sulfated cyclic analogues, and CH₃CN–TEAF (35:65) for linear analogues. Amino acid analysis was carried out by using an LKB Biochrom 4400 analyzer after hydrolysis with 6 N HCl, at 110 °C for 24 h. Mass spectra were recorded on a double-focusing VG 70-250 instrument. The FAB ionization was obtained with a FAB field source (Ion Tech Ltd., Teddington, U.K.) operated with xenon at 8 kV and 1 mA. Glycerol or cesium iodide was used for calibration. Accelerating voltage was set at 6 kV, and the resolution was 1200. The following abbreviations have been used: Z, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; Nle, norleucine (2-aminoheptanoic acid); DMF, dimethylformamide; DCU, *N,N'*-dicyclohexylurea; TFA, trifluoroacetic acid; HONSu, *N*-hydroxysuccinimide; HOBt, 1-hydroxybenzotriazole; *t*_R, retention time in HPLC; FAB, fast atom bombardment. Other abbreviations used are those recommended by the IUPAC–IUB Commission.³⁰

Boc-Glu(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-OCH₃ (11). To a chilled solution of H-Tyr-Nle-D-Lys(Z)-Trp-OCH₃·TFA²¹ (1.4 g, 1.60 mmol) in DMF (10 mL) containing Et₃N (224 μL, 1.60 mmol) were added Boc-Glu(OBzl)-OH (0.59 g, 1.76 mmol), HONSu (184 mg, 1.60 mmol), and DCC (395 mg, 1.92 mmol). The reaction mixture was stirred under N₂ for 1 h, and overnight at room temperature. The DCU was filtered off, and the filtrate was concentrated in vacuo. The oily residue was precipitated from EtOAc–ether and was purified by flash chromatography on silica gel with CHCl₃–MeOH (95:5) to yield 1.3 g (75%): mp 170–172 °C; *R*_f (A) 0.24. Anal. (C₅₈H₇₃O₁₃N₇) C, H, N.

Boc-Glu-Tyr-Nle-D-Lys-Trp-OCH₃ (13). Compound 11 (1.2 g, 1.11 mmol) in MeOH (40 mL) was hydrogenated in the presence of 10% Pd/C catalyst (135 mg) for 3 h to give, after filtration and evaporation of MeOH, 900 mg (95%) of Boc-Glu-Tyr-Nle-D-Lys-Trp-OCH₃ (12): mp 133–135 °C; *R*_f (I) 0.17. This compound was used in the next step without further purification. A solution of 12 (900 mg, 1.05 mmol) in dry DMF (210 mL) containing Et₃N (294 μL, 2.1 mmol) was treated at –40 °C with DPPA (272 μL, 1.26 mmol) in DMF (5 mL) through dropwise addition over 1 h. The resulting mixture was stirred under N₂ for 48 h at –25 °C and for 48 h at 4 °C. During this time, the apparent pH was maintained between 7 and 7.5 through addition of Et₃N (moist pH paper, range 6–8). The DMF was evaporated in vacuo, and the residual oil was purified by flash chromatography on silica gel by eluting with CHCl₃–MeOH (95:5) and then with CHCl₃–MeOH (90:10) to yield 0.35 g (40%); mp 151–155 °C; *R*_f (B) 0.29; *R*_f (J) 0.63; FAB-MS (MH⁺) calcd 834, found 834.

Boc-Glu-Tyr-Nle-D-Lys-Trp-OH (14). A solution of 13 (0.31 g, 0.37 mmol) in MeOH (5 mL) at 0 °C was treated with 1 N NaOH (0.85 mL, 0.85 mmol). The mixture was stirred under N₂ for 2 h at 0 °C and 24 h at room temperature. After evaporation of MeOH, the residue was acidified with cold 1 N HCl, and the precipitate was filtered and washed with water to neutrality. The resulting solid was dried in vacuo over KOH to yield 237 mg (79%): mp 123–125 °C; *R*_f (J) 0.33. Anal. (C₄₂H₅₇O₁₆N₇) C, H, N.

Boc-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp(OBzl)-Phe-NH₂ (15). A solution of H-Nle-Asp(OBzl)-Phe-NH₂·TFA²¹ (150 mg, 0.25 mmol) in DMF (3 mL) containing Et₃N (35 μL, 0.25 mmol) was treated with 14 (210 mg, 0.25 mmol), HONSu (35 mg, 0.3 mmol), and DCC (61.8 mg, 0.3 mmol). The reaction mixture was stirred for 1 h at 0 °C and 48 h at room temperature. After filtration of DCU and evaporation in vacuo, the residue was precipitated from EtOAc–ether to yield 0.33 g (95%): mp 237–238 °C; *R*_f (K) 0.60. Anal. (C₆₈H₉₁O₁₄N₁₁) C, H, N.

Boc-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (1). Compound 15 (315 mg, 0.24 mmol) was hydrogenated in MeOH (5 mL) and DMF (3 mL) in the presence of 10% Pd/C catalyst (20 mg) for 4 h to give after filtration of catalyst, evaporation, and chromatography on silica gel with EtOAc–pyridine–AcOH–H₂O (100:20:6:11) 216 mg (75%): mp 140–143 °C; *R*_f (E) 0.68; *R*_f (I) 0.53; HPLC (*t*_R = 9.6 min), eluent CH₃CN–TEAP (40:60); FAB-MS (MH⁺) calcd 1196, found 1196. Amino acid analysis: Asp 1.03, Glu 1.01, Nle 2.0, Tyr 0.92, Phe 0.98, Lys 0.96. Anal. (C₆₁H₈₃O₁₄N₁₁) C, H, N.

Boc-Glu-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (2). A solution of 1 (180 mg, 0.15 mmol) in dry DMF (1.5 mL) and dry pyridine (4.5 mL) was treated with a SO₃–pyridine complex (0.8 g) overnight under N₂ at room temperature. After evaporation in vacuo, the residue was taken up in a cold saturated NaHCO₃ solution and stirred at 0 °C for 3 h with the pH maintained at about 7. After lyophilization, the inorganic salts were precipitated from MeOH, and the filtrate was evaporated in vacuo. The residue was purified by chromatography on silica gel with EtOAc–pyridine–AcOH–H₂O (60:20:6:11) to give 75 mg (38%): *R*_f (F) 0.58; *R*_f (G) 0.45; HPLC (*t*_R = 6.6 min), eluent CH₃CN–TEAP (35:65); FAB-MS (MH⁺) calcd 1320, found 1320. Amino

acid analysis: Asp 1.01, Glu 1.0, Nle 2.0, Tyr 0.95, Phe 0.96, Lys 0.97.

Boc-D-Glu(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-OCH₃ (16). A chilled solution of H-Tyr-Nle-D-Lys(Z)-Trp-OCH₃-TFA²¹ (3 g, 3.4 mmol) in DMF (15 mL) containing Et₃N (0.47 mL, 3.4 mmol) was treated with Boc-D-Glu(OBzl)-OH (1.2 g, 3.56 mmol), HONSu (400 mg, 3.4 mmol), and DCC (0.85 g, 4 mmol) as described for the preparation of 1 to give 2.84 g (79%): mp 80–84 °C; *R_f* (A) 0.25. Anal. (C₅₈H₇₃O₁₃N₇) C, H, N.

Boc-D-Glu-Tyr-Nle-D-Lys-Trp-OCH₃ (18). Compound 16 (2 g, 1.9 mmol) was hydrogenated as described for the preparation of 12 to give 1.5 g (94%) of Boc-D-Glu-Tyr-Nle-D-Lys-Trp-OCH₃ (17), *R_f* (I) 0.20. Compound 17 (1.45 g, 1.7 mmol) was cyclized with DPPA as described for the preparation of 13 to yield after flash chromatography 0.6 g (42%): mp 160–163 °C, *R_f* (J) 0.64; *R_f* (B) 0.35; FAB-MS (MH⁺) calcd 834, found 834.

Boc-D-Glu-Tyr-Nle-D-Lys-Trp-OH (19). Compound 18 (0.50 g, 0.59 mmol) was saponified as described for the preparation of 14 to yield after the same working up 0.36 g (75%): mp 220–222 °C; *R_f* (J) 0.33. Anal. (C₄₂H₅₇O₁₀N₇) C, H, N.

Boc-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp(OBzl)-Phe-NH₂ (20). A solution of H-Nle-Asp(OBzl)-Phe-NH₂-TFA²¹ (250 mg, 0.42 mmol), in DMF (5 mL) containing Et₃N (59 μL, 0.42 mmol), was treated with 19 (0.34 g, 0.42 mmol), HONSu (48.3 mg, 0.42 mmol), and DCC (103 mg, 0.50 mmol) as described for the preparation of 15 to give 485 mg (90%): mp 197–199 °C; *R_f* (J) 0.63. Anal. (C₆₈H₉₁O₁₄N₁₁) C, H, N.

Boc-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (4). Compound 20 (0.40 g, 0.31 mmol) was hydrogenated as described for the preparation of 1 to yield 340 mg (92%): mp 185–187 °C; *R_f* (E) 0.75; *R_f* (I) 0.55; HPLC (*t_R* = 14.6 min), eluent CH₃CN-TEAP (40:60); FAB-MS (MH⁺) calcd 1196, found 1196. Amino acid analysis: Asp 0.98, Glu 0.96, Nle 2.0, Tyr 0.93, Phe 0.96, Lys 0.92.

Boc-D-Glu-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (5). Compound 4 (0.27 g, 0.23 mmol) in dry DMF (2.5 mL) and dry pyridine (7.5 mL) was sulfated with SO₃-pyridine complex (1.8 g) as described for the preparation of 2, to give after chromatography with AcOEt-pyridine-AcOH-H₂O (60:20:6:11) 91 mg (30%): *R_f* (F) 0.64; *R_f* (G) 0.46; HPLC (*t_R* = 15.6 min), eluent CH₃CN-TEAP (35:65); FAB-MS (MH⁺) calcd 1320, found 1320. Amino acid analysis: Asp 0.98, Glu 0.97, Nle 2.0, Tyr 0.93, Phe 1.10, Lys 0.91.

Boc-γ-D-Glu(α-OBzl)-Tyr-Nle-D-Lys(Z)-Trp-OCH₃ (21). A chilled solution of H-Tyr-Nle-D-Lys(Z)-Trp-OCH₃-TFA²¹ (1.29 g, 1.48 mmol) in DMF (10 mL) containing Et₃N (204 μL, 1.48 mmol) was treated with Boc-D-Glu(α-OBzl)-OH (0.49 g, 1.48 mmol), HONSu (0.17 g, 1.48 mmol), and DCC (0.36 g, 1.77 mmol) as described for the preparation of 11 to give 1.3 g (81%): mp 120–123 °C; *R_f* (B) 0.48. Anal. (C₅₈H₇₃O₁₃N₇) C, H, N.

Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-OCH₃ (23). Compound 21 (1.27 g, 1.18 mmol) was hydrogenated as described for the preparation of 12 to give 0.95 g (95%) of Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-OCH₃ (22): mp 155–157 °C; *R_f* (D) 0.37. Compound 22 was cyclized with DPPA as described for the preparation of 13 to yield after flash chromatography 0.39 g (40%): mp 143–147 °C; *R_f* (B) 0.40; *R_f* (J) 0.66; FAB-MS (MH⁺) calcd 834, found 834.

Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-OH (24). Compound 23 (0.73 g, 0.44 mmol) was saponified as described for the preparation of 14 to yield, after the same working up, 0.29 g (81%): mp 181–182 °C; *R_f* (J) 0.33. Anal. (C₄₂H₅₇O₁₀N₇) C, H, N.

Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp(OBzl)-Phe-NH₂ (25). A solution of H-Nle-Asp(OBzl)-Phe-NH₂-TFA²¹ (266 mg, 0.38 mmol) in DMF (5 mL) containing Et₃N (53 μL, 0.38 mmol) was treated with 24 (311 mg, 0.38 mmol), HONSu (44 mg, 0.38 mmol), and DCC (94 mg, 0.45 mmol) as described for the preparation of 15 to give, after working up, 0.45 g (92%): mp 197–200 °C; *R_f* (K) 0.66. Anal. (C₆₈H₉₁O₁₄N₁₁) C, H, N.

Boc-γ-D-Glu-Tyr-Nle-D-Lys-Trp-Nle-Asp-Phe-NH₂ (8). Compound 25 (0.44 g, 0.34 mmol) was hydrogenated as described for the preparation of 1 to yield 0.39 g (95%): mp 193–195 °C; *R_f* (E) 0.80; *R_f* (I) 0.66; HPLC (*t_R* = 23 min), eluent CH₃CN-TEAP

(40:60); FAB-MS (MH⁺) calcd 1196, found 1196. Amino acid analysis: Asp 1.03, Glu 1.01, Nle 2.0, Tyr 0.93, Phe 0.99, Lys 0.95.

Boc-γ-D-Glu-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (9). Compound 8 (230 mg, 0.192 mmol) was sulfated with SO₃-pyridine complex (1.5 g), worked up, and purified by chromatography as described for the preparation of 2 to yield 80 mg (32%): *R_f* (F) 0.65; *R_f* (G) 0.48; HPLC (*t_R* = 24 min), eluent CH₃CN-TEAP (35:65); FAB-MS (MH⁺) calcd 1320, found 1320. Amino acid analysis: Asp 0.92, Glu 0.94, Nle 2.0, Tyr 0.94, Phe 1.01, Lys 0.99.

Boc-Nle-Asp-Phe-NH₂ (26). Boc-Nle-Asp(OBzl)-Phe-NH₂²¹ (2 g, 4.0 mmol) was hydrogenated in MeOH (30 mL) for 3 h to give after working up and crystallization in CH₂Cl₂ 1.53 g (89%): mp 218–220 °C; *R_f* (C) 0.25. Anal. (C₂₄H₃₆O₇N₄) C, H, N.

Boc-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp-Phe-NH₂ (28). A chilled solution of 26 (1.5 g, 3.0 mmol) in CH₂Cl₂ (4.5 mL) and TFA (4.5 mL) was stirred for 45 min at 0 °C and 45 min at room temperature to yield, after evaporation and precipitation with dry ether, 1.48 g (97%) of H-Nle-Asp-Phe-NH₂-TFA (27), *R_f* (F) 0.26.

To a solution of Boc-Tyr-Nle-D-Lys(Z)-Trp-OH²¹ (2.32 g, 2.75 mmol) and HONSu (316 mg, 2.75 mmol) in DMF (15 mL) at –10 °C was added DCC (566 mg, 2.75 mmol). This reaction mixture was stirred for 1 h at –10 °C, 1 h at 0 °C, and 3 h at room temperature. To the above mixture at 0 °C was added a solution of 27 (1.39 g, 2.75 mmol) and Et₃N (385 μL, 2.75 mmol) in DMF (5 mL). The resulting mixture was stirred for 24 h at room temperature. Filtration of DCU, evaporation of the solvent, and precipitation from EtOAc yielded 2.67 g (80%): mp 212–214 °C; *R_f* (J) 0.54. Anal. (C₆₄H₈₄O₁₄N₁₀) C, H, N.

Boc-Glu(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp-Phe-NH₂ (30). A solution of 28 (2.65 g, 2.17 mmol) in CH₂Cl₂ (4 mL) and TFA-anisole (4 mL/0.2 mL) was stirred under N₂ for 45 min at 0 °C and for 45 min at room temperature, yielding after evaporation and precipitation with dry ether 2.56 g (96%) of H-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp-Phe-NH₂-TFA (29), *R_f* (J) 0.33.

To a chilled solution of 29 (0.65 g, 0.53 mmol) in DMF (4 mL) were added successively DIEA (109 μL, 0.63 mmol), HOBt (79 mg, 0.53 mmol), and Boc-Glu(OBzl)-ONp (291 mg, 0.63 mmol). Stirring was continued under N₂ for 1 h at 0 °C and 2 h at room temperature. After evaporation in vacuo, the oily residue was triturated with EtOAc and precipitated with dry ether to yield 0.73 g (96%): mp 191–193 °C; *R_f* (J) 0.60. Anal. (C₇₆H₉₇O₁₇N₁₁) C, H, N.

Boc-Glu(Na)-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (3). Compound 30 (0.30 g, 0.21 mmol) in dry DMF (4 mL) and dry pyridine (4 mL) was sulfated as described for the preparation of 2 to yield, after filtration of the aqueous suspension and drying of the precipitate, 0.28 g of crude Boc-Glu(OBzl)-Tyr(SO₃Na)-Nle-D-Lys(Z)-Trp-Nle-Asp(Na)-Phe-NH₂ (31), *R_f* (G) 0.48. This product was used in the next step without further purification. Compound 31 (0.27 g) in DMF (2 mL) and MeOH (5 mL) was hydrogenated for 3 h in the presence of 10% Pd/C catalyst (60 mg). After filtration of the catalyst, the methanol was evaporated in vacuo. The residue was taken up in 5% NaHCO₃ and lyophilized. After precipitation of inorganic salts in MeOH followed by evaporation, the residue was purified by chromatography on silica gel with EtOAc-pyridine-AcOH-H₂O (40:20:6:11) to yield 60 mg (21%): *R_f* (F) 0.51; *R_f* (G) 0.35; HPLC (*t_R* = 5.8 min), eluent CH₃CN-TEAP (35:65); FAB-MS (MH⁺) calcd 1338, found 1338. Amino acid analysis: Asp 0.98, Glu 0.95, Nle 2.0, Tyr 0.95, Phe 1.00, Lys 0.97.

Boc-D-Glu(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp-Phe-NH₂ (32). Compound 29 (1.65 g, 1.33 mmol) in DMF (7 mL) was treated with DIEA (276 μL, 1.6 mmol), HOBt (205 mg, 1.33 mmol), and Boc-D-Glu(OBzl)-ONp (732 mg, 1.6 mL) as described for the preparation of 30 to yield 1.8 g (93%): mp 131–133 °C; *R_f* (J) 0.6. Anal. (C₇₆H₉₇O₁₇N₁₁) C, H, N.

Boc-D-Glu(Na)-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (6). Compound 32 (0.4 g, 0.28 mmol) in dry DMF (3 mL) and dry pyridine (8.5 mL) was treated with SO₃-pyridine complex (2.1 g) as described for the preparation of 3 to yield 0.3 g of crude Boc-D-Glu(OBzl)-Tyr(SO₃H)-Nle-D-Lys(Z)-Trp-Nle-Asp-Phe-NH₂ (33), *R_f* (G) 0.48. This product was used in the next step without further purification. Compound 33 (0.29 g) was

hydrogenated as described for the preparation of **3** to yield after chromatography on silica gel with EtOAc-pyridine-AcOH-H₂O (40:20:6:11) 70 mg (18%): *R_f* (F) 0.49; *R_f* (G) 0.31; HPLC (*t_R* = 6 min), eluent CH₃CN-TEAP (35:65); FAB-MS (MH⁺) calcd 1338, found 1338. Amino acid analysis: Asp 0.93, Glu 1.00, Nle 2.00, Tyr 0.96, Phe 1.02, Lys 1.01.

Boc-γ-D-Glu(OBzl)-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp(OBzl)-Phe-NH₂ (34). A chilled solution of H-Tyr-Nle-D-Lys(Z)-Trp-Nle-Asp(OBzl)-Phe-NH₂-TFA²¹ (0.59 g, 0.44 mmol) in DMF (4 mL) containing Et₃N (62.5 μL, 0.44 mmol) was treated with Boc-D-Glu(α-OBzl)-OH (0.15 g, 0.44 mmol), HONSu (51 mg, 0.44 mmol), and DCC (109 mg, 0.52 mmol) as described for the preparation of **11**, to give, after working up, 0.58 g (92%): mp 209–211 °C; *R_f* (D) 0.60. Anal. (C₈₃H₁₀₃O₁₇N₁₁) C, H, N.

Boc-γ-D-Glu(Na)-Tyr(SO₃Na)-Nle-D-Lys-Trp-Nle-Asp(Na)-Phe-NH₂ (10). Compound **34** (0.54 g, 0.37 mmol) in dry DMF (4 mL) and dry pyridine (11.5 mL) was treated with SO₃-pyridine complex (2.7 g) as described for the preparation of **3** to yield 0.29 g of crude Boc-γ-D-Glu(OBzl)-Tyr(SO₃Na)-Nle-D-Lys(Z)-Trp-Nle-Asp(OBzl)-Phe-NH₂ (**35**), *R_f* (G) 0.48. Crude compound **35** (0.28 g) was hydrogenated and worked up as described for the preparation of **3** to yield after chromatography on silica gel using AcOEt-pyridine-AcOH-H₂O (40:20:6:11) 75 mg (15%): *R_f* (F) 0.48; *R_f* (G) 0.26; HPLC (*t_R* = 6.4 min), eluent CH₃CN-TEAP (35:65); FAB-MS (MH⁺) calcd 1360, found 1360. Amino acid analysis: Asp 0.94, Glu 1.02, Nle 2.0, Tyr 0.96, Phe 0.99, Lys 0.95.

Boc-Trp-Nle-Asp(OBzl)-Phe-NH₂ (36). To a chilled solution of H-Nle-Asp(OBzl)-Phe-NH₂-TFA²¹ (5.5 g, 9.23 mmol) in DMF containing Et₃N (1.3 mL, 9.23 mmol) were added Boc-Trp-OH (1.06 g, 9.23 mmol), HONSu (1.06 g, 9.23 mmol), and DCC (2.28 g, 11 mmol) as described for the preparation of **15** 6.7 g (95%): mp 164–166 °C; *R_f* (B) 0.53. Anal. (C₄₃H₅₄O₈N₆) C, H, N.

Boc-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂ (38). A chilled solution of Boc-Trp-Nle-Asp(OBzl)-Phe-NH₂ (**36**)²¹ (3.2 g, 4.16 mmol) in CH₂Cl₂ (6.5 mL) and TFA-anisole (6.5 mL, 0.65 mL) was stirred under N₂ for 45 min at 0 °C and for 45 min at room temperature, yielding after evaporation and precipitation with dry ether 3.15 g (97%) of H-Trp-Nle-Asp(OBzl)-Phe-NH₂-TFA (**37**), *R_f* (B) 0.21.

To a chilled solution of **37** (3.12 g, 4.0 mmol) in DMF (30 mL) containing Et₃N (555 μL, 4.0 mmol) were added Boc-D-Nle-OH (921 mg, 4.0 mmol), solubilized in CH₂Cl₂ (5 mL), HONSu (458 mg, 4.0 mmol), and DCC (985 mg, 4.8 mmol) as described for the preparation of **15** to yield 2.61 g (75%): mp 182–183 °C; *R_f* (B) 0.42. Anal. (C₄₈H₆₃O₉N₇) C, H, N.

Boc-Nle-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂ (40). A chilled solution of **38** (2.6 g, 2.95 mmol) in CH₂Cl₂ (4.5 mL) and TFA-anisole (4.5 mL, 0.45 mL) was treated as described for the preparation of **29** to yield 2.23 g (84.5%) of H-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂-TFA (**39**), *R_f* (B) 0.18.

To a chilled solution of **39** (2.11 g, 2.47 mmol) in DMF (13 mL) were added successively Et₃N (344 μL, 2.47 mmol), Boc-Nle-OH (571 mg, 2.47 mmol), HONSu (284 mg, 2.47 mmol), and DCC (610 mg, 2.98 mmol) as described for the preparation of **15** to yield 2.35 g (96%): mp 196–197 °C; *R_f* (B) 0.45. Anal. (C₅₄H₇₄O₁₄N₈) C, H, N.

Boc-Tyr-Nle-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂ (42). A chilled solution of **40** (2.34 g, 2.35 mmol) in CH₂Cl₂ (4 mL) and TFA-anisole (4 mL, 0.4 mL) was treated as described for the preparation of **29**, yielding 2.14 g (90%) of H-Nle-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂-TFA (**41**), *R_f* (B) 0.21.

A solution of **41** (2.12 g, 2.11 mmol) in DMF (11 mL) was stirred at 0 °C with Et₃N (294 μL, 2.11 mmol), Boc-Tyr-OH (59.3 mg, 2.11 mmol), HONSu (243 mg, 2.11 mmol), and DCC (521 mg, 2.54 mmol) as described for the preparation of **15**, yielding 2.27 g (93%): mp 232–233 °C; *R_f* (B) 0.41.

Boc-D-Glu(OBzl)-Tyr-Nle-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂ (44). A chilled solution of **42** (2.26 g, 1.95 mmol) in CH₂Cl₂ (4 mL) and TFA-anisole (4 mL, 0.4 mL) was treated as described for the preparation of **29**, yielding 2.22 g (97%) of H-Tyr-Nle-D-Nle-Trp-Nle-Asp(OBzl)-Phe-NH₂-TFA (**43**), *R_f* (B) 0.20.

To a chilled solution of **43** (2.21 g, 1.89 mmol) in DMF (10 mL) containing Et₃N (263 μL, 1.89 mmol) were added Boc-D-Glu(OBzl)-OH (636 mg, 1.89 mmol), HONSu (217 mg, 1.89 mmol),

and DCC (467 mg, 2.23 mmol) as described for the preparation of **15**, yielding 2.548 g (95%): mp 222–223 °C; *R_f* (B) 0.37. Anal. (C₇₄H₉₄O₁₅N₁₀) C, H, N.

Boc-D-Glu(Na)-Tyr(SO₃Na)-Nle-D-Nle-Trp-Nle-Asp(Na)-Phe-NH₂ (7). Compound **44** (800 mg, 0.58 mmol) in MeOH (10 mL) was hydrogenated for 24 h in the presence of 10% Pd/C catalyst (35 mg). After filtration of the catalyst, the ethanol was evaporated in vacuo. The residue was precipitated in dry ether to yield 648 mg (93%) of Boc-D-Glu-Tyr-Nle-D-Nle-Trp-Nle-Asp-Phe-NH₂ (**45**), *R_f* (B) 0.40.

Compound **45** (635 mg, 0.53 mmol) in dry DMF (5.33 mL) and dry pyridine (16 mL) was sulfated as described for the preparation of **2** by SO₃-pyridine complex (4 g). The residue was purified by chromatography on silica gel with EtOAc-pyridine-AcOH-H₂O (60:20:6:11), yielding 259 mg (33%): *R_f* (G) 0.37; HPLC (*t_R* = 17 min), eluent CH₃CN-TEAP (40:60), FAB-MS (MH⁺) calcd 1323, found 1323. Amino acid analysis: Asp 0.98, Glu 1.02, Nle 3.0, Tyr 0.90, Phe 0.95.

Guinea Pig Brain Membrane Preparation. Guinea pig brain cortex was dissected on ice and homogenized (12 mL/g of tissue, wet weight) in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂. The homogenate was incubated for 30 min at 35 °C and centrifuged at 4 °C for 35 min at 100000g, and the resulting pellet was rehomogenized in a large excess of ice-cold buffer and centrifuged under the same conditions. The final pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, supplemented with 0.2 mg/mL bacitracin and 5 mM MgCl₂ (6–7 mg of protein/mL) for binding experiments. Protein concentration was determined by the method of Lowry et al.²⁶ using bovine serum albumin standards.

Guinea Pig Pancreatic Membranes Preparation. Male guinea pigs (250–350 g) were sacrificed by cervical dislocation, and the pancreases were quickly dissected and placed into ice-cold 10 mM Pipes-HCl buffer, pH 6.5, containing 30 mM MgCl₂ (Pipes-MgCl₂ buffer). After careful removal of the fat, pancreases of seven guinea pigs were homogenized in 25 volumes of Pipes-MgCl₂ buffer at 4 °C with a Brinkmann Polytron PT10, the homogenate was filtered on gauze, and the filtrate was centrifuged twice at 50000g for 10 min with an intermediate rehomogenization of the pellet in fresh buffer. The final pellet was resuspended in 2 volumes of fresh buffer and stored frozen at –80 °C until use. This preparation led usually to 12–14 mL of a membrane suspension containing 25–30 mg of protein/mL.

Binding Assays. [³H]Boc[Nle^{28,31}]CCK_{27–33} ([³H]BDNL, 100 Ci/mmol) was synthesized as previously described.²⁷ [³H]-Propionyl-CCK₈ ([³H]pCCK₈, 60 Ci/mmol) was purchased from Amersham.

Binding experiments with [³H]BDNL and [³H]pCCK₈ were performed as described previously^{19,28} with some modifications. Briefly, incubations (final volume 1 mL) were carried out in 50 mM Tris-HCl buffer, pH 7.4, 5 mM MgCl₂, and 0.2 mg/mL bacitracin for 60 min at 25 °C, in the presence of brain membranes (0.6 mg of protein per tube), or in 10 mM Pipes-HCl buffer pH 7.4, 30 mM MgCl₂, 0.2 mg/mL bacitracin, and 0.2 mg/mL soybean trypsin inhibitor for 120 min at 25 °C in the presence of pancreatic membranes (0.2 mg of protein per tube). For displacement experiments, the radiolabeled probes (0.2 and 0.1 nM for brain and pancreatic membranes, respectively) were incubated in the presence of varying concentrations of the competitor. Nonspecific binding was determined in the presence of 1 μM CCK₈ in all cases. Incubation was terminated by filtration through Whatman GF/B filters precoated by incubation in buffer (brain membranes: 50 mM Tris-HCl, 5 mM MgCl₂, pH 7.4; pancreatic membranes: 10 mM Pipes-HCl, pH 6.5) containing 0.1% bovine serum albumin, filters were rinsed with 2 × 5 mL of ice-cold buffer and dried, and the radioactivity was counted in 5 mL of Ready-solv EP scintillation cocktail (Beckman). The K_i values were calculated according to the Cheng-Prusoff equation.²⁹

(27) Sasaki, N. A.; Funakoshi, S.; Potier, P.; Morgat, J. L.; Genet, R.; Gacel, G.; Charpentier, B.; Roques, B. P. *J. Labelled Compd. Radiopharm.* 1985, 22, 1123.

(28) Durieux, C.; Coppey, M.; Zajac, J. M.; Roques, B. P. *Biochem. Biophys. Res. Commun.* 1986, 137, 1167.

(29) Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* 1973, 22, 3099.

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Registry No. 1, 120059-43-0; 2, 120059-44-1; 2 (free acid), 115410-35-0; 3, 120059-45-2; 3 (free acid), 120085-46-3; 4, 120059-46-3; 5, 120059-47-4; 5 (free acid), 115397-08-5; 6, 120059-48-5; 6 (free acid), 120059-73-6; 7, 120059-49-6; 7 (free acid), 120059-74-7; 8, 115295-07-3; 9, 120059-50-9; 9 (free acid), 115308-76-4; 10, 120085-33-8; 10 (free acid), 116925-35-0; 11, 120085-34-9; 12, 120085-35-0; 13, 120059-51-0; 14, 120059-52-1;

15, 120085-36-1; 16, 120059-53-2; 17, 120085-37-2; 18, 120085-38-3; 19, 120059-54-3; 20, 120085-39-4; 21, 115295-03-9; 22, 120085-40-7; 23, 115295-05-1; 24, 120059-55-4; 25, 115295-07-3; 26, 120059-56-5; 27, 120059-57-6; 28, 120059-58-7; 29, 120059-60-1; 30, 120059-61-2; 31, 120059-62-3; 32, 120059-63-4; 33, 120059-64-5; 34, 120059-65-6; 35, 120085-41-8; 36, 117903-30-7; 37, 117903-31-8; 38, 120059-66-7; 39, 120059-68-9; 40, 120085-42-9; 41, 120085-44-1; 42, 120059-69-0; 43, 120059-71-4; 44, 120085-45-2; 45, 120059-72-5; BOC-Glu-(OBzl)-OH, 13574-13-5; H-Tyr-Nle-D-Lys(Z)-Trp-OMe-TFA, 107326-82-9; H-Nle-Asp(OBzl)-Phe-NH₂-TFA, 107326-87-4; BOC-D-Glu(OBzl)-OH, 35793-73-8; H-Tyr-Nle-D-Lys(Z)-Trp-OMe, 107326-81-8; BOC-D-Glu-OBzl, 34404-30-3; BOC-Nle-Asp(OBzl)-Phe-NH₂, 65864-24-6; BOC-Tyr-Nle-D-Lys(Z)-Trp-OH, 107326-91-0; BOC-Glu(OBzl)-ONp, 7536-59-6; BOC-D-Glu(OBzl)-ONp, 76379-00-5; BOC-Trp-OH, 13139-14-5; BOC-D-Nle-OH, 55674-63-0; BOC-Nle-OH, 6404-28-0; BOC-Tyr-OH, 3978-80-1.

(30) IUPAC-IUB Commission *Eur. J. Biochem.* 1984, 138, 9-37.

Synthesis and Structure-Activity Relationships of a Novel Class of 5-Lipoxygenase Inhibitors. 2-(Phenylmethyl)-4-hydroxy-3,5-dialkylbenzofurans: The Development of L-656,224

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The synthesis of a series of 2-(phenylmethyl)-4-hydroxy-3,5-dialkylbenzofurans and their inhibitory effects against leukotriene biosynthesis and 5-lipoxygenase activity in vitro are described. Many compounds in this series were found to be potent inhibitors of LTB₄ production by human polymorphonuclear leukocytes with IC₅₀ values ranging from 7 to 100 nM. Structure-activity relationships of the series are presented. Within this series, 2-[(4'-methoxyphenyl)methyl]-4-hydroxy-3-methyl-5-propyl-7-chlorobenzofuran (L-656,224) showed extremely potent activity, inhibiting leukotriene biosynthesis in intact human leukocytes (IC₅₀ = 11 nM), as well as the 5-lipoxygenase reaction catalyzed by cell-free preparations from rat leukocytes (IC₅₀ = 36 nM), human leukocytes (IC₅₀ = 0.4 μM), and the purified enzyme from porcine leukocytes (IC₅₀ = 0.4 μM). The compound also shows oral activity in a number of animal models in vivo.

The leukotrienes are a class of potent biologically active mediators derived from arachidonic acid through the action of the enzyme known as 5-lipoxygenase.¹ The peptido-leukotrienes LTC₄, LTD₄, and LTE₄ are potent spasmogenic agents that make up the active components of the "slow reacting substance of anaphylaxis" (SRS-A).² These leukotrienes are thought to be important mediators of human bronchial asthma and allergic diseases and may be involved in the induction of nonspecific bronchial hyper-reactivity.^{3,4} They are also potent vasoconstrictors in a variety of vascular beds including the coronary and cerebral circulations.⁵ On the other hand, leukotriene B₄ is a dihydroxyeicosatetraenoic acid that possesses potent chemotactic, chemokinetic, and neutrophil aggregation properties.⁶ In addition, LTB₄ induces vascular permeability changes^{7,8} and modulation of pain responses, suggesting that this leukotriene is an important mediator of inflammation. Thus a 5-lipoxygenase inhibitor should have utilities in the treatment of pain, certain inflammatory conditions such as psoriasis and ulcerative colitis, and various allergic diseases such as asthma.

The present paper reports on the synthesis and the structure-activity relationship of a new class of 5-lipoxygenase inhibitors, having as its basic structure the 4-hydroxy-2-(phenylmethyl)benzofuran. The activity of this class of compounds was discovered through the screening of our sample collection.

Chemistry

Most of the 3,5-dialkyl-4-hydroxy-2-(phenylmethyl)-benzofuran analogues (5) were prepared as shown in Scheme I (method A). Reaction of substituted acyl resorcinols 1 with various substituted phenacyl bromide 2 in the presence of 0.5 molar equiv of cesium carbonate in acetonitrile at room temperature gave the monoalkylated products 3. Products 3 can be isolated but in most cases

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(1) Samuelsson, B. *Science* 1983, 220, 568-575.

(2) Broekelhurst, W. E. *J. Physiol.* 1953, 120, 16.

(3) Broekelhurst, W. E. *Prog. Allergy* 1962, 6, 539-558.

(4) Austen, K. J. *Immunol.* 1978, 121, 798-805.

(5) Piper, P. J. In *Leukotrienes*; Buckle and Smith, Eds.; Butterworths: London, 1984; p 55-72.

(6) Ford-Hutchinson, A. W.; Bray, M. A.; Doing, M. V.; Shipley, M. E.; Smith, M. J. H. *Nature* 1980, 286, 264.

(7) Bray, M. A.; Cunningham, F. M.; Ford-Hutchinson, A. W.; Smith, M. J. H. *Br. J. Pharmacol.* 1981, 72, 483.

(8) Wedmore, C. V.; Williams, T. J. *Nature* 1981, 289, 646.