N-Methylated Analogs of Ac[Nle^{28,31}]CCK(26-33): Synthesis, Activity, and Receptor Selectivity†

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A series of singularly N-methylated analogs of Ac[Nle^{28,31}]CCK(26-33) were synthesized by the solid-phase methodology, and their biological activity was tested in three different in vitro bioassays. The bioassays employed were the guinea pig gallbladder (GPGB), stomach (GPS), and ileum (GPI). All *N*-methyl analogs were agonists in all three bioassays. N-Methylation at either N- or C-terminals did not affect potency and selectivity, whereas N-methylation of internal residues [Nle^{28},(*N*-Me)Nle^{31}]- and [Nle^{28,31},(*N*-Me)Trp^{30}]CCK(26-33) in the sequence resulted in analogs which were 10-fold less potent than Ac[Nle^{28,31}]CCK(26-33) in all three preparations. Different rank orders of potencies observed for [Nle^{28,31},Sar^{29}]- and [Nle^{28,31},(*N*-Me)Asp^{32}]CCK(26-33) analogs correspond to increased selectivity to either GPGB or GPS, respectively. We propose that systematic N-methylation of single amide bonds in a bioactive peptide should be conducted as an additional routine to probe structure-activity relationships.

Introduction

Cholecystokinin (CCK) is a linear peptide found in the peripheral and central nervous systems acting as a neurotransmitter/neuromodulator and a peptide hormone.[1-3] Its wide distribution and wide spectrum of physiological activities suggest the possibility for a multiplicity of CCK receptor subtypes. Indeed, based on different rank order of potencies of fragments of CCK and gastrin in different in vitro assays, three receptor subtypes have been pharmacologically characterized. The three are CCK-A receptor (also known as the "peripheral receptor"),[4-6] CCK-B receptor (also known as the "central receptor"),[4,5,7] and CCK/gastrin receptor.[8-10] Evidently, highly potent and selective CCK analogs are essential tools for elucidating the roles of different CCK receptors in physiological and pathophysiological mechanisms and in various pharmacological assays.

The prevailing hypothesis assumes that specific "bioactive conformations" of a peptide are binding to different receptor subtypes and transducing intracellular signals.[11] These putative "bioactive conformations" represent predominant and characteristic low-energy conformations. A flexible ligand, such as a linear peptide, forms a number of coexisting rapidly equilibrating low-energy conformations. Only some of these conformations may have biological relevance either as ligands for corresponding receptors (agonist or antagonist) or substrates for proteolytic enzymes.[12] Therefore, introduction of structural constraints may lead to receptor selective ligands due to either the elimination of some of the low-energy conformers and/or stabilizing a limited number of them. In contrast to the unique composition and arrangement of side chains selected through extensive evolutionary processes, the non-paleondromic peptide backbone presents a uniform and common structural entity in different bioactive peptides. Therefore, structural modifications of peptide backbone may provide a powerful approach to modify profiles of biological activity. In substance P, N-methylation of peptide bonds proved to be one of the more powerful means to obtain not only enhanced metabolic stability but also outstanding selectivity to receptor subtypes. N-Methylation of specific peptide bonds is considered to be one of the local and subtle modes of conformational constraint.[13] It reduces the energy barrier to rotation around the N-methylated peptide bond, thus eliminating the predominance of trans vs cis peptide bond

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† Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* 1984, *219*, 345. The following other abbreviations were used: Boc, *tert*-butyloxycarbonyl; GPGB, guinea pig gallbladder; GPI, gunea pig ileum; GPS, guinea pig stomach; CCK, cholecystokinin; OcHx, cyclohexyl ester; For, *N*-formyl; OBzl, benzyl ether; OdiClBzl, 2,4-dichlorobenzyl ether; pMBHA, *p*-methylbenzhydrylamine resin hydrochloride; DCC, dicyclohexylcarbodiimide; BOP, (benzotriazol-1-yloxy)tris(diethylamino)phosphonium hexafluorophosphate; DMS, dimethyl sulfide; TFA, trifluoroacetic acid; DCM; dichloromethane, DMF, *N*,*N*-dimethylformamide; RP-HPLC, reverse-phase high-performance liquid chromatography; VLC, vacuum liquid chromatography; RP%, relative potency (percent); Boc_2O, di-*tert*-butyl dicarbonate; DIPE, *N*,*N*-diisopropylethylamine; EDT, 1,2-ethylenedithiol; Pyr·SO_3 complex, pyridine·SO_3 complex; IPA, isopropyl alcohol; FAB, fast atom bombardment.

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configuration characteristic to the nonalkylated peptide bond.[14,15] N-Methylation of a peptide bond also eliminates its capacity to donate a hydrogen bond.[14,16]

Until recently, N-methylation of a specific peptide bond, in a bioactive peptide, was used only to achieve protection against proteolytic degradation. For example, N-methylation of the following peptides resulted in metabolically stable analogs: enkephalin,[17] LHRH,[18] angiotensin,[19] substance P,[20] and CCK.[21] Nevertheless, a systematic study carried by us which included synthesis and pharmacological evaluation of singularly N-methylated analogs of [pGlu^6]SP(6–11) revealed that this structural modification can lead to highly potent and selective agonists.[22] Two N-alkylated analogs, namely [pGlu^6,Sar^9]SP(6–11) and [pGlu^6,(*N*-Me)Phe^8]SP(6–11), displayed significant potency and high selectivity toward the tachykinin NK-1 and NK-3 receptor subtypes, respectively.[22] These analogs led to the synthesis of Septide[23] and Senktide,[24] which are among the most selective and potent agonists in the field of neuropeptides.

Although selective CCK agonists have been recently synthesized,[25,26] a systematic study of N-methylated analogs of CCK-8 has not yet been undertaken. Because of the growing interest in understanding the modes of CCK activity in vitro and in vivo we have chosen Ac[Nle^{28,31}]-CCK(26–33), an analog of CCK octapeptide (CCK-8), as the subject of our studies. In this work we tested our hypothesis that N-methylation of a single amide bond in linear bioactive peptides may provide a general and convenient approach to probe the effect of subtle conformational constraints on the pharmacological profiles. Therefore, N-alkylation of a particular peptide bond can offer leads for the design of receptor-selective agonists.

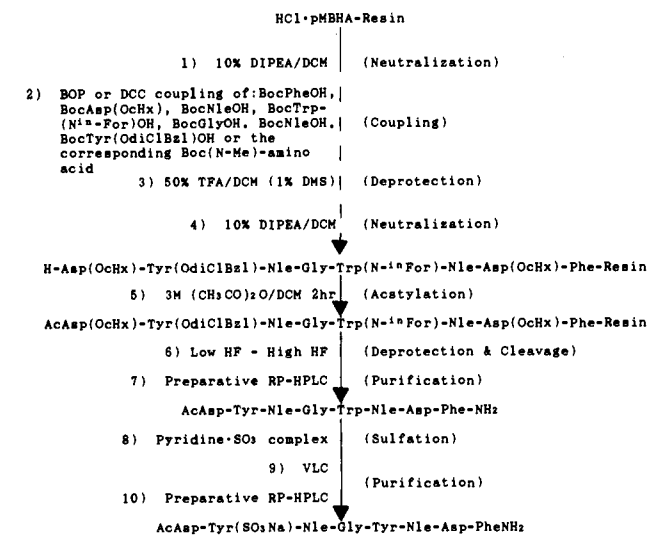

Figure 1. General synthetic scheme for the synthesis of *N*-methyl analogs of Ac[Nle^{28,31}]CCK(26–33).

Synthesis

The CCK-8 analog Ac[Nle^{28,31}]CCK(26–33) was used as the parent peptide. Substitution of the methionine residues with norleucine renders a peptide analog which is equipotent to CCK-8 and is resistant to spontaneous oxidation.[27-29] N-Methylation of the *N-tert*-butyloxycarbonyl (Boc) amino acids *N*-BocPheOH, *N*-BocAsp(OcHx)OH, *N*-BocNleOH, and *N*-BocTyr(OBzl)OH was accomplished according to the procedure of Cheung and Benoiton.[30] *N*-BocSarOH and *N*-Boc(*N*-Me)TrpOH were prepared by introducing Boc groups to the corresponding L-(*N*-Me)amino acids according to Preso et al.[31]

The analogs containing *N*-methyl amino acids were synthesized by a modification of the solid-phase peptide synthesis methodology.[32] The following side chain protecting groups were used for the synthesis: cyclohexyl ester (OcHx) for β-COOH of Asp, *N*-formyl (For) for *N*-indole of Trp, and either benzyl (OBzl) or 2,4-dichlorobenzyl ether (OdiClBzl) for Tyr.

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Table I. Physicochemical Characterization of N-Methylated Analogs of [Nle[28,31]]CCK(26–33) (1–7)

analog of CCK(26–33)[a]	TLC[b] R_f[d]	TLC[b] R_f[e]	HPLC[b] t_R[b]	HPLC[b] t_R[a]	HPLC[b] t_R[c]	amino acid analysis[c] (cal) obtained	FAB/MS $(m/z)^+$
Ac[Nle[28,31],(*N*-Me)Phe[33]]							
1a	0.8	0.36	6.4	4.4		Asp(2) 2.03, Tyr(1) 0.91, Nle(2) 2.06, Gly(1) 1	
1b	0.57	0.17	4.6	1.7	15.3		1161 (M)
Ac[Nle[28,31],(*N*-Me)Asp[32]]							
2a	0.8	0.4	7.2	4.4		Asp(1) 1.15, Tyr(1) 0.94, Nle(2) 2.03, Gly(1) 0.98, Phe(1) 1.03	
2b	0.62	0.23	4.7	1.6	17		1233 (M + 3Na + 2H)
Ac[Nle[28],(*N*-Me)Nle[31]]							
3a	0.8	0.38	7.1	4.7		Asp(2) 2.09, Tyr(1) 0.8, Nle(1) 1, Gly(1) 1.14, Phe(1) 1.06	
3b	0.62	0.22	4.4	1.6	16.6		1207 (M + 2Na)
Ac[Nle[28,31],(*N*-Me)Trp[30]]							
4a	0.9	0.45	7.3	4.7		Asp(2) 2.06, Tyr(1) 0.9, Nle(2) 2.1, Gly(1) 1.05, Phe(1) 1.1	
4b	0.63	0.28	4.9	1.6	15.4		1161 (M)
Ac[Nle[28,31],Ser[29]]							
5a	0.8	0.38	6.7	4.3		Asp(2) 2.06, Tyr(1) 0.9, Nle(2) 2.06, Phe(1) 0.99	
5b	0.69	0.14	4.85	1.8	16.4		1201 (M + H + K)
Ac[Nle[28,31],Pro[29]]							
6a	0.8	0.4	7.1	4.5		Asp(2) 1.93, Tyr(1) 0.98, Nle(2) 2, Pro(1) 1.05, Phe(1) 1.03	
6b	0.75	0.17	4.6	1.6	15.8		1211 (M + H + Na)
Ac[Nle[28,31],(*N*-Me)Tyr[27]]							
7a	0.61	0.42	7.0	4.7		Asp(2) 2.06, Nle(2) 2.3, Gly(1) 1.06, Phe(1) 1.08	
7b	0.42	0.25	4.7	1.6	15.7		1161 (M)

[a] **1a–7a**, nonsulfated analogs; **1b–7b** sulfated analogs. [b] Subscripts a–e refer to TLC/HPLC conditions detailed in the Experimental Section. [c] Amino acid analysis was performed on the nonsulfated analogs **1a–7a**.

Table II. Biological Activities of the *N*-Methylated Analogs of [Nle[28,23]]CCK(26–33) in the in Vitro Assays

analog	GPGB EC_{50} ± SEM (nM)	GPGB *n*	GPGB RP%[a]	GPI EC_{50} ± SEM (nM)	GPI *n*	GPI RP%[a]	GPS EC_{50} ± SEM (nM)	GPS *n*	GPS RP%[a]
Ac[Nle[28,31]]CCK(26–33)	1.2 ± 0.5	10	100	1.4 ± 0.5	10	100	13.5 ± 0.7	10	100
Ac[Nle[28,31],(*N*-Me)Phe[33]]CCK(26–33) (**1b**)	3 ± 0.3	6	42	4 ± 2.5	6	35	19 ± 1.2	3	71
Ac[Nle[28,31],(*N*-Me)Asp[32]]CCK(26–33) (**2b**)	2 ± 0.5	4	60	0.9 ± 0.09	4	155	9 ± 0.3	5	150
Ac[Nle[28],(*N*-Me)Nle[31]]CCK(26–33) (**3b**)	20 ± 0.3	3	6	37 ± 2.4	3	4	140 ± 40	3	10
Ac[Nle[28,31],(*N*-Me)Trp[30]]CCK(26–33) (**4b**)	53 ± 0.3	4	2	26 ± 0.8	3	5	265 ± 30	4	5
Ac[Nle[28,31],Sar[29]]CCK(26–33) (**5b**)	0.1 ± 0.15	4	1250	0.5 ± 0.20	3	280	6 ± 2	7	225
Ac[Nle[28,31],Pro[29]]CCK(26–33) (**6b**)	60 ± 2	4	2	104 ± 34	3	1.3	54 ± 12	5	25
Ac[Nle[28,31],(*N*-Me)Tyr[27]]CCK(26–33) (**7b**)	1.4 ± 0.2	3	90	3.5 ± 0.9	6	40	12 ± 0.9	3	112

[a] The relative potency (RP%) was calculated according to the EC_{50} of Ac[Nle[28,31]]CCK(26–33) in the three bioassays.

Manual solid-phase peptide synthesis was carried out using a wrist action shaker. Synthesis was accomplished on a *p*-methylbenzhydrylamine resin hydrochloride (pMBHA) as the solid support, following procedures outlined in Figure 1. Dicyclohexylcarbodiimide (DCC) was used as the coupling reagent,[32] except for the coupling steps of a N^{α}-Boc-amino acid to either a free *N*-methyl amino acyl or a free *N*-methyl peptidyl. These difficult coupling steps were accomplished using (benzotriazol-1-yloxy)tris(diethylamino)phosphonium hexafluorophosphate (BOP) reagent.[33] To ensure complete couplings, these difficult coupling cycles were repeated two to three times, followed by a capping step with 3 M acetic anhydride in DCM for 2 h in order to block any residual free amino end group. The deprotections of *N*-Boc protecting groups following the incorporation of Boc(*N*-Me)TrpOH were done in the presence of scavengers using 20% DMS/10% EDT/2% anisole/50% TFA/18% DCM (v/v). At the final step of the solid-phase synthesis, acetylation of the N-terminal amino acid residues was performed with 3 M acetic anhydride in DCM for 2 h.

The peptides (0.5–1 mmol) were cleaved from the resin with concomitant removal of side chain protecting groups, by following the low/high HF protocol.[34] The crude peptides were extracted from the resin with 10% AcOH (2 × 20 mL) and DMF (2 × 20 mL), followed by evaporation under reduced pressure, dilution with water, and lyophilization. The crude peptides were purified by preparative RP-HPLC on a C-18 column and then were subjected to the sulfation reaction employing Pyr·SO_3 complex as the sulfation reagent. The sulfation reaction was carried out on the unprotected peptide. Therefore, in order to decrease the side reactions and side product, the sulfation reaction was carefully monitored with HPLC and TLC. The workup of the sulfation reaction provided the crude peptide analogs in the sulfated sodium salt form (see Figure 1) which were firstly purified using the vacuum liquid chromatography method[35] (VLC) on RP-18 silica. The VLC method proved to be an easy, fast, and efficient way to get rid not only of the water-soluble inorganic salts but also the very hydrophobic side products. The final purification step of the sulfated analogs was carried out by preparative RP-HPLC. Table I includes the physicochemical characterization of analogs **1b–7b** and their nonsulfated precursors **1a–7a**.

Results and Discussion

The three in vitro smooth muscle contraction assays that were used to evaluate the biological activity of the N-methylated analogs consist of three different CCK receptor populations. The guinea pig gallbladder (GPGB) represent a "monoreceptor assay" for CCK-A receptor subtype.[6] Based on rank order of potencies and binding affinities, it was suggested that the guinea pig stomach preparation

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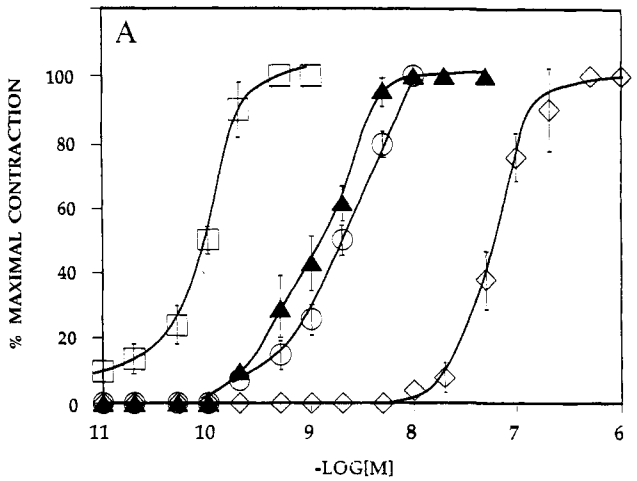

Figure 2. The concentration-response curves of CCK-8 (▲) and the following analogs: Ac[Nle^{28,31},(*N*-Me)Asp^{32}]CCK(26–33) (**2b**) (○), Ac[Nle^{28,31},Sar^{29}]CCK(26–33) (**5b**) (□), Ac[Nle^{28,31},Pro^{29}]-CCK(26–33) (**6b**) (◇) in three in vitro bioassays: The GPGB (A), the GPI (B), and the GPS (C). Each point was determinated in duplicate. Each point represents the mean ± SEM of 10 experiments for Ac[Nle^{28,31}]CCK(26–33) and at least three experiments for the analogs (see the *n* value in Table II).

(GPS) consists predominantly of CCK/gastrin receptor subtype.[8,10,36–40] The guinea pig ileum (GPI) contains two or even three CCK receptor subtypes.[41] The use of the GPGB and GPS assays allowed us to assess not only the relative potency of the N-methylated analogs of CCK-8 but also to characterize their selectivity toward the different CCK receptor subtypes.

The EC_{50} of Ac[Nle^{28,31}]CCK(26–33) in each of the in vitro assays was taken as 100% and the relative potency (RP%) of each analog (**1b–7b**) was calculated accordingly. Table II and Figure 2 summarize the EC_{50} and RP% values of the N-methylated analogs in the three bioassays. The *N*-methyl group has been introduced in the past at different peptide bonds along the CCK-8 sequence.[21,26,42,43] Analogs such as [Sar^4]-, [Pro^4]-, [(*N*-Me)Trp^5]-, and [(*N*-Me)Phe^8]CCK(26–33) were studied by Spatola and co-workers searching for metabolically stable analogs.[43] Analogs of CCK(26–33) CCK(27–33), and CCK(28–33) were 6–8-fold more potent than CCK-8 in the GPGB assay.[43] Nadzan and co-workers reported [desamino-Tyr^{27},Nle^{28,31},(*N*-Me)Asp^{32}]CCK(27–33) to be selective toward CCK-A receptors.[26] Reduced potency was reported by Roques and co-workers for Boc[Nle^{28},(*N*-Ne)Nle^{31}]-CCK(27–33).[45] Lack of a common reference compound, pesence of additional structural modifications, and variations in the protocols of the in vitro assays do not allow simple and coherent conclusion on the effect of N-methylation on selectivity. Therfore, we have conducted a systematic study to test the effect of N-methylation of single peptide bonds on the biological activity and selectivity of analogs derived from Ac[Nle^{26,31}]CCK(26–33). All analogs (**1b–7b**) were CCK agonists in all three bioassays. A small decrease of potency in all three preparations was observed for analog **1b**, which was methylated at Phe^{33}. However, N-methylation of Asp^{32} (analog **2b**) resulted in increased potency, compared to Ac[Nle^{28,31}]CCK(26–33), in both the GPI (RP = 155%) and GPS (RP = 150%). The relative potencies of analogs **3b** and **4b**, methylated at Nle^{31} and Trp^{30}, respectively, were both smaller than that of Ac[Nle^{28,31}]CCK(26–33) by 1 order of magnitude in all three assays. Analog **5b** in which Gly^{29} was substituted with Sar was the most potent analog obtained in this series. We found that the activity was not uniformly enhanced among the different preparations. Analog **5b** was only 2-fold more potent in the GPS (RP = 225%) and GPI (RP = 280%) preparations, whereas in the GPGB it was 10-fold more potent than Ac[Nle^{28,31}]CCK(26–33) (RP = 1250%).

Analog **6b** was synthesized in order to further examine the effect of N-alkylation in position 29. However, substitution by Pro^{29} combines N-alkylation with greater conformational constraint imposed by the pyrolidine ring and allowed the comparison with analog **5b** (substituted by Sar^{29}). The pharmacological profile observed for analog **6b** was very different from that obtained for analog **5b**. Substitution with Pro^{29} resulted in reduced potency in all three preparations. Analog **6b** was a weak agonist in both the GPGB (RP = 2%) and GPI (RP = 1.3%) but retained 25% of Ac[Nle^{28,31}]CCK(26–33) activity in the GPS. The larger conformational constraint imposed by Pro^{29} prevented the potentiation effect obtained through N-methylation and resulted in reduced activity and reversed selectivity.

These results show that substitution of Ac[Nle^{28,31}]-CCK(26–33) with N-methylated amino acid residues at different sites in the sequence not only changes the potency but also the selectivity patterns toward the different CCK receptor subtypes. N-Methylation of Asp^{32} (as in analog **2b**) confers, to a small extent, selectivity toward the CCK receptor in GPS (analog **2b** was 1.5 and 0.5 times more potent than Ac[Nle^{28,31}]CCK(26–33) in the GPS and GPGB assays). The opposite selectivity pattern was observed for analog **5b**. It was only twice more potent than

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Ac[Nle^{28,31}]CCK(26–33) in the GPS but 1 order of magnitude more potent in the GPGB, which indicates preference toward the CCK-A receptor subtype. Further studies furnished additional evidence that relative potencies observed in this series of N-methylated analogs could not be attributed to differences in metabolic stability (manuscript in preparation).

Interestingly, the enhanced selectivity toward CCK-A and CCK/gastrin receptor subtypes of some of the N-methylated analogs, described in this study, supports a model of the bioactive conformation concluded from conformational analysis studies employing fluorescence and NMR techniques.[27] Accordingly, the putative bioactive conformation of CCK-8 is highly folded and it is comprised of two β-turns spanning residues Asp^{26} through Gly^{29} and Gly^{29} through Asp^{32}. Thus, N-methylation on Asp^{32} or Gly^{29} residues can interfere with hydrogen bonds stabilizing these turns to the extent of preventing their formation. Earlier reports showed that replacing the Gly residue with Aib or L-Ala caused a complete loss of activity whereas replacing it with β-Ala or D-Ala resulted in analogs which retained their activity on the pancreatic receptors but not on the GPI and GPGB receptors.[27] These observations provide further evidence that the Gly^{29} residue plays an important role in the biologically active conformation of CCK-8.

N-Methylation of residues found in the middle of the sequence, namely Trp^{30} (analog **4b**) and Nle^{31} (analog **3b**), caused a 10-fold decrease in potency in all preparations. The decrease of potency observed for analog **4b** is not surprising since earlier structure–activity studies showed that this residue is essential for the biological activity and that modifications on this residue resulted in substantial decrease in potency.[44]

Conformational constraints at either the C- or N-termini (analogs **1b** and **7b**, respectively) did not alter much the relative potency and had no significant effect on the selectivity compared to Ac[Nle^{28,31}]CCK(26–33). For example, the relative potencies obtained for Ac[Nle^{28,31},(*N*-Me)Tyr^{27}]CCK(26–33) (**7b**) were 90% and 112% in the GPGB and GPS, respectively. Only in the GPI did analog **7b** show decreased potency (RP = 40%).

The potencies of the various N-methylated analogs observed in the GPI assay were not consistent with the results obtained for either the GPGB or the GPS assays. For example, analog **1b** was found to have the same relative potency in the GBGP and GPI but slightly different in the GPS, and analogs **2b** and **5b** both had the same pattern of relative potencies in the GPS and GPI but differnt ones from that observed in the GPGB. However, analog **7b** was found to have the same relative potency in the GPGB and GPS but different in the GPI (see Table II). Moreover, differences between potencies of CCK analogs in GPI and characteristic CCK-A receptor subtype containing tissues were reported previously.[45,46] Taken together, these studies suggest that the CCK receptor population in the GPI is not identical to either one of the two CCK receptors characteristic to GPGB and GPS and strongly support our view that this receptor population is heterogenic.[41] Hence, the results obtained in this assay cannot be easily interpreted.

In conclusion, N-methylation is a subtle local structural modification which leads to biologically active analogs of CCK-8 with altered selectivity. Analogs Ac[Nle^{28,31},(*N*-Me)Asp^{32}]CCK(26–33) (**2b**) and Ac[Nle^{28,31},Sar^{29}]CCK-(26–33) (**5b**), which show different trends of selectivity, can provide new directions for the synthesis of more selective agonists for the CCK/gastrin and CCK-A receptor subtypes, respectively. Interestingly, the substitution of sarcosine (in analog **5b**) with proline (analog **6b**) reversed the selectivity trend from CCK-A for analog **5b** to CCK/gastrin for analog **6b**. Thus, we postulated that N-methylation at the Asp^{32} and Gly^{29} results in structural constraints which enrich the population of bioactive conformations which show preference toward the CCK/gastrin and CCK-A receptor subtypes, respectively. Therefore, systematic N-methylation of single peptide bonds in Ac-[Nle^{28,31}]CCK(26–33) sequence changed not only the relative potency of the analogs but also in some cases the selectivity tendencies toward the different CCK receptor subtypes.

This approach may provide a routine and simple procedure to assess the structural latitude of the biologically active peptide backbone to conformational hindrance, cis–trans isomerization, and the role of hydrogen bond formation patterns. Systematic single *N*-methyl peptide bond replacements side by side with the more traditional systematic substitutions of each of the residues with Gly, Ala, or D-Ala will provide a more comprehensive set of procedures for screening almost any biologically interesting peptide for structural modifications contributing not only to enhanced metabolic stability but also to modified selectivity.

Experimental Section

Materials. N^{α}-Boc derivatives of L-PheOH, L-GlyOH, and L-NleOH were prepared in our laboratory by standard procedure.[47] Ac[Nle^{28,31}]CCK(26–33), BocAsp(OcHx)OH, and Boc(O-diClBzl)TyrOH were purchased from Bachem (Torrence, CA). BocTrp(N^{in}-For)OH was purchased from Sigma (St. Louis, MO). The *p*-methylbenzhydrylamine resin·HCl (pMBHA) was purchased from Applied Biosystem Inc. (Foster City, CA). Trifluoroacetic acid (TFA) and di-*tert*-butyl dicarbonate (Boc_2O) were purchased from E. Merck (Darmstadt, Germany). (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and *N*,*N*-diisopropylethylamine (DIPEA) were purchased from Chemical Dynamic Co. (South Plainfield, NJ). *N*,*N*-Dicyclohexylcarbodiimide (DCC), 1,2-ethylenedithiol (EDT), and anisole were purchased from Fluka (Buchs, Switzerland). Pyridine·SO_3 complex (Pyr·SO_3 complex) was purchased from Aldrich (Milwaukee, WI).

Solvents and Reagent Purification. *N*,*N*-Dimethylformamide (DMF) was distilled under reduced pressure from CaH_2, redistilled from ninhydrin, and stored over 4-Å molecular sieves. Pyridine was distilled from potassium hydroxide.

HPLC. HPLC analysis was performed on Merck-Hitachi Liquid Chromatograph using a Merck Hibar Lichrosorb RP-18 column (5 μm, 4 × 250 mm). The effluent was monitored at 220 and 280 nm by a Merck-Hitachi 655A-II variable-wavelength UV detector. The isocratic and gradient systems used were as follows: flow rate 1 mL/min; eluents, (A) 0.05% TFA in H_2O, (B) 0.05% TFA in CH_3CN, A:B 60/40 (a), flow rate 1.5 mL/min, linear gradient system 30%–70% B in 10 min (b), flow rate 1.5 mL/min, linear gradient system 85%–15% B in 30 min (c). Preparative HPLC was performed on a Whatman RP-18 column Partisil M9 ODS-3 (10 μm, 9 × 500 mm).

TLC. TLC analysis was performed on precoated silica gel plastic and glass plates 60 F_{254} (0.2 mm) purchased from E. Merck and on precoated silica gel aluminum cards (0.2 mm) purchased from Riedel-de Haen (Seelze, Germany). The solvent systems used were IPA/NH_4OH/H_2O 3/1/1 (d) and EtOAc/Pyr/

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AcOH/H_2O 60/20/6/11 (e). The plates were developed with the following reagent sprays: (1) 4% ninhydrin in *n*-BuOH, (2) chlorine/*o*-tolidine test,[32] (3) Ehrlich test,[32] and (4) Pauly test.[32]

The homogeneity of the purified products was confirmed by TLC/HPLC, and the structural integrity was assessed by amino acid analysis and FAB-MS.

Vacuum-Driven Liquid Chromatography. VLC was performed on E. Merck RP-18 Lichroprep silica gel (15–25 μm).

Amino Acid Analysis (AAA). The analyses were performed on LKB 4400 amino acid analyzer, equipped with a Spectra-Physics 4100 Printer/Plotter Computing Integrator, using a four-component sodium buffer system. Hydrolysis of peptide samples for AAA was carried out on 1-mg samples with 6 N constant-boiling HCl (0.5 mL), which were degassed and sealed under vacuum and then heated for 24h at 110° C.

Mass spectrometry was performed by Dr. Jorg Metzger at the Institute of Organic Chemistry, University of Tubingen, Tubingen, Germany, or at Merck Sharp and Dohme Research Laboratories, West Point, PA, using a fast atom bombardment (FAB) ion source.

Representative Synthetic Procedures. The procedures employed in the solid-phase synthesis of analog **1a** and its sulfation to **1b** were chosen as representative examples for the synthesis of the rest of the corresponding peptides.

AcAsp-Tyr-Nle-Gly-Trp-Nle-Asp-(*N*-Me)PheNH$_2$ (1a). The protected acetyl-octapeptide AcAsp(OcHx)-Tyr(O-diClBzl)-Nle-Gly-Trp(N^{in}For)-Nle-Asp(OcHx)-(*N*-Me)PheNH$_2$ was synthesized on pMBHA resin (0.55 g, 0.5 mmol) following the procedures outlined in Figure 1. A synthetic cycle involving DCC coupling was comprised of the following consecutive steps: **Deprotection**: 1% DMS in dichloromethane (DCM) 1 × 1 min, 50% TFA in 1% DMS/DCM 1 × 1 min, 50% TFA in 1% DMS/DCM 1 × 20 min, DCM 2 × 1 min, MeOH 1 × 1 min. **Neutralization**: DCM 2 × 1 min, 10% DIPEA in DCM 1 × 5 min, DCM 2 × 1 min, MeOH 2 × 1 min, DCM 2 × 1 min. **Coupling**: 1.25 mmol of N^{α}-Boc-amino acid dissolved in DCM or DMF followed by 1.25 mmol of DCC dissolved in DCM 1 × 60 min, 1.5% DIPEA in DCM was added to the coupling mixture for an additional 15 min. **Recoupling**: DCM 1 × 1 min, and repetition of the steps described under Coupling, DCM 1 × 1 min, MeOH 2 × 1 min followed by sampling of the resin-bound peptide and confirming the completion of coupling by a negative ninhydrin test,[32] DCM 2 × 1 min. The incorporation of N^{α}-Boc-(*N*-Me)-amino acid was carried out by BOP-mediated coupling replacing the above-mentioned coupling stage. **BOP-Mediated Coupling**: 1 mmol of N^{α}-Boc-(*N*-Me)PheOH in either DCM or DMF followed by 1 mmol of BOP in DCM and 2.5 mmol of DIPEA 1 × 60 min. **Liquid HF Deprotection and Cleavage**: The resin-bound peptide was deprotected from side chain protecting groups and cleaved from the resin by following the "low HF/high HF" protocol.[34] The "low HF" step was accomplished with HF, *p*-thiocresol, *p*-cresol, and DMS (25%/2.5%/7.5%/65% v/v) with stirring for 2 h at −5 °C and followed by the removal of HF under vacuum. The residue was extrated consecutively with petroleum ether (40–60 °C) and ether and dried under vacuum. It was followed by the "high HF" step employing HF, *p*-cresol, and *p*-thiocresol (90%/7.5%/2.5% v/v) for 1 h at −5 °C. After the evaporation of the HF, the residue obtained was washed consecutively with petroleum ether (40–60 °C) and ether. The crude peptide was extracted from the resin-peptide mixture with 10% AcOH (2 × 2 mL) and DMF (2 × 2 mL), followed by evaporation and lyophilization. The crude peptide was then purified on preparative RP-HPLC using a C-18 column at a flow rate of 3 mL/min and monitored at 280 nm employing a two-phase linear gradient (10–30% B in acetonitrile for the first 15 min followed by 30–70% B for the next 85 min). The solvent system consisted of the following components: (A) 0.05% TFA in H_2O; (B) 0.05% TFA in CH_3CN (0.05% TFA). Fractions were collected at 1-min intervals. The fractions were analyzed by an analytical RP-HPLC yielding 154 mg (28.5%) of the pure **1a** peptide analog. The physicochemical characterization of **1a** is summarized in Table I.

AcAspTyr(SO$_3^-$Na$^+$)-Nle-Gly-Trp-Nle-Asp-(*N*-Me)-PheNH$_2$ (1b). A 60-fold excess of Pyr·SO_3 complex (480 mg) was added at 0 °C to a solution of peptide **1a** (50 mg, 0.046 mmol) dissolved in a mixture of dry pyridine–dry DMF (0.5 mL, 1/1). The reaction mixture was stirred under N_2 for 24–48 h and its progress was carefully monitored by analytical RP-HPLC and TLC. Upon completion of the sulfation reaction, the excess of reagent was destroyed by adding H_2O dropwise at 0 °C. The residue, obtained after removal of the solvents from the reaction mixture under reduced pressure, was dissolved in water and the pH was adjusted to 7 with 5% Na_2CO_3 at 0 °C. The solution was stirred for 30 min at 0 °C at pH 7 and lyophilized. The crude sodium salt of the sulfated peptide was partially purified using the VLC method described below: The crude peptide was loaded on RP-18 silica packed into a sinter glass (1/30, crude peptide to silica gel, w/w). The silica gel was then washed consecutively with H_2O (3 × 20 mL), to remove mostly the inorganic salts, followed by H_2O– CH_3CN (1/1) which washed off the crude sulfated peptide. After evaporation and lyophilization, the sulfated peptide was purified on preparative RP-HPLC using a C-18 column at a flow rate of 3 mL/min and monitored at 280 nm employing a biphasic linear gradient (5–20% B for the first 15 min followed by 20–50% B for the next 85 min), yielding 12 mg (22%) of pure sulfated peptide **1b**. The physicochemical characterization of **1b** is summarized in Table I.

Biological Assays. Animals and Drugs. Male guinea pigs (200–400 g) were purchased from Levenstien, Yokneam, Israel. CCK-8 was purchased from Bachem (Torrance, CA).

Solution: standard Krebs solution containing (concentration in mM) NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.18, $NaHCO_3$ 25, $NaH_2PO_4 \cdot 2H_2O$ 1.18, glucose 11.5, $CaCl_2$ 2.5; bubbled with 95% O_2 and 5% CO_2.

Tissue Preparation. The guinea pigs were stunned by a blow to the head and bled through the neck. The gallbladder, the ileum, and the fundus part of the stomach were immediately removed and immersed in Krebs solution. Muscle strips (3 × 10 mm) were obtained from the circular muscle layers of the gastric fundus and from the longitudinal muscle layers of the ileum. The gallbladder was washed with Krebs solution and was taken as a whole. The gastric mucosa was removed by gentle scraping with fine forceps. The strips were suspended in 10-mL chambers containing Krebs solution maintained at 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2. The muscle strips were stretched by a tension of 1 g (ileum, stomach) and 0.5 g (gallbladder). The preparation was allowed to equilibrate for 90 min, during which the tension was periodically readjusted and the bath volume was replaced several times. Force was measured with an isomeric transducer (Gould UC2) and was recorded on a Gould chart recorder (Model 2200S).

Concentration–Response Curve. Concentration–response curves were produced in the gallbladder, ileum, and stomach by applications of increasing concentrations of the peptide. After each dose of peptide, the strips were washed three times with 5–15-min intervals between doses. Before, during, and after completing the experiment, the response to a test dose of carbachol (100 nM) was measured as control.

Statistical Analysis. The results are expressed as means ± SEM (standard error of the mean) and statistical significance was evaluated with the Student's *t*-test for paired samples. P values < 0.05 were considered significant.

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Registry No. **1a**, 141611-94-1; **1b**, 141612-01-3; **2a**, 141611-95-2; **2b**, 141612-02-4; **3a**, 141611-96-3; **3b**, 141634-68-6; **4a**, 141611-97-4; **4b**, 141612-03-5; **5a**, 141611-98-5; **5b**, 141612-04-6; **6a**, 141611-99-6; **6b**, 141612-05-7; **7a**, 141612-00-2; **7b**, 141612-06-8; Ac[Nle^{28,31}]-CCK(26–33), 103494-23-1; AcAsp(OcHx)-Tyr(OdiClBzl)-Nle-Gly-Trp(N^{in}For)-Nle-Asp(OcHx)-(*N*-Me)PheNH$_2$, 141612-07-9; $N\alpha$-Boc-(*N*-Me)PheOH, 37553-65-4.