Cyclodextrin as Carrier of Peptide Hormones. Conformational and Biological Properties of β-Cyclodextrin/Gastrin Constructs

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Abstract: The C-terminal tetrapeptide amide of gastrin, the shortest sequence of this gastrointestinal hormone capable of exhibiting all the biological properties even though at reduced potency, and the related heptapeptide amide were covalently linked to mono-(6-succinylamino-6-deoxy)-β-cyclodextrin to analyze the effect of the bulky cyclic carbohydrate moiety on recognition of the peptides by the G-protein-coupled CCK−B/gastrin receptor and on their signal transduction potencies. With the four-carbon succinyl spacer and particularly with the additional tripeptide spacer in the heptapeptide/β-cyclodextrin conjugate, full recognition by the receptor was obtained with binding affinities identical to those of the unconjugated tetrapeptide and with a potency comparable to that of full agonists. Docking of this conjugate onto a structure of the human CCK−B receptor derived by homology modeling indicates sufficient free space of the peptide moiety for intermolecular interaction at the putative gastrin binding site, whereby additional interactions of the cyclodextrin with the receptor surface apparently suffice for stabilizing the complex and thus for triggering the full hormonal message. The host/guest complexation of the peptide moiety by the β-cyclodextrin which seems to occur at least in the case of the tetrapeptide conjugate does not suffice in its strength for competing with the receptor recognition. However, multiple presentation of the tetragastrin by its covalent linkage to the heptakis-(6-succinylamino-6-deoxy)-β-cyclodextrin leads to peptide/peptide and/or peptide/cyclodextrin collapses with strong interferences in the receptor recognition process. Retention of full agonism by suitably designed monoconjugates of bioactive peptides with cyclodextrins suggests a highly promising approach for targeting host/guest complexed or covalently bound cytotoxic drugs to specific tumor cells for receptor-mediated internalization.

Introduction

Cyclodextrins are cyclic oligosaccharides consisting of a hydrophobic cavity that is capable of including a variety of hydrophobic compounds via host−guest complexation.[1] This property has been extensively exploited in the past to change the physicopharmaceutical properties of lipophilic drugs such as water-solubility, bioavailability, improved stability, and effectiveness.[2] Covalent linkage of bioactive peptides to cyclodextrins has also been proposed[3,4] to possibly take advantage of this complexation in terms of solubility and reduced catabolism, although such conjugates with the relatively large cyclodextrin carrier were expected to impair recognition processes at a molecular level. NMR and X-ray analysis of β-cyclodextrin and its methylated form monosubstituted at the C6 of the carbohydrate with aromatic chromophores, amino acids, and related derivatives as well as dipeptides[5−9] clearly revealed that self-inclusion of the grafted molecules is critically depending upon the spacer used.[6,7] Similarly, the spacer is expected to play a decisive role for the accessibility of grafted bioactive components to molecular recognition events. This was fully confirmed in our recent studies on the application of β-cyclodextrin/peptide aldehyde constructs as inhibitors of cysteine proteinases where only suitably sized spacers allowed to retain both the carrier effects and the inhibitory properties of the covalently bound molecules.[10] Conversely, direct attachment of a hormone molecule, i.e., of the enkephalin analogue DPDPE, to β-cyclodextrin led to a significantly reduced receptor affinity and selectivity.[11]

For the family of G-protein-coupled receptors (GPCR) a topology has been proposed that consists of a bundle of seven transmembrane helices tethered by a series of extracellular and cytoplasmatic loops of variable lengths.[12−15] The footprint of

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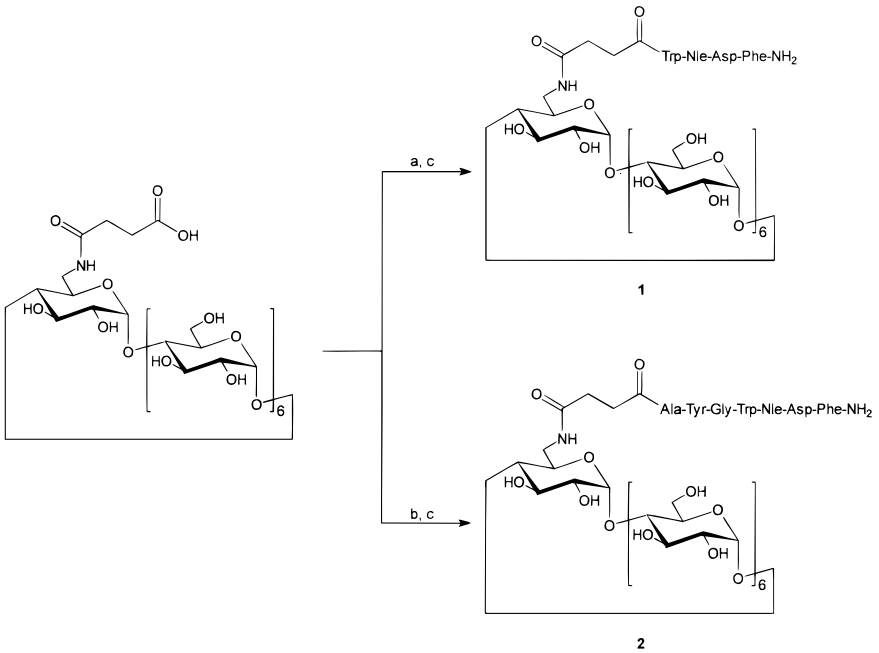

Figure 1. Synthesis of the monoconjugates of the gastrin peptides [Nle[15]]-HG-[14−17] and [Nle[15]]-HG-[11−17] with 6-amino-6-deoxy-β-cyclodextrin using the succinyl moiety as spacer: (a) H-Trp-Nle-Asp(OtBu)-Phe-NH_2/EDC/HOBt/DMF (39%); (b) H-Ala-Tyr(tBu)-Gly-Trp-Nle-Asp(OtBu)-Phe-NH_2/EDC/HOBt/DMF (18%); (c) 95% aqueous TFA containing 1% 1,2-ethanedithiol (60−85%).

ligand binding derived from mutagenesis studies is spatially rather conserved and involves residues of the extracellular loops as well as residues located in more hydrophobic compartments of the transmembrane domain.[15−17] Using receptor binding data of lipo-derivatized gastrin peptides and mutagenesis data as experimental constraints, docking of the peptide hormone gastrin to the CCK−B/gastrin receptor led to a similar picture of the ligand binding mode with a large portion of the peptide spanning the extracellular surface of the receptor, but with the C-terminal tetrapeptide of the ligand, i.e., the message portion of the hormone,[18] penetrating into the helix bundle.[19,20]

CCK−B/gastrin receptors are predominantly present throughout the central nervous system where they regulate anxiety/panic attacks and dopamine release implicated in the pathogenesis of dopaminergic related behavioral disorders in humans. In the periphery these receptors regulate acid and histamine secretion as well as growth in the gastric mucosa and gastrointesinal motility.[21−23] Thus the CCK−B/gastrin receptor represents an attractive target for drug development.

For a rational drug design a more precise delineation of the ligand binding sites of the receptor is required. In this context, we have investigated in the present study how covalent linkage of the tetra- and heptagastrin peptides [Nle[15]]-HG-[14−17] and [Nle[15]]-HG-[11−17] to β-cyclodextrin is affecting receptor recognition and signal transduction, since with the large carbohydrate moiety the interaction mode of the ligand, according to modeling experiments, should be significantly constrained. Correspondingly, such conjugates were expected to represent promising pharmacological tools to derive structurally relevant information on the nature of the hormone-receptor complex. As the multifunctional β-cyclodextrin offers the additional option of an oligo-presentation of ligands, the heptakis-[Nle[15]]-HG-[14−17]/β-cyclodextrin conjugate was synthesized to analyze the effect of an *in loco* artificially enhanced concentration on the signal transduction efficiency.

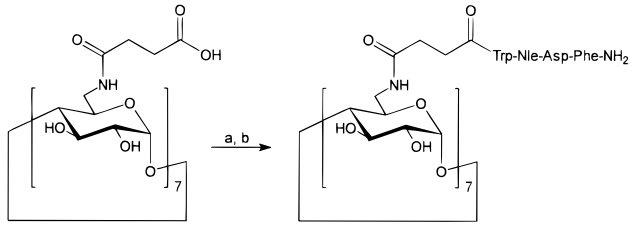

Figure 2. Synthesis of the heptaconjugates of the gastrin peptide [Nle[15]]-HG-[14−17] with heptakis-(6-amino-6-deoxy)-β-cyclodextrin using the succinyl moiety as spacer: (a) H-Trp-Nle-Asp(OtBu)-Phe-NH_2/PyBOP/NEt_3/DMF and (b) 95% aqueous TFA containing 1% 1,2-ethanedithiol (33%).

Results

Synthesis of the Gastrin/β-Cyclodextrin Conjugates. By applying known methods of cyclodextrin chemistry, we have recently elaborated efficient synthetic routes for mono- and oligo-functionalization of β-cyclodextrin with linear and flexible carboxyalkyl spacers for the synthesis of peptide conjugates.[10,24] As shown in Figure 1, in the present study we used the mono-(6-succinylamino-6-deoxy)-β-cyclodextrin[10] for coupling the two

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Table 1. Chemical Shifts (ppm), Amide Proton Temperature Coefficients ($-d\delta/dT$) and $^3J_{N\alpha}$ Coupling Constants (Hz) of [Nle^{15}]-HG-[14-17]/β-Cd (**1**) in D_2O/H_2O (1:9) at 300 K, pH 7.0

amino acid residue	NH	CαH	CβH	others	$-d\delta/dT$	$^3J_{N\alpha}$
sugar unit A	7.67			C6H 3.84, C5H 2.94, C4H 3.56, C3H 3.30, C2H 3.86, C1H 4.92		
spacer				$-CH_2-CH_2-$ 2.34	7.44	
Trp	8.10	4.33	2.97, 3.14	N1H 9.63, C2H 7.12, C4H 7.44, C5H 6.97, C6H 7.05, C7H 7.32	7.67	6.61
Nle	7.74	3.97	1.41	CγH 0.88, CδH 1.06, CϵH 0.66	6.78	6.89
Asp	8.00	4.47	2.55, 2.68		7.89	7.45
Phe	7.88	4.37	2.84, 3.02	ring 7.10, 7.16	7.51	7.51
C term. NH2	6.91, 7.16				5.43, 4.38	

Table 2. Chemical Shifts (ppm) and $^3J_{N\alpha}$ Coupling Constants (Hz) of [Nle^{15}]-HG-[11-17]/β-CD (**2**) in D_2O/H_2O (1:9) at 300 K, pH 7.0

amino acid residue	NH	CαH	CβH	others	$^3J_{N\alpha}$
sugar unit A	7.83				
spacer				$-CH_2-CH_2-$ 2.54	
Ala	8.23	4.01	1.16		5.15
Tyr	8.08	4.27	285, 2.90	ring C2,6H, 7.00 C3,5H 6.79	7.17
Gly	7.99	3.77, 3.88			*
Trp	7.73	4.57	3.21	N1H 10.01, C2H 7.19, C4H 7.52, C5H 7.06, C6H 7.14, C7H 7.39	*
Nle	7.73	4.00	1.42, 1.48	CγH 0.94, CδH 1.13, CϵH 0.74	*
Asp	8.00	4.51	2.60, 2.78		*
Phe	7.93	4.46	2.95, 3.10	ring 7.19, 7.29	6.81
C term. NH_2	7.24				

side chain-protected gastrin peptide derivatives H-Trp-Nle-Asp-(OtBu)-Phe-NH_2[25] and H-Ala-Tyr(tBu)-Gly-Trp-Nle-Asp(OtBu)-Phe-NH_2[25] via the EDC/HOBt procedure to the carbohydrate carrier. Acidolytic removal of the protecting groups from the peptidic moieties with 90% aqueous trifluoroacetic containing 1% 1,2-ethanedithiol as scavenger, followed by RP-chromatography led to the β-cyclodextrin monoconjugates of [Nle^{15}]-HG-[14−17] (**1**) and [Nle^{15}]-HG-[11−17] (**2**) as analytically well characterized compounds. In similar manner (Figure 2) the heptakis-(6-succinylamino-6-deoxy)-β-cyclodextrin was coupled with H-Trp-Nle-Asp(OtBu)-Phe-NH_2 by the PyBOP method to yield upon acidolytic deprotection the β-cyclodextrin heptaconjugate of [Nle^{15}]-HG-[14−17] (**3**) which was purified by gel filtration. Analytical characterization of the monoconjugates by chromatographic and spectroscopic methods was straightforward; for the heptaconjugate, however, it proved to be difficult since the multiplicity of peptide−peptide and peptide−carrier interactions simulates aggregation-type phenomena. In this context, capillary zone electrophoresis was found to be the most useful analytical method.

Analytical and Conformational Analysis of the Gastrin/β-Cyclodextrin Conjugates. From 2D-COSY (or DQF−COSY), TOCSY, and NOESY spectra recorded in DMSO-d_6 and water all the resonances of the peptide components of conjugates **1** and **2** including the succinyl spacer were identified. The carbohydrate signals of compound **1** and **2** overlap severely in DMSO (see Experimental Section), while in water at least the signals of the subunit derivatized at the C6 in compound **1** were unambiguously assigned (Tables 1 and 2). The resonances related to all other subunits showed strong overlaps even in water.

Besides the interresidue sequential NOEs (Figure 3) obtained for compound **1** in water at 300 K, two weak CαH(i)/NH(i+2) and CαH(i)/CβH(i+3) NOEs were detected that suggest a

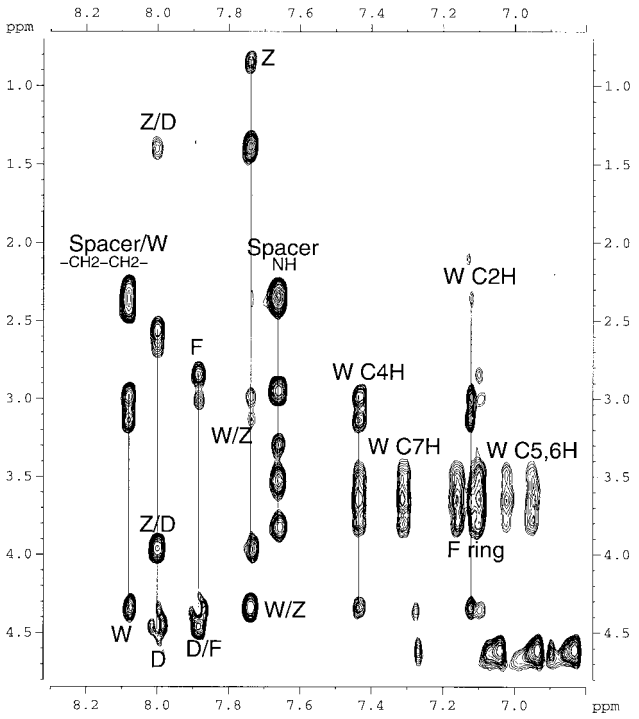

Figure 3. NH-aliphatic region of the NOESY spectrum of [Nle^{15}]-HG-[14−17]/β-cyclodextrin (**1**) in D_2O/H_2O (1:9) at 300 K; Z = norleucine.

bended conformation of the tetrapeptide moiety in this solvent (Table 3). This kink in the peptide backbone is further supported by the observed through-space NOEs from the aromatic side chains of both Trp and Phe as well as from the C-terminal amide protons to the carbohydrate carrier which are not observable in the NOESY spectrum at 330 K. Analysis of

Table 3. Interresidue and Peptide/Carbohydrate NOEs Derived from the NOESY Spectrum of [Nle[15]]-HG-[14-17]*β*-CD (**1**) in D_2O/H_2O (1:9), 10 μM, τ_m = 150 ms at 300 K[a]

Peptide-Sugar NOEs		Intensity
Trp ring:	N1H	Medium
	C2H	Weak
	C4H	Strong
	C5H	Medium
	C6H	Medium
	C7H	Strong
Phe ring:	Ar 2,6	Strong
	Ar 3,5	Strong
C terminal	NH_2	Weak

[a] The thickness of the bars are related to the NOEs intensities.

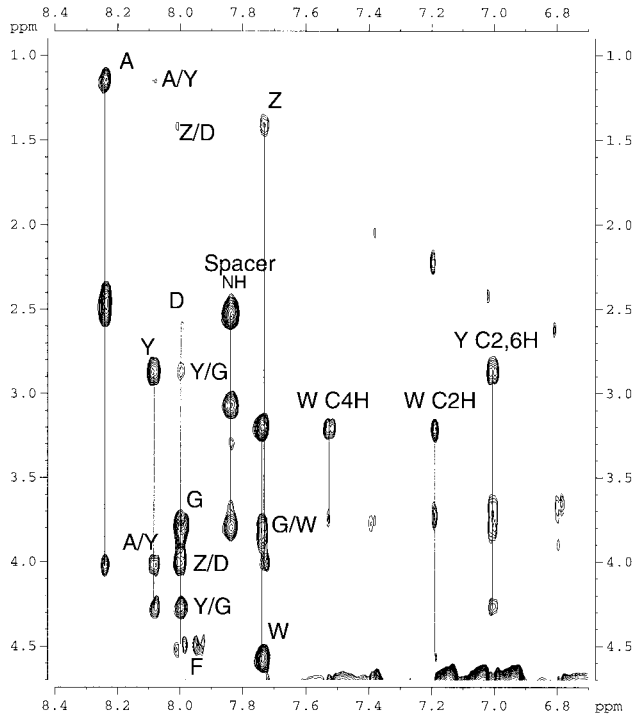

Figure 4. NH-aliphatic region of the NOESY spectrum of [Nle[15]]-HG-[11−17]*β*-cyclodextrin (**2**) in D_2O/H_2O (1:9) at 300 K; Z = norleucine.

the temperature dependency of the chemical shifts of the amide protons showed the usual linear upfield shift but with a smaller slope for the C-terminal amide protons that are apparently more shielded from the bulk solvent. The lack of an ordered conformation like a *β*- or *γ*-type turn of the tetrapeptide moiety in the conjugate, as possibly induced by self-complexation of the hydrophobic Trp or Phe side chain, is confirmed by the $^3J_{N\alpha}$ values which are all close to the standard random coil values of about 7 Hz.

For compound **2** the NOESY spectrum shows again the expected sequential interresidue connectivities (Figure 4). The NOEs, however, were found to be of weak intensity suggesting an even greater flexibility of the heptapeptide than that of the

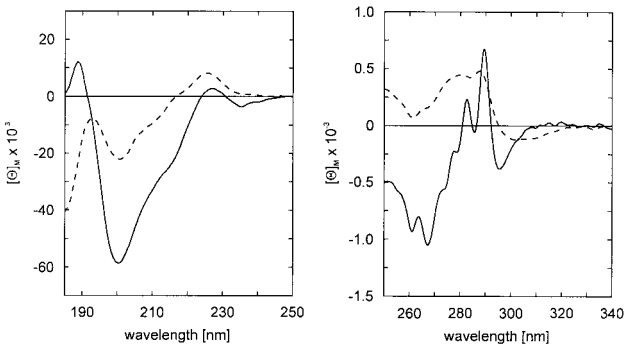

Figure 5. CD spectra of [Nle[15]]-HG-[14−17]/*β*-cyclodextrin (**1**) (—) and Ac-Trp-Nle-Asp-Phe-NH_2 (- - -) in 5 mM phosphate buffer (pH 7.0) at 20 °C in the near and far UV.

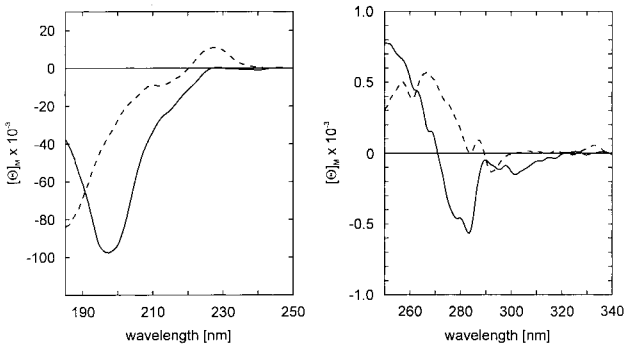

Figure 6. CD spectra of [Nle[15]]-HG-[11−17]/*β*-cyclodextrin (**2**) (—) and Ac-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 (- - -) in 5 mM phosphate buffer (pH 7.0) at 20 °C in the near and far UV.

tetrapeptide chain in the conjugate, a fact that is fully supported by the negative signs of all the cross-peaks.

The information derived from the ^{1}H NMR experiments agrees fully with the dichroic properties of the two conjugates. The far UV CD spectrum of the tetragastrin conjugate **1** in aqueous solution (Figure 5) shows a significantly increased intensity if compared to that of the acetylated tetrapeptide with a negative maximum at 200 nm and a shoulder around 215 nm that could reflect the tendency for a bended conformation. In the near UV the sharp L_b bands of Trp (at 282 and 289 nm) and the well resolved vibronic structure of the phenyl L_b transitions would indicate significant rigidity of the two aromatic groups as resulting from their interaction with the carbohydrate carrier. A shielding of both the Trp and Phe side chains by the carrier is also supported by the increased stability of the conjugate **1** toward chymotryptic digestion ($t_{1/2}$ = 140 min) if compared to the rate of enzymatic digestion of the reference tetrapeptide Ac-Trp-Nle-Asp-Phe-NH_2 ($t_{1/2}$ = 50 min). For cleavage of the Nle-Asp bond with endoproteinase Asp-N the $t_{1/2}$ was again increased by a factor of 3 upon conjugation of the tetrapeptide to the *β*-cyclodextrin.

The CD spectra of the conjugate **2** in the far and near UV region (Figure 6) are supportive for more flexibility of the aromatic side chains and for random coil conformation of the peptide backbone. Full accessibility of the conjugated heptapeptide in compound **2** as suggested by the spectroscopic data is further confirmed by the rates of enzymatic digestion with chymotrypsin and endoproteinase Asp-N which were practically superimposable to those of the reference heptapeptide Ac-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2.

The ^{1}H NMR spectrum of the heptaconjugate **3** in DMSO-d_6 fully agrees with the 7-fold symmetric structure of the construct (Figure 7), and 2D NOESY and TOCSY experiments

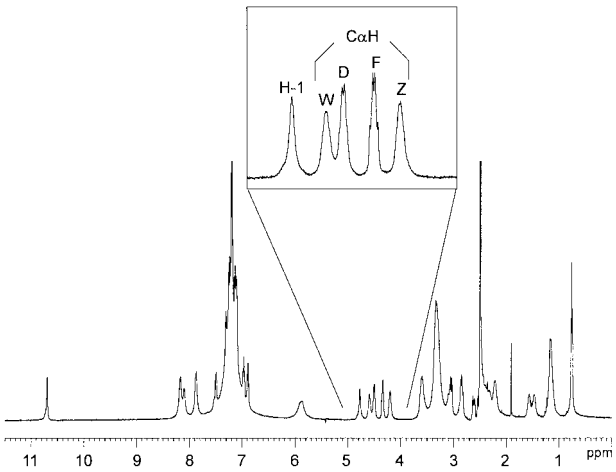

Figure 7. ^{1}H NMR spectrum of the heptaconjugate ([Nle^{15}]-HG-[14−17])$_7$/β-cyclodextrin (**3**) in DMSO-d_6 at 300 K; Z = norleucine.

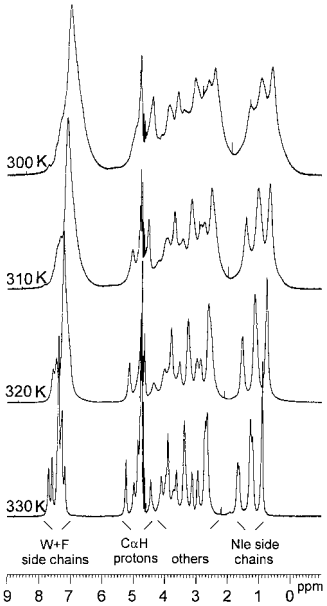

Figure 8. ^{1}H NMR spectrum of the heptaconjugate ([Nle^{15}]-HG-[14−17])$_7$/β-cyclodextrin (**3**) in D_2O/ND_3 (500:1) at increasing temperature. Due to the overlap of the signals of the identical residues in the seven peptide moieties as well as the overlap of the peaks in the sugar moiety only a partial 1D spectrum-based assignment was possible.

were used to perform the assignments. Conversely, in water a strong broadening of the signals in the 1D spectrum was observed which becomes better resolved by raising the temperature as shown in Figure 8. This would be consistent with a system of multiple conformers deriving from fast interconversions of peptide/peptide and particularly, peptide/carrier interactions (host−guest complexes) in the time scale of the NMR experiments. The presence of multiple conformers is also supported by the coalescence of satellite peaks in the CE electropherogram upon raising the temperature from 24 °C to 60 °C. A similar collapse of the symmetry due to conformational mobility of the host−guest complexes has previously been reported for the cyclodextrin derivatives heptakis-(2,3-di-*O*-benzyl-6-*O*-(2-methoxy-6-naphthoyl)-β-cyclodextrin[26] and hexakis-2,6-di-*O*-methyl-3-*O*-benzyl)-β-cyclodextrin.[27]

Table 4. Binding and Functional Properties of the β-Cyclodextrin Conjugates **1**−**3** for the CCK−B/Gastrin Receptor Expressed in CHO Cells in Comparison to the Unconjugated Peptides[a]

	binding to CCK−B/ gastrin receptor		inositol phosphate production	
peptides	IC_{50} (nM)	F_a	EC_{50} (nM)	F_b
[Thr, Nle]-CCK-9	0.33		0.09	
Ac-[Nle^{15}]-HG-[14−17]	4.8		1.7	
[Pyr,^{10}Nle^{15}]-HG-[10−17]	1.6		0.12	
[Nle^{15}]-HG-[14−17]/β-CD (**1**)	26.3	5.5	1.53	0.9
[Nle^{15}]-HG-[11−17]/β-CD (**2**)	5.7	3.9	0.11	1.0
([Nle^{15}]-HG-[14−17])$_7$/β-CD (**3**)	25.4	5.2	17.3	10.4

[a] F_a and F_b are the related potent factors.

Binding and Functional Properties of Gastrin/β-Cylodextrin Conjugates. The binding affinitites of the β-cyclodextrin/gastrin-peptide conjugates **1**, **2**, and **3** for the CCK−B/gastrin receptor expressed in CHO cells are compared in Table 4 with those of the reference gastrin-peptide derivatives Ac-[Nle^{15}]-HG-[14−17] and [Pyr,^{10}Nle^{15}]-HG-[10−17] as well as of [Thr, Nle]-CCK-9 as a full agonist of the CCK−B/gastrin receptor. Replacement of the Met-15 residue in gastrin with norleucine is known to be without any effect on the biological properties of this hormone.[28] The receptor binding affinities clearly show that the β-cyclodextrin moiety causes a significant decrease in the affinity of the peptides conjugates relative to the unconjugated peptide ligands. This decrease was of 5.5-fold for [Nle^{15}]-HG-[14−17]/β-CD and 3.9-fold for [Nle^{15}]-HG-[11−17]/β-CD. It is known from previous studies that N-terminal extension of the gastrin tetrapeptide in sequence mode leads to enhanced receptor affinities.[20,28] This was confirmed even in the present case with [Pyr,^{10}Nle^{15}]-HG-[10−17] exhibiting a 3.3-fold higher affinity than Ac-[Nle^{15}]-HG-[14−17] for the CCK−B/gastrin receptor. This contribution of the tripeptide portion Ala-Tyr-Gly to the binding is largely suppressed in the conjugate **2** where it apparently serves more as additional spacer of the C-terminal tetrapeptide from the carrier moiety that leads to an IC_{50} value similar to that of the unconjugated tetrapeptide.

Most interesting was the observation that the heptaconjugate **3** exhibited a binding affinity almost identical to that of the monoconjugate **1**. This would exclude significant steric clashes as expected from the bulky polyvalent ligand unless these are compensated by the *in loco* enhanced ligand concentration. Interconversion of the host−guest complexes has been postulated in heptacylodextrin conjugates to explain the broadening of the NMR signals. A similar continuous exchange of the ligand could possibly occur even at the receptor level. In this context it is worthy to note that heptapresentation of peptide aldehydes as inhibitors of cysteine proteinases led in comparison to the monopresentation to both weaker and stronger inhibition depending upon the enzyme.[24] An alternative explanation for the binding affinity of the heptaconjugate **3** could be a per se weaker binding, as resulting from the impaired access to the ligand binding site, compensated by unspecific interactions of the additional peptide moieties with the receptor surface.

In the functional assay, as monitored by inositol phosphate production in the intact CHO cells, the monotetragastrin and monoheptagastrin conjugates **1** and **2** were as efficacious and

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potent as the parent unconjugated peptides (Table 4). Thereby, the [Nle[15]]-HG-[11−17]/β-CD conjugate **2** was found to be a full agonist despite its 17-fold lower affinity than [Thr,Nle]-CCK-9. Conversely, oligopresentation of the tetragastrin with compound **3** led to a pronounced decrease of potency if compared both with the unconjugated tetrapeptide (10.4-fold) and the monoconjugate **1** (11.3-fold). Interestingly, compound **3** binds to the receptor with the same affinity as compound **1** but induces inositol phosphate production with an about 10-fold lower potency. This result suggests that structural requirements for agonist binding are not exactly identical to those for agonist activity and that the relatively high binding affinity of **3** may result from strong unspecific binding contributions. In a previous study on the dimeric presentation of the gastrin peptide HG-[5−17] on a peptide scaffold conformational studies combined with immunological responses clearly confirmed a collapse of the two peptide chains as responsible for the strongly reduced accessibility of the construct to recognition by the CCK−B/gastrin receptor.[29]

Discussion

Multiple antigen-presentation on lysine dendrimers to the recognition by immune competent cells has been successfully employed in the development of synthetic immunogens.[30] Similarly, multiple presentation of peptide hormones covalently grafted to the surface of tobacco mosaic virus led to surprisingly strong enhancements of receptor-mediated bioactivities per unit of peptide.[31] This enhanced bioactivities related to signal transductions via GPCRs has fostered intensive research on modulation of agonist activity via dimerizations or multiple presentations on templates.[32−34] Controversial and mostly negative results were obtained which in view of the present day knowledge about the transmembrane structure of GPCRs can find rational explanations. Cross-linking of monospecific agonists or antagonists to exploit affinity increase derived from entropic effects of multivalent ligands[35−37] requires sufficiently long spacers (>40 Å) to induce microaggregation of GPCRs.[38] In this context the limiting factor could well be the local concentration of receptor molecules. Our attempt to present on the relatively small β-cyclodextrin template a higher population of agonists to artificially enhance *in loco* their concentration, failed since the binding affinity calculated per unit of tetragastrin is 10-fold lower than that of the monotetragastrin conjugate **1**. Sterical interferences of the peptide moieties in the recognition process as well as a collapse of the chains on the template as deduced from the NMR spectrum of the conjugate **3** in aqueous solution could explain the negative results and thus seriously question the perspectives of oligopresentations even for immunological purposes.

Lipo-derivatization of the hormones gastrin and CCK with di-fattyacyl-glycerol moieties and, correspondingly, a forced membrane interaction allowed us to demonstrate that a membrane-bound pathway for hormone recognition and binding by the receptor is indeed possible.[39] Conformational studies of these membrane-bound hormones using DMPC vesicles as mimic of cell membranes excluded a preferential folding of the peptides at the water/lipid interface[40,41] to facilitate thermodynamically a complexation by the receptor as foreseen by the theory of a membrane-bound recognition process of hormones proposed by Schwyzer.[42]

In the present study an opposite approach was taken via derivatization of the gastrin peptides with the hydrophilic β-cyclodextrin that should prevent strong interactions of the ligands with hydrophobic compartments of the cell membrane. Thus recognition has to occur upon statistical collisional events depending solely on the concentration of the ligand in the aqueous phase. Although the spectroscopic analysis of the monogastrin peptide conjugates suggested that self-complexation is occurring at least to some extent in the case of the tetragastrin conjugate **1**, the stability of such complexes is known to be relatively low. Therefore, the host/guest complexation in peptide/β-cyclodextrin constructs should not interfere significantly with binding of the peptide moieties to the receptor unless the carrier itself is not responsible of steric clashes. The succinyl spacer in compound **1** is apparently sufficient to guarantee recognition of the tetragastrin moiety, the shortest sequence of this hormone capable of activating the receptor. Thereby sterical interferences of the bulky β-cyclodextrin moiety is apparently not critical since even the signal transduction process is largely retained. In this context it is worthy to note that N-terminal glycosylation of tetragastrin with deoxyfructosyl or glucosyl-deoxyfructosyl was found to increase the hormonal potency *in vivo* significantly more than simple acetylation.[43] With the present day knowledge about receptor structures the steady increase in potency of tetragastrin from the monosaccharide to disaccharide and finally to the β-cyclodextrin derivative **1** strongly suggests beneficial interactions of the carbohydrate moieties with the receptor surface. With insertion of the tripeptide Ala-Tyr-Gly as additional spacer in compound **2** the binding affinity was remarkably enhanced, and full hormonal potency was recovered.

Docking experiments of the conjugate **2** into a homology modeling-derived structure of the human CCK−B receptor protein[20,44] were performed in order to visualize the potential role of the cyclodextrin moiety in the interaction of this ligand with the receptor. The receptor-agonist complex was manually constructed in analogy to the putative binding mode of gastrin[20] and was then subjected to extensive molecular dynamics simulations not only for efficient energetic relaxation after the model building procedure but also for estimating the stability and thereby the relevance of the generated molecular assembly. Special emphasis was laid on the explicit treatment of the environment conditions of the transmembrane entity in that the

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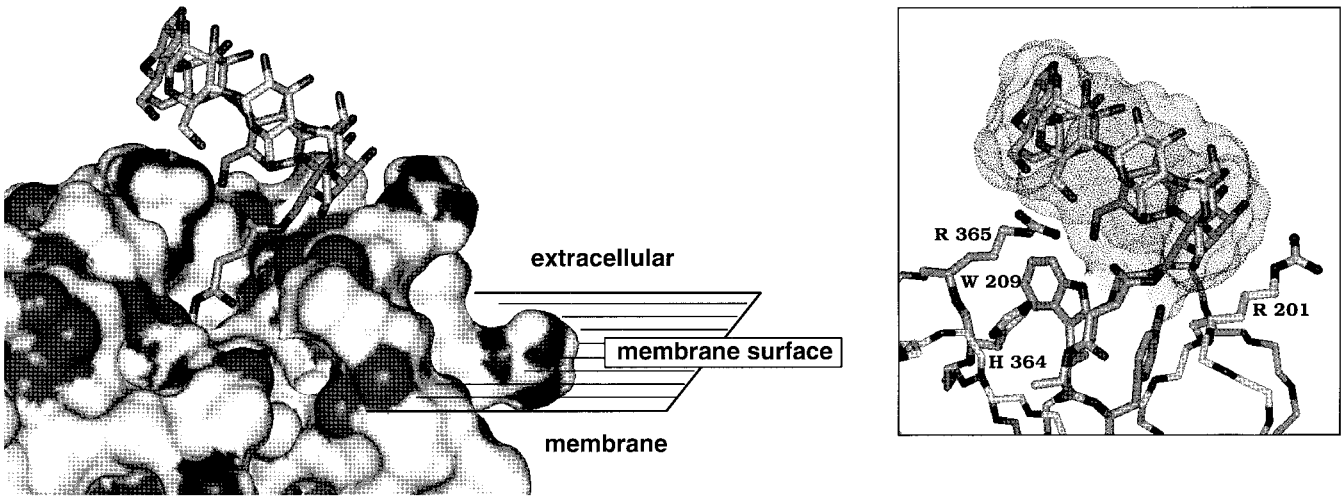

Figure 9. [Nle[15]]-HG-[11−17]/β-cyclodextrin docked to the CCK−B/gastrin receptor model protein; *left panel*: side view of the receptor in a solvent accessible surface presentation while the ligand molecule is depicted in the stick mode; *right panel*: hydrophilically interacting residues of the extracellular portion of the receptor with the cyclodextrin moiety.

simulations were carried out in a triphasic solvent system comprising a central hydrophobic compartment mimiking the lipid phase of a native membrane, flanked by two aqueous phases representing the extracellular and cytoplasmic liquids, respectively.[44] The structure obtained after 500 ps of MD simulation within this solvent mimic shows the β-cyclodextrin moiety significantly protruding from the protein surface into the extracellular aqueous compartment (Figure 9). However, a fluctuating interaction pattern of various hydroxyl groups of the cyclic carbohydrate with charged and hydrophilic side-chain functionalities of protein loop residues is observed in the time course of the molecular dynamics simulation. Especially the guanido groups of Arg-201 (extracellular loop 2) and Arg-365 (extracellular loop 3) as well as the indole NH of Trp-209 (extracellular loop 2) are frequently found in hydrogen bond interactions with the β-cyclodextrin. Thereby the β-cyclodextrin cone is confined to a spatial orientation with a tilt angle of approximately 30° with respect to the extracellular protein surface and the outer membrane interface. In this docking model the peptide moiety is intensively engaged in intermolecular interactions involving residues of the extracellular loops as well as of the transmembrane helices in good agreement with the mutagenesis data (a detailed description of these docking experiments will be reported elsewhere).

In conclusion, upon monoconjugation of gastrin peptides to β-cyclodextrin the receptor binding affinity is largely retained, and the signal transduction efficacy and potency is comparable to that of fully active CCK−B/gastrin receptor agonists. Conversely, oligo-presentation of these bioactive peptides on the cyclic carbohydrate carrier significantly impairs the ligand receptor recognition process. The surprisingly high hormonal potency of the monogastrin peptide constructs can only be explained by unspecific interactions of the carbohydrate moiety with the receptor surface that is apparently confirmed by docking experiments of the β-cyclodextrin/gastrin peptide **2** on a CCK−B/gastrin receptor model. These properties make such conjugates with β-cyclodextrin promising compounds for targeting host/guest complexed or covalently bound cytotoxic drugs to specific tumor cells for receptor-mediated internalization. Indeed, internalization of receptor-bound ligands besides representing a general physiological process has been well documented in the case of the CCK−A and CCK−B receptors.[45−48]

Experimental Section

Materials and Methods. All reagents and solvents used in the synthesis were of the highest quality commercially available. Chymotrypsin was from Sigma (München) and Asp-N (sequence grade) from Böhringer Mannheim. CE was performed on a Spectra Phoresis 1000 capillary electrophoresis apparatus (TSP, Darmstadt) at 25 kV using an underivatized fused silica capillary (67 cm × 75 μm; length × ID) and 50 mM sodium borate buffer (pH 8.5); HPLC was carried out with Waters equipment (Eschborn, Germany) on Nucleosil 300/C18 (Machery & Nagel, Düren) using a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min. Amino acid analyses of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid; 110 °C; 24 h) were performed on a Biotronic amino acid analyzer (LC 6001). FAB-MS spectra were recorded on Finnigan MAT 900 and MALDI-TOF-MS on Bruker Reflex II.

The synthesis of [Thr,Nle]-CCK-9,[49] ^{125}I−BH-[Thr,Nle]-CCK-9,[50] and [Pyr,[10]Nle[15]]-HG-[10−17][51] were described previously.

Synthesis of the Gastrin Peptides/β-Cyclodextrin Conjugates. [β-CD]-NHCO−$(CH_2)_2$CO-Trp-Nle-Asp-Phe-NH_2 (1). Mono-(6-deoxy-6-succinylamino)-β-cyclodextrin[10] (0.2 g; 0.16 mmol) and H-Trp-Nle-Asp(O*t*Bu)-Phe-NH_2[25] (0.1 g; 0.16 mmol) in 2.5 mL of DMF were reacted overnight at room temperature with equivalent amounts EDC/HOBt. The solvent was evaporated, and the crude product was purified on Lichroprep RP-18 (Merck, Darmstadt) using a linear gradient of water/acetonitrile from 80:20 to 20:80 in 3 h. Yield of the protected conjugate: 0.12 g (39%); HPLC: t_R = 17.75 min; FAB-MS: m/z = 1850.1 $[M + H]^+$, 1833.1 $[M + H - NH_3]^+$; calcd for $C_{80}H_{119}N_7O_{42}$: 1849.7.

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An aliquot of the protected conjugate (10.0 mg; 0.005 mmol) was dissolved in ice-cold 90% aqueous TFA containing 1% 1,2-ethandithiol. After 2.5 h stirring at room temperature the TFA was evaporated, and the residue was purified by preparative HPLC on Nucleosil C18 (Macherey & Nagel, Düren) by isocratic elution with 0.1% aqueous TFA/acetonitrile (95:5; 5 min) followed by the linear gradient from 95:5 to 82:18 (5 min) and 82:18 to 40:60 (80 min). The product was obtained as a lyophilisate; yield: 7.0 mg (78%); HPLC: t_R = 13.52 min; amino acid analysis of the acid hydrolysate: Asp 1.00 (1), Nle 0.99 (1), Phe 1.00 (1), Trp 0.28 (1); peptide content: 86.2%; the low recovery or Trp is due to reaction with carbohydrate (deep red colored hydrolysate); ^{1}H NMR (DMSO-d_6) δ 0.83 (t, 3H, J = 6.87 Hz, ϵCH_3 Nle), 1.15 (m, 1H, γ_2CH_2 Nle), 1.21 (m, 1H, γ_1CH_2 Nle), 1.22 (m, 2H, δCH_2 Nle), 1.49 (m, 1H, β_2CH_2 Nle), 1.58 (m, 1H, β_1CH_2 Nle), 2.28 (m, 4H, NHCO(CH_2)$_2$CO), 2.47 (m, 1H, β_2CH_2 Asp), 2.66 (m, 1H, β_1CH_2 Asp), 2.85 (m, 1H, β_2CH_2 Phe), 2.93 (m, 1H, β_2CH_2 Trp), 3.03 (m, 1H, β_1CH_2 Phe), 3.12 (m, 1H, β_1CH_2 Trp), 3.15−3.75 (br m, 42H, H-2, H-3, H-4, H-5, H-6a, H-6b, overlapping with water signal of the solvent), 4.17 (m, 1H, αCH Nle), 4.35 (m, 1H, αCH Phe), 4.50 (m, 1H, αCH Asp), 4.52 (m, 1H, αCH Trp), 4.83 (m, 7H, H-1), 5.80 (br s, 14H, C(2)−OH, C(3)−OH), 6.94 (t, 1H, J = 7.45 Hz, C5H Trp), 7.04 (t, 1H, J = 7.54 Hz, C6H Trp), 7.13 (s, 1H, C2H Trp), 7.14 (s, 1H, CONH_2), 7.20 (m, 5H, C_6H_5 Phe), 7.23 (s, 1H, CONH_2), 7.31 (d, 1H, J = 8.13 Hz, C7H), 7.57 (d, 1H, J = 7.99 Hz, C4H), 7.59 (m, 1H, NHCO(CH$_2$)$_2$CO), 7.77 (d, 1H, J = 8.19 Hz, NH Phe), 8.00 (d, 1H, J = 7.57 Hz, NH Nle), 8.04 (d, 1H, J = 7.73 Hz, NH Trp), 8.14 (d, 1H, J = 7.32 Hz, NH Asp), 10.71 (s, 1H, N1H Trp), 12.37 (br. s, 1H, COOH Asp); FAB-MS: m/z = 1794.7 [M + H]$^+$; calcd for $C_{76}H_{111}N_7O_{42}$: 1793.6.

[β-CD]-NHCO(CH$_2$)$_2$CO-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH$_2$ (2). Mono-(6-deoxy-6-succinylamino)-β-cyclodextrin[10] (0.2 g; 0.16 mmol) and H-Ala-Tyr(tBu)-Gly-Trp-Nle-Asp(OtBu)-Phe-NH$_2$ × 2.5H$_2$O[25] (0.17 g; 0.16 mmol) in 5 mL of DMF were reacted with EDC/HOBt, and the crude product was purified as described above for the tetrapeptide conjugate. Yield: 64 mg (18%); HPLC: t_R = 19.92 min; FAB-MS: m/z = 2197.3 [M + H]$^+$, 2180.0 [M + H − NH$_3$]$^+$; calcd for $C_{98}H_{144}N_{10}O_{46}$: 2196.9.

An aliquot of the protected conjugate (15.5 mg; 7 μmol) was exposed to ice-cold 90% aqueous TFA (2 mL) containing 1% 1,2-ethanedithiol for 5 h at room temperature. TFA was evaporated, and the residue was purified by HPLC as described for **1**. Yield: 8.7 mg (59%); HPLC: t_R = 14.62 min; amino acid analysis of the acid hydrolysate: Asp 0.98 (1), Gly 1.10 (1), Ala 1.00 (1), Tyr+Nle 1.99 (2), Phe 0.89 (1), Trp 0.41 (1); for the low recovery of Trp see compound **1**; peptide content: 87.5%; ^{1}H NMR (DMSO-d_6) δ 0.81 (t, 3H, J = 6.7 Hz, ϵCH_3 Nle), 1.10 (d, 3H, J = 6.7 Hz, CH_3 Ala), 1.15 (m, 1H, γ_2CH_2 Nle), 1.19 (m, 2H, δCH_2 Nle), 1.21 (m, 1H, γ_1CH_2 Nle), 1.49 (m, 1H, β_2 CH_2 Nle), 1.57 (m, 1H, β_1CH_2 Nle), 2.34 (m, 4H, NHCO(CH_2)$_2$CO), 2.48 (m, 1H, β_2CH_2 Asp), 2.67 (m, 1H, β_1CH_2 Asp), 2.68 (m, 1H, β_2CH_2 Tyr), 2.85 (m, 1H, β_2CH_2 Phe), 2.89 (m, 1H, β_1CH_2 Tyr), 2.94 (m, 1H, β_2CH_2 Trp), 3.02 (m, 1H, β_1CH_2 Phe), 3.14 (m, 1H, β_1CH_2 Trp), 3.20−3.75 (br m, 42H, H-2, H-3, H-4, H-5, H-6a, H-6b, overlapping with the water signal of the solvent), 3.60, 3.70 (2 m, 2H, αCH Gly), 4.18 (m, 1H, αCH Nle), 4.21 (m, 1H, αCH Ala), 4.34 (m, 1H, αCH Tyr), 4.35 (m, 1H, αCH Phe), 4.50 (m, 1H, αCH Asp), 4.56 (m, 1H, αCH Trp), 4.80−4.90 (m, 7H, H-1), 5.62−5.80 (br s, 14H, C(2)−OH, C(3)−OH), 6.60, 6.97 (2 d, 4H, J = 6.7 Hz, C_6H_4OH Tyr), 6.94 (t, 1H, J = 7.6 Hz, C5H Trp), 7.03 (t, 1H, J = 7.6 Hz, C6H Trp), 7.12 (s, 1H, C2H Trp), 7.14 (s, 1H, CONH_2), 7.19 (m, 5H, C_6H_5 Phe), 7.24 (s, 1H, CONH_2), 7.30 (d, 1H, J = 8.9 Hz, C7H), 7.57 (d, 1H, J = 8.9 Hz, C4H), 7.64 (m, 1H, NHCO(CH$_2$)$_2$CO), 7.79 (d, 1H, J = 8.9 Hz, NH Phe), 7.84 (d, 1H, J = 7.6 Hz, NH Tyr), 7.93 (d, 1H, J = 7.6 Hz, NH Trp), 7.98 (d, 1H, J = 7.6 Hz, NH Ala), 8.04 (d, 1H, J = 7.6 Hz, NH Nle), 8.07 (m, 1H, NH Gly), 8.15 (d, 1H, J = 7.6 Hz, NH Asp), 9.11 (s, 1H, C_6H_4OH Tyr), 10.73 (s, 1H, N1H Trp), 12.32 (br. s, 1H, COOH Asp); FAB-MS: m/z = 2086.1 [M + H]$^+$; calcd for $C_{90}H_{128}N_{10}O_{46}$: 2084.7.

[β-CD]-(NHCO(CH$_2$)$_2$CO-Trp-Nle-Asp-Phe-NH$_2$)$_7$ (3). Heptakis-(6-deoxy-6-succinylamino)-β-cyclodextrin[24] (20 mg; 0.011 mmol) and H-Trp-Nle-Asp(OtBu)-Phe-NH$_2$[25] (73 mg; 0.115 mmol) were reacted in 3 mL of DMF at room temperature with PyBOP (60 mg; 0.115 mmol) and equivalent amounts of triethylamine. After 18 h the bulk of the solvent was evaporated, and the product precipitated with water (MALDI-TOF-MS: m/z = 6147.02 [M + H]$^+$, 6169.43 [M + Na]$^+$, 6185.60 [M + K]$^+$; calcd for $C_{308}H_{413}N_{49}O_{84}$: 6144.9).

The product was dissolved in 50 mL of ice-cold 95% aqueous TFA containing 2% 1,2-ethanedithiol; after 4 h the TFA was evaporated, and the resulting crude product chromatographed on fractogel TSK HW-40S using 0.1% ammonia as eluent. Yield: 21 mg (33%); CE (T = 60 °C): t_M = 4.72 min; amino acid analysis of the acid hydrolysate: Asp 7.00 (7), Nle 7.17 (7), Phe 6.77 (7), Trp 4.05 (7); for the low recovery of Trp see compound **1**; peptide content: 74%; ^{1}H NMR (DMSO-d_6) δ 0.80 (s, 3H, ϵCH_3 Nle), 1.18 (m, 4H, γCH_2 Nle, δCH_2 Nle), 1.49 (m, 1H, β_2CH_2 Nle, 1.58 (m, 1H, β_1CH_2 Nle), 2.23 (br s, 4H, NHCO(CH_2)$_2$CO), 2.47 (m, 1H, β_2CH_2 Asp), 2.62 (m, 1H, β_1CH_2 Asp), 2.85 (m, 1H, β_2CH_2 Phe), 2.87 (m, 1H, β_2CH_2 Trp), 3.04 (m, 1H, β_1CH_2 Phe), 3.10 (m, 1H, β_1CH_2 Trp), 3.15−3.70 (br m, 6H, H-2, H-3, H-4, H-5, H-6a, H-6b, overlapping with the water signal of the solvent), 4.20 (m, 1H, αCH Nle), 4.35 (m, 1H, αCH Phe), 4.50 (m, 1H, αCH Asp), 4.60 (m, 1H, αCH Trp), 4.78 (s, 1H, H-1), 5.90 (br s, 2H, C(2)−OH, C(3)−OH), 6.70−7.70 (br m, 12H, CONH_2 Phe, C_6H_5 Phe, C2H, C4H, C5H, C6H, C7H), 7.87 (br s, 2H, NH Phe, NHCO-(CH$_2$)$_2$CO), 8.10 (br s, 1H, NH Trp), 8.15 (br s, 2H, NH Asp, NH Nle), 10.70 (s, 1H, N1H); MALDI-TOF-MS: m/z = 5775.19 [M + Na]$^+$, 5791.98 [M + K]$^+$; calcd for $C_{280}H_{357}N_{49}O_{84}$: 5752.5 (most aboundant isotope).

Synthesis of Reference Gastrin Peptides. Ac-Trp-Nle-Asp-Phe-NH$_2$ (4). H-Trp-Nle-Asp(OtBu)-Phe-NH$_2$[25] (0.1 g; 0.16 mmol) was reacted in DMF (3 mL) with acetic acid anhydride (18 μL; 0.19 mmol) in the presence of pyridine (15 μL; 0.19 mmol). After 1 h the solution was evaporated to dryness, and the residue was dissolved in ice-cold 95% aqueous TFA containing 1% 1,2-ethanedithiol. The solution was kept in the ice-bath for 3 h, diluted with water, and lyophilized. The crude product was purified on Lichroprep RP-18 (Merck, Darmstadt) using a linear gradient of 0.1% aqueous TFA/acetonitrile from 90:10 to 20:80 in 3 h. Yield: 70 mg (70%); peptide content as determined by amino acid analysis: 92.8%; HPLC: t_R = 16.5 min; FAB-MS: m/z = 621.3 [M + H]$^+$; calcd for $C_{32}H_{40}N_6O_7$: 620.3.

Ac-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH$_2$ (5). H-Ala-Tyr(tBu)-Gly-Trp-Nle-Asp(OtBu)-Phe-NH$_2$ × 2.5H$_2$O[25] (0.1 g; 0.1 mmol) was acylated with acetic acid anhydride, deprotected with TFA, and purified on Lichroprep RP-18 as described above. Yield: 58 mg (65%); peptide content as determined by amino acid analysis: 90.3%; HPLC: t_R = 16.7 min; FAB-MS: m/z = 912.4 [M + H]$^+$; calcd for $C_{46}H_{57}N_9O_{11}$: 911,4.

Enzymatic Degradation of Gastrin Peptides/β-Cyclodextrin Conjugates. To solutions of the peptides **1**-**5** (0.1 μmol) in 0.395 mL of 50 mM sodium phosphate buffer (pH 7.6) a solution of chymotrypsin (5 μg) in 5 μL 10 mM phosphate and 0.15 mM $CaCl_2$ buffer (pH 7.6) was added, and the resulting mixtures were incubated at 25 °C. Aliquots were taken at time intervals and quenched with 1 M phosphoric acid, and digestion rates were determined by quantification of the peak areas of the parent peptides in HPLC: $t_{1/2}$ = 140 min (**1**), 16 min (**2**), 50 min (**4**), and 13 min (**5**).

Digestion of peptides **1**−**5** with Asp-N proteinase was performed under identical conditions: $t_{1/2}$ = 16 min (**1**), 3 min (**2**), 5 min (**4**), and 2.5 min (**5**).

Circular Dichroism. CD spectra were recorded on a Yobin-Yvon dichrograph Mark IV equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. All data are averages of 10 scans, and the spectra were taken at 20 °C employing quartz cells of 0.2 cm optical path length. The spectra are reported in terms of ellipticity units per mole of compounds ([Q]$_M$). The spectra were recorded in 5 mM phosphate buffer (pH 7.0), and concentrations were determined by weight and peptide content as determined by quantitative amino acid analysis.

NMR Spectroscopy. NMR spectra were recorded at 500 MHz in DMSO-d_6 (5 mM) and water (10 mM, 10% D_2O/90% H_2O) at 300 K unless stated otherwise on a Bruker AMX500 spectrometer equipped with a Z-gradient unit for pulsed field gradient spectroscopy. For 2D-^{1}H NMR spectra of compound **1** and **2** in DMSO-d_6 the following parameters were used. TOCSY:[52,53] mixing time for MLEV17 50 ms,

trim pulse 2.5 ms, size 2K, sweep width 7042.3 Hz, 64 scans, 340−512 increments, phase-sensitive according to States et al.;[54] NOESY:[55] mixing time 150 ms, size 2K, sweep width 7042.3 Hz, 64−128 scans, 320−380 increments, phase-sensitive;[54] gas-COSY:[56] sweep width 7042.3 Hz, size 2K, 16 scans, 256 increments, gradient ratio 1:1. For 2D-^{1}H NMR spectra of compound **3** in DMSO-d_6 the following parameters were used. TOCSY: mixing time for MLEV17 55 ms, trim pulse 2.5 ms, size 2K, sweep width 7042.3 Hz, 128 scans, 256 increments, phase-sensitive according States *et al.*[54] NOESY: mixing time 150 ms, size 2K, sweep width 7042.3 Hz, scans 128, increments 300, phase-sensitive according to States *et al.*[54]

For the 2D-^{1}H NMR spectra in water the suppression of the water peak in the DQF−COSY experiments[57] was achieved with presaturation by continuous low power irradiation during the relaxation delay, while in the TOCSY and NOESY experiments the WATERGATE selective single-echo scheme[58,59] was applied to remove the water trace. DQF−COSY of compound **1**: phase-sensitive according to States *et al.*,[54] size 2K, sweep width 7042.3, scans 128, increments 256; compound **2**: as for **1** except sweep width 6024.1. TOCSY of compound **1**: mixing time for MLEV17 50 ms, trim pulse 1.5 ms, size 2K, sweep width 7042.3 Hz, 64 scans, 256 increments; phase-sensitive according to States-TPPI,[60] number of gradient pulses 2, ratio 1:1; compound **2**: as for **1** except sweep width 6024.1 Hz. NOESY of compound **1**: mixing time 150 ms; size 2K, sweep width 7042.3 Hz, scans 64, increments 256, phase-sensitive (TPPI),[61] number of gradient pulses 2, ratio 1:1; compound **2**: as for **1** except mixing time 200 ms, 300 ms, sweep width 6024.1 Hz, scans 128, number of gradient pulses 3; gradient ratio 1.2:1:1. NOESY of compound **1** at 330 K: mixing times 150 ms, 250, 350, and 400 ms, size 2K, sweep width 6024.1 Hz, scans 128, increments 300, phase-sensitive (TPPI), number of gradient pulses 2, gradient ratio 1:1.

Data were zero-filled in the second dimension to yield frequency-domain matrixes of 2048 × 1024 real data points, giving a digital resolution of 2.9−3.4 Hz/point in F2 and 5.8−6.8 Hz/point in F1. Prior to Fourier transformation Gaussian or shifted square sine-bell window functions were used in both dimensions. All data processing was performed using XWINNMR software on a Silicon Graphics computer.

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Cell Line. CHO-DG44 were acquired from the American Type Collection Culture (Rockville, MD) and grown on Falcon plastic ware in F12 medium suplemented with 5% fetal calf serum. Cells were passaged twice weekly. The cDNA encoding the CCK−B/gastrin receptor cloned from a human pancreas[20] was inserted in pRFENeo vector[62] and transfected into the CHO-DG44 cells using lipofectin. Stable transfectants were selected using Geneticin and individual geneticin-resistant clones were isolated and further characterized for the number of expressed receptors. B8−CHO clone stably expressed 650,000 CCK−B/gastrin receptors/cell.

Receptor Binding Assay on B8−CHO Cells. Approximately 24 h after the transfer of transfected CHO cells to 24-wells plates, the cells were washed with phosphate-buffered saline (pH 6.95) containing 0.1% BSA and were incubated for 60 min at 37 °C in 0.5 mL of Dublelcco's Modified Eagle's Medium/0.1% BSA with 71 pM ^{125}I−BH-[Thr,Nle]-CCK-9 in the presence or absence of competing compounds. The cells were washed two times with phosphate-buffered saline (pH 6.95) containing 2% BSA, and cell-associated ^{125}I−BH-[Thr,Nle]-CCK-9 was collected with 0.1 N NaOH added to each well. The radioactivity was directly counted in a gamma counter (Auto-Gamma, Packard, Downers Grove, IL). Nonspecific binding determined in the presence of 1 μM [Thr,Nle]-CCK-9 was always less than 10% of total binding. Binding data from at least three separated experiments from different batches of transfected cells were analyzed using the EBDA LIGAND program[63] or GraphPad Prism program. The relative affinity of each compound corresponds to the concentration which inhibited 50% of the specific radioligand binding.

Assay for Inositol Phosphate Production by B8−CHO Cells. Approximately 24 h after the transfer of transfected cells to 24-wells plates, the cells incubated overnight in complete medium containing 2 μCi/mL of myo-2-[^{3}H]inositol were washed with Dubelcco's Modified Eagle's Medium and incubated 30 min in 2 mL/well Dubelcco's Modified Eagle's Medium containing 20 mM LiCl at 37 °C. The cells were washed by PI buffer at pH 7.45 (phosphate-buffered saline containing 135 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA). The cells were incubated 60 min at 37 °C in 0.5 mL of PI buffer with or without ligands at various concentrations. The reaction was stopped by adding 1 mL MeOH/HCl to each well, and the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad, Hercules, CA) to release the extraction of inositol phosphates. The columns were washed two times with 5 mL of distilled water and then two times with 2 mL of 5 mM sodium tetraborate/60 mM sodium formate. Each column was eluted with 4 mL of 1 M ammonium formate/100 mM formic acid. 0.5 mL of elution were added to scintillant and beta-radioactivity was counted**.** Data from at least three separate experiments from different batches of transfected cells were analyzed using GraphPad Prism program (Software). The potency of each stimulant corresponded to the concentration which caused a response equal to 50% of the maximal response.

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