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Structure−Activity Relationships and Characterization of Highly Selective, Long-Acting, Peptide-Based Cholecystokinin 1 Receptor Agonists

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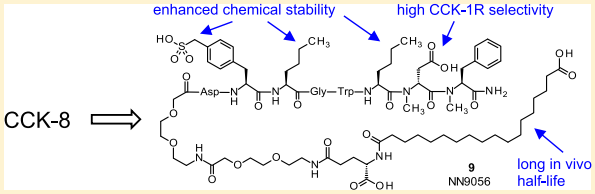
Supporting Information

ABSTRACT: A group of peptide-based, long-acting, stable, highly selective cholecystokinin 1 receptor (CCK-1R) agonists with the potential to treat obesity has been identified and characterized, based on systematic investigation of synthetic CCK-8 analogues with N-terminal linkage to fatty acids. Sulfated Tyr in such compounds was stable in neutral buffer. CCK-1R selectivity was achieved mostly by introducing D-*N*-methyl-Asp instead of Asp at the penultimate position of CCK-8. Our compound **9** (NN9056) showed similar in vitro CCK-1R potency and CCK-1R affinity as CCK-8, very high selectivity for CCK-1R over the cholecystokinin 2 receptor (CCK-2R), strong reduction of food intake in lean pigs for up to 48 h after one subcutaneous injection without adverse effects, a plasma half-life of 113 h in minipigs after intravenous injection, and acceptable chemical stability in a neutral liquid formulation. In addition, we found a highly selective CCK-2R agonist by replacing Gly in a CCK-8 derivative with Glu.



INTRODUCTION

Cholecystokinin (CCK) is a gut and brain peptide hormone that has been known for many decades. It plays an important role in the regulation of energy balance and is therefore of potential interest in view of the globally increasing obesity epidemic.[1] The biological effects of CCK are mediated by its two G-protein-coupled receptors named CCK 1 receptor (CCK-1R) (CCK-A receptor) and CCK 2 receptor (CCK-2R) (CCK-B receptor or gastrin receptor). Both CCK-1R and CCK-2R are present in the gastrointestinal tract and in the central nervous system. Appetite regulation by CCK is mediated by CCK-1R present at gastrointestinal vagal afferent neurons.[2] Other effects mediated by CCK-1R activation include gallbladder contraction, pancreatic enzyme secretion, and delay of gastric emptying.[3] CCK-2R plays a role in anxiety, pain, attention, and memory.[4] Stimulation of gastric acid secretion by gastrin is mediated by CCK-2R activation.[5] Chronic activation of CCK-2R located on thyroid C cells can lead to elevated calcitonin levels.[6,7] Endogenous CCK and synthetic CCK analogues increase β-cell mass and potentiate insulin secretion.[1,8−12] Both CCK receptors seem to be involved in these actions.[8] Combinations of CCK peptides with GLP-1 receptor agonists have shown significant potential in rodents for the treatment of both type 2 diabetes and obesity.[13−15]

No antiobesity drug targeting the CCK-1R is on the market, although there is rich evidence that CCK or synthetic CCK-1R agonists can reduce food intake in different species including humans,[2] especially when combined with other antiobesity agents,[1] and CCK-1R antagonists can increase food intake.[16] GI181771, an orally administered small-molecule CCK-1R agonist and CCK-2R antagonist, failed in clinical trials because of the lack of efficacy.[17] GI181771 showed poor oral bioavailability[17] and much lower in vitro CCK-1R affinity than the most well-known native CCK-1R ligand, CCK-8 (Asp-sTyr-Met-Gly-Trp-Met-Asp-Phe-NH_2, **1**).[18,19] CCK peptides might be an alternative to small-molecule drugs, but CCK-8 and its longer native analogues show an in vivo half-life of only few minutes[20] and are similarly potent at both CCK receptors.[21] The goal of our work has been to identify a series of peptide-based, long-acting, stable, highly selective CCK-1R agonists with the potential to treat obesity by subcutaneous injection. Herein are described our results on such a compound series with a pharmacokinetic profile apparently suitable for once daily and possibly even once weekly subcutaneous injection.[22] Special emphasis has been placed on maximum selectivity for CCK-1R to avoid any unwanted side effects related to centrally or peripherally located CCK-2R.

Major results from the relevant previous literature on CCK peptides and structure−activity studies aiming at selective CCK-1R agonist peptides are mentioned below. Several fully bioactive forms of CCK are known. They share the C-terminal

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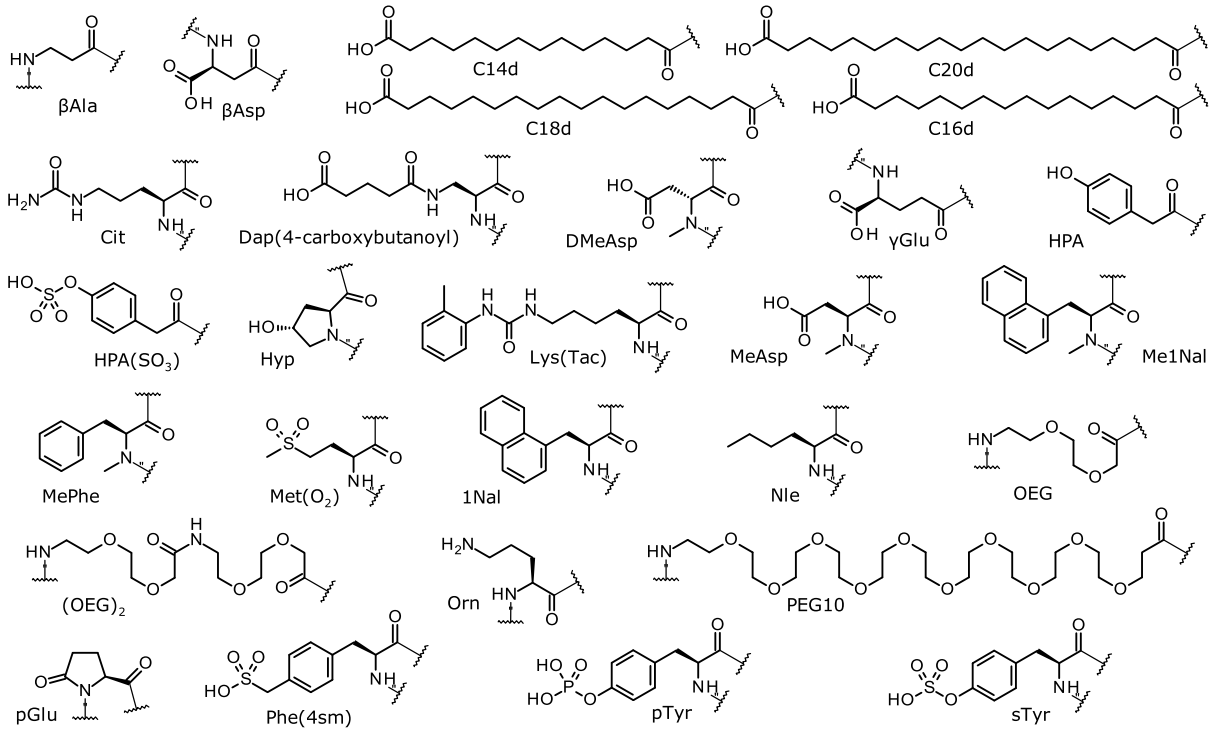

Figure 1. Abbreviations used for nonstandard amino acids, residues, and linker fragments in CCK peptides, in alphabetical order.

sequence including a C-terminal amide and a sulfated tyrosine (sTyr) residue and are named according to their number of amino acid residues, such as CCK-58, CCK-33, CCK-22, and CCK-8. Molecular models for the three-dimensional structure of the complex between CCK-1R and CCK-8 have been suggested. Fourmy et al. used site-specific mutagenesis data for building a refined model of the CCK-CCK-1R complex in which the C-terminal part of CCK is embedded between transmembrane (TM) helices of CCK-1R and in particular interacting with TM-6.[23,24] In contrast, Miller et al. suggested several models based on photoaffinity labeling experiments and known structures of other G-protein-coupled receptors, wherein CCK binds CCK-1R only at the extracellular domains and wherein the C-terminus of CCK is located far from TM-6.[25,26] The sTyr residue in position 27 (residue numbering based on CCK-33) is crucial for CCK-1R binding, whereas nonsulfated CCK binds selectively to CCK-2R.[27] sTyr residues in other peptides were reported to be chemically unstable under acidic conditions.[28] sTyr in CCK analogues can be replaced with an artificial and stable 4-sulfomethylphenylalanine residue[29] [Phe(4sm); see Figure 1 for the structure of abbreviated nonstandard residues] or with O-sulfated 4-hydroxyphenylacetic acid (HPA(SO_3)),[30,31] with the latter replacing 26-Asp and the N-terminus, too. Substitutions of 28-Met with non-natural norleucine (Nle),[32] Leu, and Pro, but not DNle, were well-tolerated by CCK-1R.[33] Almost any reported replacement of 29-Gly resulted in major loss of CCK-1R affinity or potency,[32−40] with the exception of one example with *N*-methylglycine (sarcosine)[38,40] and one with the fluorescent artificial amino acid Aladan.[41] Even minor changes of the 30-Trp residue were not tolerated well by CCK-1R, unless 30-Trp was replaced with 2-naphthylalanine[42] or certain monofluorinated Trp derivatives.[43] Replacement of 31-Met with Nle did not alter biological activity,[32] whereas Ile[30] and Leu[33] substitutions gave slightly reduced CCK-1R affinity and several other amino acids led to lower CCK-1R affinity.[33,44] Incorporation of a special N^{ϵ}-modified Lys residue, Lys(*o*-tolylcarbamoyl) or Lys(Tac), in position 31 gave potent and CCK-1R selective compounds even in the absence of a sulfated residue in position 27.[31,45,46] Substituting 32-Asp with *N*-methyl-Asp (MeAsp) is also known to give high CCK-1R selectivity.[47] One single CCK-peptide with N-methylated DAsp (DMeAsp) in position 32 was reported. This peptide, HPA(SO_3)-Nle-Gly-Trp-Nle-DMeAsp-Phe-NH_2 (**2**), seems to be the most selective CCK-1R agonist peptide ever disclosed but showed lower anorectic potency than the corresponding 32-MeAsp diastereomer (ARR15849) after intranasal administration to dogs[30] and has not gained any attention in later publications. Some residues in position 32 such as Glu and *β*Ala resulted in severe loss of activity,[48] whereas others like Asn,[49] O-sulfated Ser or Hyp,[50] and the sterically hindered proline analogue R-Dtc were reported to work well, the latter with very high selectivity for CCK-1R.[49] Replacement of 33-Phe with *N*-methyl-Phe (MePhe) was reported to slightly increase CCK-1R selectivity by lowering CCK-2R affinity[33] and to enhance CCK-1R affinity and selectivity when combined with DAsp in position 32.[40] Position 33 seems to tolerate a range of aromatic amino acid substitutions[51] including 1Nal,[52−54] whereas diverging results were published for analogues with cyclohexylalanine in position 33.[52−54] However, most of these conclusions are based on very different reported experiments performed 20−30 years ago with partly inconsistent results. A systematic reinvestigation of the CCK peptide sequence to optimize CCK-1R potency and selectivity seemed therefore necessary and is an essential part of the work described here.

We chose pig models for our in vivo experiments with selected compounds, as rodents are prone to pancreatitis induced by high doses of CCK.[55−57] CCK-induced pancreatitis was attributed to CCK-1R stimulation in the pancreatic acinar

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Table 1. Sequence,[a] in Vitro CCK-1R and CCK-2R Affinity and Potency of CCK Peptides

No.	Name or Sequence[a]	Affinity (pIC_{50})[b]		Potency (pEC_{50})[b]	
		CCK-1R	CCK-2R	CCK-1R	CCK-2R
1	CCK-8; CCK-33(26-33); Asp-sTyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	9.95	9.54	10.36	9.30
2	HPA(SO_3)-Nle-Gly-Trp-Nle-DMeAsp-Phe-NH_2	10.35	5.13	10.60	<6
3	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2	9.61	9.68	9.89	9.36
4	Gastrin I; pGlu-Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	6.07	8.39	6.57	9.34
5	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-Asp-Phe-NH_2	9.56	9.78	9.89	9.30
6	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-MeAsp-Phe-NH_2	10.05	6.61	9.77	8.81
7	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-Phe-NH_2	10.01	5.25	9.35	6.88
8	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-MeAsp-MePhe-NH_2	9.50	5.06	9.97	<6
9	NN9056; C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.28	<5	9.60	<6
10	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-DAsp-MePhe-NH_2	9.58	7.18	9.30	8.57
11	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-Asn-Phe-NH_2	7.72	6.51	7.93	8.24
12	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-Hyp-Phe-NH_2	8.52	6.34	8.95	7.59
13	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DPro-MePhe-NH_2	7.39	5.27	6.61	<6
14	C14d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	9.81	<5	9.74	<6
15	C16d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	9.70	<5	9.72	<6
16	C20d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.39	<5	9.43	<6
17	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.22	5.75	9.54	6.98
18	C18d-γGlu-PEG10-PEG10-Asp-sTyr-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	9.96	<5	9.50	<6
19	C18d-γGlu-$(OEG)_2$-Lys-OEG-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.20	6.89	9.67	<6
20	C18d-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	9.97	5.11	9.14	<6
21	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-Asp-Phe-NH_2	9.61	9.05	10.17	9.35
22	C18d-γGlu-$(OEG)_2$-Asp-Tyr-Nle-Gly-Trp-Nle-MeAsp-MePhe-NH_2	6.78	<5	8.48	<6
23	C18d-γGlu-$(OEG)_2$-Asp-pTyr-Nle-Gly-Trp-Nle-Asp-Phe-NH_2	7.44	8.73	7.93	9.48
24	C18d-γGlu-$(OEG)_2$-Asp-Dap(4-carboxybutanoyl)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2	6.08	8.24	6.64	9.42
25	C18d-γGlu-$(OEG)_2$-Asp-Tyr-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	8.99	5.31	9.81	<6
26	HPA-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	9.30	5.08	10.22	<6
27	HPA-Lys(C18d-γGlu-$(OEG)_2$)-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	9.28	5.43	9.80	<6
28	C18d-γGlu-$(OEG)_2$-Asp-Trp-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	8.57	5.13	9.67	<6
29	C18d-γGlu-$(OEG)_2$-Asp-Ile-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	8.37	<5	9.41	<6
30	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.13	5.34	9.44	6.91
31	Ac-Asp-sTyr-Lys(C18d-γGlu-$(OEG)_2$)-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.19	6.24	9.61	6.50
32	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Glu-Gly-Trp-Nle-Asp-Phe-NH_2	8.90	9.80	9.78	9.53
33	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Glu-Trp-Nle-Asp-Phe-NH_2	5.40	9.82	<6	9.36
34	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Lys-Trp-Nle-Asp-Phe-NH_2	5.59	7.87	6.04	9.08
35	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Pro-Trp-Nle-Asp-Phe-NH_2	6.03	9.23	6.22	9.42
36	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-βAla-Trp-Nle-Asp-Phe-NH_2	6.66	9.40	6.93	9.50
37	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Ile-Asp-Phe-NH_2	9.10	8.67	9.96	9.20
38	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Gln-Asp-Phe-NH_2	9.03	8.69	9.79	9.38
39	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Met-Gly-Trp-Met-DMeAsp-MePhe-NH_2	10.30	5.18	9.53	6.76
40	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Gln-DMeAsp-MePhe-NH_2	10.08	<5	9.87	<6
41	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Ala-DMeAsp-MePhe-NH_2	9.21	<5	9.78	6.06
42	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Phe-DMeAsp-MePhe-NH_2	7.79	<5	8.79	<6
43	C18d-γGlu-$(OEG)_2$-Asp-Tyr-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2	9.47	6.12	9.97	8.30
44	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Lys(Tac)-MeAsp-MePhe-NH_2	9.32	<5	9.94	<6
45	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-Asp-MePhe-NH_2	10.06	9.54	9.94	9.34
46	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-Asp-1Nal-NH_2	10.12	10.03	9.79	9.39
47	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-MeAsp-1Nal-NH_2	10.10	6.94	9.30	8.73
48	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-Me1Nal-NH_2	10.37	5.69	9.39	6.82
49	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-DMeAsp-1Nal-NH_2	10.13	6.00	9.44	7.05
50	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-MeAsp-Me1Nal-NH_2	9.94	6.22	9.30	7.78
51	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-Asp-Trp-NH_2	8.65	9.88	9.49	9.38
52	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-Asp-Tyr-NH_2	8.04	9.88	9.01	9.49
53	HPA-Nle-Gly-Trp-Nle-DMeAsp-Phe-NH_2	8.15	<5	8.81	<6
54	HPA-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2	7.81	5.52	8.04	6.71
55	HPA-Nle-Gly-Trp-Nle-MeAsp-MePhe-NH_2	7.37	<5	8.07	<6
56	C18d-γGlu-$(OEG)_2$-βAsp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	10.10	<5	9.50	<6

[a]Color code: brown = fatty chain, purple = linker between the peptide and fatty chain, red = position 27, dark blue = position 28, pink = position 29, green = position 31, dark red = position 32, and blue = position 33. [b]Negative common logarithm of the concentration in mol/L; mean values of at least three experiments; 95% CIs in Table 2.

cells and is not expected to be relevant in humans because subchronic treatment of monkeys and humans with the CCK-1R agonist GI181771 did not give any signs of pancreatitis[57] and the pancreatic acinar cells of humans have no or minimal CCK-1R expression.[58,59]

RESULTS

Using Fmoc-based solid-phase peptide synthesis, we prepared a series of CCK-8 analogues to optimize CCK-1R selectivity and potency by systematic modification of peptide residues 27−29 and 31−33. Met residues were replaced in most cases to avoid undesired oxidation. To obtain injectable compounds with prolonged in vivo half-life, we chose to attach a fatty acid with a hydrophilic linker to the N-terminus of CCK-8 analogues. The rationale of this well-known approach[60] is that the fatty acid moiety of the peptide strongly binds to albumin that is abundant in the body and has a serum half-life of about 3 weeks in humans. Mostly, we used exactly the same combination of the C18 fatty acid and linker as used for the once weekly GLP-1 analogue semaglutide.[61] The corresponding CCK-8 derivative, compound **3** in Table 1, was the starting point of this compound series.

Synthetic Chemistry. Our compounds were prepared by Fmoc-based solid-phase peptide synthesis using activation with

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Table 2. In Vitro CCK Receptor Affinity and Potency with Confidence Interval (CI) and Number of Experiments (*N*)

	affinity (pIC_{50})					potency (pEC_{50})				
compound	CCK-1R mean	CCK-1R 95% CI	CCK-2R mean	CCK-2R 95% CI	*N*	CCK-1R mean	CCK-1R 95% CI	CCK-2R mean	CCK-2R 95% CI	*N*
1	9.95	9.90−10.01	9.54	9.49−9.59	45	10.36	10.24−10.48	9.30	9.22−9.37	4
2	10.35	10.16−10.54	5.13	4.73−5.54	4	10.60	10.50−10.71	<6	NA	3
3	9.61	9.00−10.22	9.68	9.52−9.84	4	9.89	9.62−10.17	9.36	9.02−9.70	3
4	6.07	5.89−6.24	8.39	8.31−8.47	45	6.57	6.50−6.64	9.34	9.30−9.38	15
5	9.56	8.79−10.32	9.78	9.37−10.19	3	9.89	9.73−10.05	9.30	9.18−9.42	4
6	10.05	9.70−10.41	6.61	6.37−6.85	4	9.77	9.38−10.15	8.81	8.43−9.19	4
7	10.01	9.88−10.13	5.25	5.17−5.32	3	9.35	8.99−9.70	6.88	6.73−7.03	3
8	9.50	9.40−9.61	5.06	4.83−5.30	3	9.97	9.89−10.05	<6	NA	4
9	10.28	10.17−10.38	<5	NA	5	9.60	9.48−9.73	<6	NA	7
10	9.58	9.28−9.87	7.18	7.00−7.35	3	9.30	9.10−9.50	8.57	8.47−8.66	3
11	7.72	7.58−7.85	6.51	6.18−6.85	3	7.93	7.88−7.98	8.24	8.02−8.46	3
12	8.52	8.25−8.78	6.34	6.19−6.50	3	8.95	8.37−9.53	7.59	7.40−7.79	3
13	7.39	6.91−7.87	5.27	5.09−5.45	4	6.61	4.96−8.27	<6	NA	3
14	9.81	9.59−10.03	<5	NA	3	9.74	9.04−10.43	<6	NA	3
15	9.70	9.58−9.81	<5	NA	3	9.72	9.43−10.01	<6	NA	3
16	10.39	10.06−10.73	<5	NA	3	9.43	9.21−9.65	<6	NA	3
17	10.22	10.07−10.36	5.75	5.62−5.89	4	9.54	9.40−9.69	6.98	6.19−7.77	5
18	9.96	9.75−10.17	<5	NA	3	9.50	9.31−9.69	<6	NA	3
19	10.20	10.02−10.39	6.89	6.36−7.42	3	9.67	9.25−10.08	<6	NA	3
20	9.97	9.68−10.27	5.11	5.07−5.16	3	9.14	9.08−9.20	<6	NA	3
21	9.61	9.43−9.79	9.05	8.82−9.28	5	10.17	9.89−10.44	9.35	9.19−9.51	3
22	6.78	6.48−7.08	<5	NA	3	8.48	8.32−8.65	<6	NA	3
23	7.44	6.99−7.88	8.73	8.41−9.04	3	7.93	7.23−8.63	9.48	9.14−9.82	3
24	6.08	5.95−6.22	8.24	8.14−8.34	3	6.64	6.08−7.21	9.42	9.33−9.50	3
25	8.99	8.75−9.23	5.31	4.96−5.66	3	9.81	9.71−9.92	<6	NA	5
26	9.30	8.97−9.63	5.08	4.21−5.96	5	10.22	9.71−10.73	<6	NA	3
27	9.28	8.99−9.58	5.43	5.34−5.53	3	9.80	9.32−10.27	<6	NA	3
28	8.57	8.41−8.72	5.13	4.77−5.48	3	9.67	9.10−10.24	<6	NA	4
29	8.37	8.21−8.54	<5	NA	3	9.41	8.86−9.95	<6	NA	4
30	10.13	9.97−10.29	5.34	5.26−5.42	4	9.44	9.24−9.64	6.91	5.92−7.90	3
31	10.19	10.13−10.25	6.24	6.04−6.44	3	9.61	9.35−9.86	6.50	6.38−6.61	4
32	8.90	8.73−9.08	9.80	9.49−10.11	3	9.78	9.46−10.09	9.53	9.47−9.59	3
33	5.40	5.23−5.57	9.82	9.61−10.02	3	<6	NA	9.36	9.07−9.66	3
34	5.59	5.52−5.67	7.87	7.68−8.06	3	6.04	5.82−6.26	9.08	8.86−9.31	3
35	6.03	5.84−6.23	9.23	8.77−9.70	3	6.22	5.90−6.54	9.42	9.31−9.54	4
36	6.66	6.27−7.04	9.40	9.14−9.65	3	6.93	6.58−7.27	9.50	9.29−9.71	3
37	9.10	9.02−9.19	8.67	8.54−8.80	3	9.96	9.67−10.25	9.20	9.00−9.41	4
38	9.03	8.78−9.28	8.69	8.37−9.01	3	9.79	9.65−9.93	9.38	9.28−9.48	3
39	10.30	10.07−10.53	5.18	5.10−5.27	3	9.53	9.42−9.65	6.76	6.23−7.28	3
40	10.08	9.91−10.26	<5	NA	3	9.87	9.57−10.16	<6	NA	4
41	9.21	8.87−9.54	<5	NA	3	9.78	9.72−9.83	6.06	5.98−6.13	3
42	7.79	7.45−8.14	<5	NA	3	8.79	8.50−9.08	<6	NA	3
43	9.47	9.31−9.62	6.12	5.90−6.34	3	9.97	9.72−10.21	8.30	8.05−8.54	3
44	9.32	9.10−9.54	<5	NA	3	9.94	9.62−10.25	<6	NA	3
45	10.06	10.01−10.11	9.54	9.27−9.82	3	9.94	9.87−10.01	9.34	9.10−9.58	3
46	10.12	9.91−10.34	10.03	9.88−10.19	3	9.79	9.34−10.23	9.39	9.06−9.73	3
47	10.10	9.65−10.56	6.94	6.67−7.20	4	9.30	8.90−9.70	8.73	8.41−9.06	3
48	10.37	10.19−10.54	5.69	5.40−5.97	3	9.39	9.20−9.58	6.82	6.36−7.27	6
49	10.13	9.75−10.52	6.00	5.63−6.36	4	9.44	9.27−9.60	7.05	6.16−7.94	4
50	9.94	9.54−10.33	6.22	5.90−6.54	4	9.30	9.10−9.49	7.78	7.50−8.06	3
51	8.65	8.32−8.98	9.88	9.62−10.14	3	9.49	9.38−9.60	9.38	9.15−9.61	3
52	8.04	7.82−8.26	9.88	9.59−10.17	3	9.01	8.71−9.31	9.49	9.08−9.89	3
53	8.15	7.95−8.35	<5	NA	4	8.81	8.38−9.24	<6	NA	5
54	7.81	7.72−7.90	5.52	5.34−5.70	5	8.04	7.90−8.17	6.71	4.66−8.76	3
55	7.37	7.18−7.55	<5	NA	5	8.07	7.75−8.39	<6	NA	4
56	10.10	9.63−10.56	<5	NA	3	9.50	9.32−9.67	<6	NA	5

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diisopropylcarbodiimide and oxyma. For the synthesis of peptides containing a sTyr residue, we used Fmoc-Tyr(SO_3-neopentyl)-OH. After cleavage from the resin with trifluoroacetic acid (TFA), the neopentyl group was removed from the sulfo group by reaction with aqueous ammonium acetate,[62] typically overnight at 55 °C with acetonitrile being used as a cosolvent for keeping our fatty chain containing peptides in solution. Incorporation of Phe(4sm) was performed with the corresponding Fmoc-amino acid bearing a removable 2,2,2-trichloroethyl (TCE) group at the sulfonic acid. Fmoc-deprotections after the incorporation of TCE-protected Phe(4sm) were carried out using 2-methylpiperidine instead of piperidine to avoid side reactions.[63] For removing the TCE group from the crude peptide, several reductive methods are reported.[63] Our initial investigations employed zinc dust in acetic acid, which typically required overnight reaction in our hands. The reduction was found to proceed much faster in the presence of ammonium acetate. From this point forward, TCE removal was achieved using 12 equiv of freshly activated zinc dust and 2 equiv of ammonium acetate in 75% aqueous acetic acid, which resulted in complete deprotection within less than 1 h.

DMeAsp was incorporated into the peptides using different methods. First, we used racemic D/L-MeAsp and separated the resulting two products by high-performance liquid chromatography (HPLC). We later adopted N-methylation of a resin-bound DAsp residue via an intermediate 2-nitrobenzenesulfonamide that can be cleaved with thiols in the presence of a base. Methylation of the sulfonamide was carried out either with methyl 4-nitrobenzenesulfonate and the base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or by Mitsunobu reaction with methanol, similar to reported procedures.[64,65] The Mitsunobu procedure worked excellently on a multigram scale. Later, we used purchased custom-made Fmoc-DMeAsp-(OtBu)-OH. Me1Nal in position 33 of certain peptides was made in the same way as DMeAsp by N-methylation of resin-bound 1Nal. Commercially available Fmoc-amino acids were used for incorporating all the other amino acids. In the same manner, Fmoc-protected building blocks such as Fmoc-OEG-OH were used for assembling the hydrophilic linkers at the resin-bound peptide. Fatty acids were attached as their mono-*tert*-butyl-protected α,ω-dicarboxylic acids. Alternatively, the semaglutide side-chain C18d-γGlu-OEG-OEG was introduced by acylation with its unprotected *N*-hydroxysuccinimide ester[66] either in aqueous solution or on solid phase. Lys and Dap residues modified at the side chain were introduced by using the corresponding Fmoc-amino acid with 4-methyltrityl (Mtt) protection at the side chain, followed by removal of the Mtt group from the side chain and acylation on the solid support.

Purifications of our peptides were typically performed in one step by preparative neutral HPLC using acetonitrile in water containing 0.01 M ammonium acetate. Subsequent freeze-drying removed excess ammonium acetate and afforded the corresponding peptide ammonium salt.

In Vitro Receptor Biology. Binding affinity of the test peptides to human CCK-1R and CCK-2R was measured by way of their ability to displace radio-labeled ^{125}I-CCK-8 from the receptors in a plasma membrane-based scintillation proximity assay (SPA). The in vitro potency of the peptides was determined by measuring the accumulation of inositol 1 phosphate (IP1) in cells expressing human CCK-1R or CCK-2R. CCK-8 (**1**) and nonsulfated gastrin-17 (**4**) were used as reference compounds. The following structure−activity relationships (SARs) were found (Tables 1 and 2).

The residue in position 32 is discussed first because it has a crucial impact on CCK-1R potency and selectivity. Replacing Asp in position 32 with MeAsp gave compounds with improved CCK-1R selectivity. However, DMeAsp in this position gave even more CCK-1R selective compounds (compounds **5**−**7**). CCK-2R binding and activity of these compounds with DMeAsp in position 32 were hardly measurable. MeAsp and DMeAsp gave equal CCK-1R affinity if the peptide contained a sulfated residue in position 27 and a nonmethylated residue in position 33. DMeAsp gave slightly higher CCK-1R affinity in the presence of 33-MePhe and a sulfo group in position 27 (compounds **8** and **9**). DAsp in position 32 combined with 33-MePhe gave some CCK-1R selectivity as well (compound **10**). Incorporation of other residues in position 32, such as Asn, hydroxyproline (Hyp), DPro (compounds **11**−**13**), Pro, *N*-methyl-βAsp, Glu, DGlu, Gly, *N*-methylglycine, and *N*-benzylglycine (data not shown), resulted in partial or full loss of CCK-1R affinity and potency. Thus, DMeAsp is the residue of choice at position 32 in terms of CCK-1R selectivity.

As for the N-terminus, compounds with and without the N-terminal fatty acid gave similar results. The length of the fatty acid (compounds **14**−**16**) and the linker between the fatty acid and peptide (compounds **17**−**20**) did not largely change CCK-1R binding. However, compound **19** with a positive charge in the linker was less selective for CCK-1R than compound **9** with a different linker and the same backbone.

Replacing sTyr in position 27 with Phe(4sm) gave equal CCK-1R affinity and potency (compounds **5**, **9**, **17**, and **21**), whereas Tyr, O-phosphorylated Tyr, or aliphatic carboxy-containing residues in position 27 led to loss in activity (compounds **22**−**24**). However, the presence of a sulfo group at residue 27 does not seem to be a strict requirement if the peptide contains both DMeAsp in position 32 and MePhe in position 33. In this case, high CCK-1R affinity, potency, and selectivity were observed with Tyr or nonsulfated HPA in position 27 (compounds **25**−**27**), whereas other hydrophobic residues in position 27 gave reduced CCK-1R affinity but retained CCK-1R selectivity (compounds **28** and **29**).

In position 28, Nle and Leu gave equal results (compounds **3**, **5**, **17**, and **30**) as well as Met, Ile, Ala, Pro, Ser, His, Phe, Lys, and Arg, at least in combination with 32-DMeAsp and 33-MePhe (data not shown). A Lys residue modified with a hydrophilic linker and a fatty acid at its side chain was also well-tolerated (compounds **27** and **31**). Glu in position 28, combined with the native residues 32-Asp and 33-Phe, gave slightly lower or equal CCK-1R affinity (compound **32**).

Substitution of Gly in position 29 with Glu, Lys, Pro, and βAla (compounds **33**−**36**) as well as Ala, Ser, DSer, and *N*-methylglycine (data not shown) resulted in major loss of CCK-1R affinity and potency. L-Amino acids, especially Glu and Pro, as well as βAla gave selective CCK-2R agonists, where compound **34** with 29-Lys notably exhibited the lowest CCK-2R affinity. Compound **33** with 29-Glu showed the highest selectivity for CCK-2R.

For the investigation of position 31, two compound series were made. In the first series of analogues of nonselective peptide **3** modified only in position 31, CCK-1R affinity was highest for Nle and equal or slightly lower for Ile, Gln (compounds **37** and **38**), and Leu, whereas Phe, Ser, Ala, Lys, and Glu led to loss in affinity and potency (data not shown).

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Table 3. Pharmacokinetics of Selected Compounds in Female Minipigs after iv Injection of 5 nmol/kg

Compound	Name or Sequence[a]	$T_{1/2}$ Mean (h)[b]	$T_{1/2}$ Range (h)[b]	n
7	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-Phe-NH_2	115	106-126	2
8	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-MeAsp-MePhe-NH_2	145	143-147	3
9	NN9056; C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	113	105-123	3
14	C14d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	2.0	2.0-2.1	2
15	C16d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	37	30-51	3
16	C20d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	153	141-166	2
17	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	103	90-120	2
18	C18d-γGlu-PEG10-PEG10-Asp-sTyr-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	161	156-166	2
19	C18d-γGlu-$(OEG)_2$-Lys-OEG-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	36	36-36	2
20	C18d-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	2.6	2.0-3.6	2
25	C18d-γGlu-$(OEG)_2$-Asp-Tyr-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	45	42-49	2
27	HPA-Lys(C18d-γGlu-$(OEG)_2$)-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	7.1	6.7-7.4	2
30	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	112	101-124	2
48	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Leu-Gly-Trp-Nle-DMeAsp-Me1Nal-NH_2	147	146-147	2
49	C18d-γGlu-$(OEG)_2$-Asp-sTyr-Nle-Gly-Trp-Nle-DMeAsp-1Nal-NH_2	140	133-148	2
56	C18d-γGlu-$(OEG)_2$-βAsp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2	106	101-112	2

[a]Color code: brown = fatty chain, purple = linker between the peptide and fatty chain, red = position 27, dark blue = position 28, pink = position 29, green = position 31, dark red = position 32, and blue = position 33. [b]Terminal plasma half-lifes ($T_{1/2}$) are given as harmonic mean and range.

No major change of receptor selectivity was observed within this series. In the second series consisting of analogues of CCK-1R selective compound **30** modified only in position 31, the following order of CCK-1R affinity was observed: Met, Nle, Gln, Leu, Ile ≥ Pro > Val, Ala, Met(O_2) > Cit, His, Phe > Tyr, Lys(C18d-γGlu-$(OEG)_2$) (data only shown for Nle, Met, Gln, Ala, and Phe, compounds **39**−**42**). Gln and Ile in position 31 gave lower CCK-1R affinity and potency than Nle in nonsulfated compounds with DMeAsp-MePhe at the C-terminal positions 32−33 (data not shown). We also prepared few CCK analogues with a Lys(Tac) residue in position 31 and found them to be selective for CCK-1R but did not pursue this compound type further (compounds **43** and **44**).

Replacement of 33-Phe with MePhe, 1Nal, *N*-methyl-1-naphthylalanine (Me1Nal), 4-methylphenylalanine, 4-fluorophenylalanine, and 4-chlorophenylalanine, either in combination with 32-Asp or 32-MeAsp or 32-DMeAsp, gave compounds with similar CCK-1R affinity (data only shown for MePhe, 1Nal, and Me1Nal, compounds **45**−**50**). Trp and especially Tyr in position 33 diminished CCK-1R affinity and shifted selectivity toward CCK-2R (compounds **51** and **52**). An analogue of peptide **9** with exactly the same sequence but a C-terminal acid instead of an amide was inactive (data not shown).

The role of N-methylation at residue 33 for CCK-1R selectivity depends on the corresponding residues in positions 32 and 27. In the presence of Asp or MeAsp in position 32 and a sulfated residue in position 27, the *N*-methyl group of MePhe or Me1Nal in position 33 has only a minor impact, with a tendency to slightly enhance CCK-1R selectivity (compounds **5**, **45**, **47**, and **50**). For compounds containing both DMeAsp in position 32 and a sulfated residue in position 27, no impact of N-methylation at residue 33 was found (compounds **7**, **30**, **48**, and **49**). However, in nonsulfated CCK-peptides with MeAsp or DMeAsp in position 32, inclusion of an N-methyl group at residue 33 is influential. In this case, CCK-1R affinity depended on the C-terminal dipeptide at positions 32−33 in the following order: DMeAsp-MePhe > DMeAsp-Phe ≥ MeAsp-Phe > MeAsp-MePhe (compounds **26** and **53**−**55**).

Summarizing our biological in vitro results with acylated CCK-8 analogues, the following amino acid residues looked most promising in terms of CCK-1R affinity, potency, and selectivity: sTyr or Phe(4sm) in position 27, a variety of residues in position 28, Gly in position 29, Trp in position 30 (not modified), Nle or Met in position 31, DMeAsp in position 32, and Phe, MePhe, 1Nal or Me1Nal in position 33 with a C-terminal amide. Attachment of a hydrophilic linker with a fatty acid either at the N-terminus of 26-Asp or at the side chain of a Lys residue in position 28 was well-tolerated.

Chemical Stability. High chemical stability in liquid formulations has been a crucial requirement for the CCK analogues of this project. We needed to decide at an early stage whether or not to pursue compounds containing a sTyr residue because sTyr is known to hydrolyze at least under acidic conditions. Chemical stability of the acylated CCK analogue **3** in phosphate buffer solutions was therefore tested. Initially, two solutions of peptide **3** in 8 mM sodium phosphate buffer at either pH 7.4 or pH 2.2 were incubated at 37 °C for 1 week and then analyzed by ultra-HPLC (UHPLC) with UV detection. The starting peptide concentration was 0.5 mM at pH 7.4 and only about 0.03 mM in the pH 2.2 sample because of partial gelation and loss upon filtration. The initial purity of peptide **3** was 98.6%. After 1 week at 37 °C, the purity was only 22% in the pH 2.2 sample, almost entirely due to the loss of sulfate. On the other hand, the purity of the pH 7.4 sample had only decreased by 0.8% points and no loss of sulfate was detected. We subsequently deemed the sulfated CCK analogue **3** to be stable enough for being applied in a neutral liquid formulation.

Chemical stability of CCK-1R selective peptide **9** with a non-hydrolyzable Phe(4sm) residue in position 27 was tested in a similar experiment using another UHPLC method and apparatus. A 1 mM solution of peptide **9** containing 8 mM sodium phosphate buffer pH 7.4 was incubated at 37 °C, resulting in a purity decrease of 1.3% points after 1 week and of 2.2% points after 2 weeks at 37 °C. The initial purity of peptide **9** measured by this method was 95.9%. The major degradation product of compound **9** under these and similar conditions was the equally active isomer **56** with βAsp in position 26, most likely formed via the corresponding aspartimide.

Pharmacokinetics. A set of compounds was selected for investigation of pharmacokinetics in Göttingen minipigs after single intravenous (iv) administration of 5 nmol/kg. This set included compounds with different fatty chain lengths, different linkers between the fatty chain and CCK octapeptide, and different residues in position 27 (sTyr, Phe(4sm), Tyr,

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HPA), in position 28 (Nle, Leu), in position 32 (DMeAsp, MeAsp), and in position 33 (MePhe, Phe, 1Nal, Me1Nal) (Table 3).

As expected, the length of the fatty chain is of crucial importance. In trend, a longer fatty chain correlated with a longer half-life, as can be seen from the data for compounds **14**−**16**, which differ only in the length of their fatty chain, and compound **9**. C20 and C18 compounds were cleared much more slowly than the corresponding C16 and C14 compounds, with calculated half-lives ranging from 153 h for C20 (**16**) and 113 h for C18 (**9**) over 37 h for C16 (**15**) to only 2 h for the C14 compound (**14**).

The linker between the peptide N-terminus and the fatty chain also bears a significant importance. Thus, a modified linker containing a Lys residue gave significantly shorter half-life than the corresponding compound with the semaglutide linker γGlu-OEG-OEG (compounds **19** and **9**). Omission of the linker gave a compound with an even shorter half-life of less than 3 h, despite its C18 chain (**20** and **30**). A strongly elongated PEG linker gave a longer half-life than the Semaglutide linker (**18** and **17**).

To our surprise, compound **25** with nonsulfated Tyr in position 27 was cleared much faster than the corresponding sulfated compound **30**, with a half-life of 45 h for the nonsulfated versus 112 h for the sulfated compound. Another nonsulfated compound, peptide **27** with both the linker and C18 fatty chain at residue 28 and with HPA in position 27, was cleared even faster with a half-life of only 7 h.

Lower clearance and longer half-life were apparently supported by the presence of 1Nal or Me1Nal in position 33 (compounds **7**, **17**, **30** vs **48**, **49**) and by MeAsp instead of DMeAsp in position 32 (**8** and **9**). Other modifications, namely, 27-sTyr versus 27-Phe(4sm), 28-Nle versus 28-Leu, and 33-MePhe versus 33-Phe, did not have any impact on the pharmacokinetics (PK) of the investigated compounds (compounds **7**, **9**, **17**, and **30**).

In vivo metabolism of compound **9** was investigated in several species including Göttingen minipigs. Plasma samples taken post-dose were analyzed and the major species was found to correspond to compound **9**. In plasma samples from iv-dosed minipigs, three minor metabolites were observed, resulting from proteolytic cleavage of the peptide backbone. The metabolites found (**M1**−**M3**) are listed in Table 4.

Table 4. Metabolites of Compound 9 Found in Plasma Samples of Intravenously Dosed Minipigs

compound	name or sequence
9	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-MePhe-NH_2
M1	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-OH
M2	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-OH
M3	C18d-γGlu-$(OEG)_2$-Asp-Phe(4sm)-Nle-Gly-Trp-Nle-DMeAsp-OH

In vitro plasma protein binding was investigated for compound **9** in plasma from several species including Göttingen minipig. This was performed by incubating the plasma sample with immobilized human serum albumin (HSA)[60] followed by precipitation of proteins and liquid chromatography−mass spectroscopy (LC−MS) analysis of the remaining dissolved peptide. The results showed a very high degree of protein binding, exceeding 99.7% in all the tested species.

Acute Food Intake in LYD Pigs. The acute effect of seven different CCK analogues on food intake of young growing female Landrace Yorkshire Duroc (LYD) pigs after single subcutaneous injection of 30 nmol/kg was investigated. Plasma samples taken after 96 h were analyzed for remaining drug concentration. Accumulated food intake compared to baseline and plasma concentration after 96 h are given in Table 5.

One of the investigated compounds was peptide **56** with βAsp in position 26, a slowly formed degradation product of compound **9** in neutral buffer, as mentioned above. Both compounds **9** and **56** gave a strong, long lasting reduction of food intake. The effect of compound **9** was still significant at 24−48 h but not thereafter. Compound **19**, a similar compound with a different, Lys-containing linker between the peptide and fatty acid, gave an equally strong effect within the first 48 h, despite its shorter in vivo half-life reported above and its lower plasma concentration detected after 96 h. Compound **40**, which differs from peptide **9** by a Gln residue in position 31 and by sTyr instead of Phe(4sm) in position 27, gave a significant reduction of food intake, although the effect was apparently slightly weaker than that for compounds **9**, **19**, and **56**. Compounds **25** and **27**, both without a sulfated residue in position 27, did not reduce food intake under these conditions and gave very low plasma concentrations after 96 h, in line with the minipig PK results mentioned above. Interestingly, compound **8**, the isomer of compound **9** with MeAsp instead of DMeAsp in position 32, gave no significant effect, despite its high plasma concentration after 96 h (Table 5). No adverse effects were observed with any of the compounds in these studies.

DISCUSSION AND CONCLUSIONS

Starting from native CCK-33 or CCK-8 toward injectable CCK peptides suitable for the treatment of obesity, the following major drawbacks of these peptides had to be changed: lack of CCK-1R selectivity, poor pharmacokinetics, and possibly low chemical stability. From the literature and own results, we concluded that there was no need to pursue CCK peptides longer than CCK-8 and focused on acylated CCK-8 analogues.

To optimize CCK-1R selectivity and potency, we made a series of synthetic peptides and tested their binding and potency at both CCK receptors, resulting in a comprehensive SAR described above. Selectivity for CCK-1R over CCK-2R was hard to quantify in many cases when CCK-2R affinity and potency were hardly measurable. It is therefore difficult to use these CCK-2R assays for ranking our best compounds and for further optimization toward even higher CCK-1R selectivity. Our CCK receptor IC_{50} values were in some cases more diverse and better fitting with in vivo data than the EC_{50} values and seem to be more suitable for comparing and ranking our compounds. Very high CCK-1R selectivity was achieved by incorporating a DMeAsp residue in position 32, based on empiric work not structure-based design. DMeAsp in position 32 strongly hampered CCK-2R binding but was also best or at least equally good in terms of CCK-1R affinity and in vitro potency, compared to Asp or MeAsp in position 32. The combination of 32-DMeAsp and 33-MePhe is especially favorable for CCK-1R affinity, at least when CCK-1R binding of the CCK analogue is impaired, for example, by the lack of a sulfated residue in position 27 or the presence of a suboptimal

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Table 5. Food Intake (FI) in Female LYD Pigs after Single Subcutaneous Injection of 30 nmol/kg CCK Peptide

compound	FI (kg)[a] baseline per 24 h[b]	FI (kg)[a] 0−24 h	FI (kg)[a] 24−48 h	FI (kg)[a] 48−72 h	FI (kg)[a] 72−96 h	plasma concentration after 96 h (nmol/L)[a]
8	1.8 ± 0.13	1.9 ± 0.20	2.0 ± 0.09	2.3 ± 0.17	2.2 ± 0.11	93.9 ± 6.7
9	2.0 ± 0.13	1.0 ± 0.18****	1.1 ± 0.08***	1.5 ± 0.33	1.9 ± 0.24	84.9 ± 3.7
19	1.8 ± 0.24	0.9 ± 0.07****	1.2 ± 0.18***	1.6 ± 0.18	1.8 ± 0.12	8.8 ± 2.8
25	2.0 ± 0.16	2.3 ± 0.15	2.2 ± 0.15	2.6 ± 0.12	2.3 ± 0.24	8.5 ± 1.0
27	2.1 ± 0.17	2.1 ± 0.23	2.2 ± 0.24	2.1 ± 0.16	2.4 ± 0.05	<0.9
40	2.0 ± 0.10	1.4 ± 0.12**	1.3 ± 0.26***	1.8 ± 0.14	1.8 ± 0.27	91.2 ± 5.0
56	2.0 ± 0.08	1.1 ± 0.12****	1.5 ± 0.20*	1.8 ± 0.21	1.7 ± 0.12	88.7 ± 6.6

[a]Values are given as mean ± standard error of the mean (n = 3−4). Statistical comparison was done using repeated measures two-way ANOVA (analysis of variants) followed by Dunnett's multiple comparisons test. Significance levels are marked as $p < 0.0001$ (****), 0.001 (***), 0.01 (**), and 0.05 (*). [b]Baseline per 24 h was calculated from the average FI monitored during 72 h prior to compound administration.

residue in position 31. Even compounds with a nonsulfated residue in position 27 were highly CCK-1R selective, if they contained the C-terminal dipeptide DMeAsp-MePhe. It is therefore very likely that any degradation product or metabolite formed from a CCK peptide containing DMeAsp-MePhe at the C-terminus would be either CCK-1R selective or inactive. In the literature, CCK-1R selectivity of CCKR agonist peptides has been achieved typically by using 32-MeAsp[30,47] or Lys(Tac) in position 31.[31,45,46,67] The use of 32-DMeAsp for this purpose seems to have remained largely unknown, although one such compound (**2**) including solid biological data was published in 1997 by Pierson et al. In Pierson's study, peptide **2** decreased food intake less potently than its 32-MeAsp isomer ARR15849 in dogs after intranasal administration and was therefore deselected,[30] which explains the lack of later attention for this compound. The reported relatively lower anorectic potency of peptide **2** in dogs might be due to a possibly higher clearance of peptide **2** compared to its 32-MeAsp isomer, in line with our pig PK data for 32-DMeAsp compound **9** and its MeAsp isomer **8**. Our few CCK analogues with a Lys(Tac) residue in position 31 were CCK-1R selective, but an N-methyl group at 32-Asp was still necessary for maximum CCK-1R selectivity. Combination of 31-Lys(Tac) with 32-DMeAsp in one molecule would possibly have given CCK-1R specific compounds but was not attempted here. We concluded that 31-Lys(Tac) is not necessary for CCK-1R selectivity if the molecule contains 32-DMeAsp. None of the many other amino acids used for replacing 31-Met was better than Nle in terms of CCK-1R affinity. Attachment of a hydrophilic linker with an albumin-binding fatty acid at the side chain of a Lys residue in position 28 or at the N-terminus of 26-Asp gave compounds equipotent with each other and with similar compounds without the linker and fatty acid. Acylation at the N-terminus is easier and was prioritized. Substitution of Gly in position 29 gave CCK-2R selective compounds, which has not been a goal of this work. Selective CCK-2R agonists are known in the literature, among them truncated CCK mimetics.[4] However, especially compound **33** with 29-Glu might be of interest because it is an injectable CCK-2R agonist with higher CCK-2R affinity and selectivity than gastrin-17 (**4**) and with presumably extended in vivo half-life due to albumin binding. Incorporating Asn in position 32 resulted in an only weakly active compound (**11**), contrary to published results with a similar compound without fatty chain,[49] whereas the rest of our in vitro biology SAR apparently does not contradict published results.

Our results fit well with previous CCK-SAR-studies showing the crucial influence of the three C-terminal residues of CCK, especially residue 32, on CCK-1R binding and selectivity.[30,31,45−47] In the CCK-receptor/peptide complex models suggested by Fourmy's group, the C-terminal part of CCK is constrained between several transmembrane helices of CCK-1R or CCK-2R, respectively, with slightly distinct positioning of CCK within both receptors.[24] This might explain the high impact of small changes in the C-terminal part of CCK on receptor binding and selectivity. It is likely that our compounds bind to CCK-1R in a similar pose as CCK-8 does, whereas it is not clear why CCK-2R binding is disrupted so strongly by the presence of DMeAsp in position 32.

We tested the chemical stability of one acylated CCK analogue (**3**) containing a sTyr residue and concluded that desulfation of sTyr proceeded very slowly at pH 7.4 and would not prevent us from using such compounds in a neutral formulation. Balsved et al. concluded from kinetic experiments that sTyr-containing peptides are stable enough for being handled and purified at pH 1−3 and room temperature.[68] However, we also pursued peptides with Phe(4sm) in position 27 because they are not limited to neutral formulations and gave at least as good biological results as the corresponding sTyr compounds. Synthesis of our compounds proceeded generally well and usually required one additional synthetic step to remove a protecting group from either sTyr or Phe(4sm). Our CCK-peptides without sTyr or Phe(4sm) in position 27 looked more attractive from a synthetic point of view but showed comparatively low CCK-1R affinity and did not exhibit in vivo activity, as discussed below.

Because rodents are prone to CCK-induced pancreatitis, we performed our in vivo experiments with a limited set of compounds in pigs. PK experiments in Göttingen minipigs with iv dosing indicated long plasma half-lives of up to 161 h, typically 100−150 h for most of our C18-acylated compounds. For comparison, the once-weekly GLP-1 analogue semaglutide had a half-life of 46 h in intravenously dosed Göttingen minipigs.[61] The observed long plasma exposure of our compounds was due to strong albumin binding of the fatty acid chain. In line with this, longer fatty acid chains and longer linkers at the peptide correlated in trend with longer plasma half-lives. However, a C18-compound containing a Lys residue within the linker (**19**) was cleared much faster, possibly due to reduced albumin binding attributed to the positive charge in the linker or because of enzymatic cleavage of the linker. It is not clear why a nonsulfated compound like 27-Tyr peptide **25** was cleared twice as fast as the corresponding sulfated analogue **30**. The observed longer plasma half-life of compound **8** with 32-MeAsp compared to its 32-DMeAsp isomer **9** was also unexpected.

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Acute food intake experiments in LYD pigs were performed with a limited set of compounds. The best compounds gave a strong reduction of food intake, for example, of about 50% for compound **9** in the first 24 h after subcutaneous injection of 30 nmol/kg, and were efficacious for up to 48 h after one injection. The only compound with sTyr in position 27 that was tested in the pig food intake experiment was peptide **40** with a Gln residue in position 31. It had a significant and long lasting anorectic effect, although the effect seemed to be slightly weaker compared to compounds **9**, **19**, and **56**. We did not see a difference in CCK-1R affinity and in vitro potency between these compounds. The two nonsulfated compounds **25** and **27** did not show any anorectic effect. At least for peptide **25**, this must be attributed rather to its comparatively low CCK-1R affinity than to its short half-life because peptide **25** had a similar PK profile as the highly efficacious compound **19**. The CCK-1R affinity of peptide **25** was 10-fold lower, in accordance with PD results, whereas peptides **25** and **19** showed equal CCK-1R potency in our functional in vitro assay. Similarly, compound **8**, the 32-MeAsp isomer of peptide **9**, failed to reduce food intake significantly, in accordance with about 5-fold lower CCK-1R affinity of peptide **8** compared to peptide **9**.

Because compound **9** had performed best at reducing food intake in pigs, it was investigated further. The observed in vitro plasma protein binding of **9** was very high, in line with the expectation of strong albumin binding through the fatty acid chain and resulting long in vivo plasma half-life. Most of the circulating drug-related material in minipigs was compound **9**. Three inactive minor metabolites of peptide **9** were formed by proteolytic cleavage of the peptide backbone and characterized in plasma from dosed minipigs. Chemical stability of compound **9** in a neutral buffer was tested and considered as sufficiently high for a liquid formulation, with the equally active 26-βAsp isomer **56** being the only major degradation product.

We were able to minimize the risk of CCK-2R-mediated adverse effects by developing highly CCK-1R selective compounds. However, there is also a potential risk of adverse effects caused by CCK-1R activation. Pancreatitis mediated by CCK-1R on pancreatic acinar cells is at least a theoretical risk, as mentioned above. Chronic activation of CCK-1 receptors on the gallbladder wall may lead to constant contraction of the gallbladder and altered gallbladder motility in relation to food consumption. If this results in drug tolerance and reduced CCK sensitivity, the consequences would be gallbladder hypomotility, larger gallbladder volume, and increased risk of gallstone formation.[69] On the other hand, treatment with CCK-1R agonists has been suggested as a new way to prevent gallstone formation by stimulating gallbladder emptying.[70]

In summary, we have described the SARs of a series of CCK-8 analogues with fatty acids linked to the N-terminal part of the peptide. A group of peptide-based, long-acting, stable, highly selective CCK-1R agonists with the potential to treat obesity was identified, among them peptides with a sTyr residue. Very high CCK-1R selectivity was achieved mostly by introducing DMeAsp instead of Asp at the penultimate position of CCK-8. In pigs, strong reduction of food intake for up to 48 h after single subcutaneous injection and plasma half-lives of about 100−160 h after iv dosing were observed for some peptides. Compound **9**, now referred to as NN9056, was selected for further development toward an injectable antiobesity drug. Compound **9** contains the following modifications compared to CCK-8: an N-terminal C18 fatty acid derivative with a free carboxy group and linker to give strong albumin binding and thereby long in vivo half-life, Phe(4sm) in position 27 to ensure high chemical stability also under acidic conditions, two Nle residues to replace chemically unstable Met, and the C-terminal dipeptide DMeAsp-MePhe to ensure maximum CCK-1R selectivity, affinity, and potency. In addition, we found a highly selective CCK-2R agonist by replacing Gly in a CCK-8 derivative with Glu.

EXPERIMENTAL SECTION

CCK-8 (**1**) and nonsulfated gastrin-17 (**4**) were purchased from Bachem Holding AG, Bubendorf, Switzerland. Fmoc-DMeAsp-(OtBu)-OH was purchased from ChemPep, Inc., Wellington, FL, USA. The investigated compounds do not belong to known classes of pan assay interference compounds (PAINS), according to an analysis performed with a publicly available filter.[71] All in vivo studies were conducted in accordance with national regulations in Denmark, which are fully compliant with internationally accepted principles for the care and use of laboratory animals, and with animal experimental licenses granted by the Danish Ministry of Justice.

Peptide Synthesis. Fmoc-based solid-phase peptide synthesis was performed manually or on an automated peptide synthesizer, typically starting with 0.1 mmol Rink Amide AM polystyrene resin with a loading of about 0.6 mmol/g. Fmoc deprotection was performed with 20% piperidine in *N*-methylpyrrolidone (NMP), or, after incorporation of Phe(4sm), with 20% 2-methylpiperidine in NMP (typically 2 × 5 min or 2 × 10 min or 1 × 20 min), followed by washings with NMP and dichloromethane (DCM). Couplings were performed using an excess of 0.3 M Fmoc-aa/oxyma/DIC/collidine in NMP without preactivation and with reaction times of 1 h or 2 h. Double coupling for 2 × 1 h was used for introducing Arg and γGlu residues.

Removal of Mtt from side chain amino groups was performed after extensive DCM washings with 1,1,1,3,3,3-hexafluoro-2-propanol (e.g., 1 × 2 min and 2 × 20 min), followed by several washings with DCM, NMP, and a DCM−NMP−collidine mixture. After completing synthesis, the resin was repeatedly washed with DCM.

N-Methylation of Resin-Bound DAsp. In a 5 mL plastic reactor (5 mL plastic syringe with internal frit, outlet stoppered during reactions), the peptide resin containing N-terminal Fmoc-DAsp-(OtBu) [0.1 mmol, obtained by coupling of Fmoc-DAsp(OtBu)-OH to the peptide resin] was shaken with 20% piperidine in NMP for 20 min, then drained, and washed repeatedly with NMP and DCM. A solution of 2,4,6-collidine (0.171 mL, 1.28 mmol) in DCM (1 mL) was added to the resin, followed by a solution of 2-nitrobenzenesulfonyl chloride (0.171 g, 0.77 mmol) in DCM (1.5 mL). The reactor was capped and shaken for 1 h. The resin was washed with NMP/DCM 1:1 (5 × 4 mL). A solution of methyl 4-nitrobenzenesulfonate (261 mg, 1.2 mmol) in NMP (2 mL) was added to the resin, followed by addition of DBU (0.119 mL, 0.8 mmol). The reactor was capped and shaken for 1 h. The resin was drained and washed with NMP (3 × 2 mL), tetrahydrofuran (THF) (3 × 2 mL), and NMP (2 × 2 mL). NMP (2 mL), 2-mercaptoethanol (0.070 mL, 1.0 mmol), and DBU (0.112 mL, 0.75 mmol) were added to the resin. The reactor was capped and shaken for 1 h. The resin was drained (yellow filtrate) and washed with THF (5 × 2 mL) to give a resin with N-terminal DMeAsp(OtBu) ready for further peptide elongation.

Cleavage from the resin was performed with TFA/water/triisopropylsilane (95:2.5:2.5) typically for 2 h, followed by ether precipitation and ether washing.

Removal of Neopentyl from Peptides Containing Neopentyl-Protected sTyr. The crude peptide obtained from 0.1 mmol resin after ether precipitation was treated with a mixture of acetonitrile (7.5 mL) and 2 M aq ammonium acetate solution (7.5 mL), resulting in two liquid phases. The mixture was stirred overnight at 55 °C. The resulting deprotected peptide was then isolated by HPLC as described below.

Removal of TCE from Peptides Containing TCE-Protected Phe(4sm). The crude peptide obtained from 0.1 mmol resin after

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ether precipitation was dissolved in acetic acid (2.25 mL). In a separate test tube with a magnetic stirrer, zinc dust (81 mg, 1.24 mmol) was stirred with 2 mL of 1 M aq HCl for 5 min, resulting in moderate gas evolution. The liquid phase was removed by means of a syringe. To the resulting freshly activated zinc dust, the peptide solution in acetic acid was added. A solution of ammonium acetate (15 mg, 0.2 mmol) in water (0.75 mL) was added. The resulting mixture was stirred for 1 h. The mixture was treated with water (20 mL) and acetonitrile (7 mL). pH was adjusted to 7.6 with 26% aq ammonia (approx. 5 mL). The resulting peptide solution was diluted with water to a final volume of 100 mL and then purified by HPLC as described below.

Purification and Quantification. Crude deprotected peptides were dissolved in aq ammonium acetate and acetonitrile at neutral pH and purified by reversed-phase HPLC (Waters Delta Prep 4000) on a column containing C18-silica gel. Elution was performed with an increasing gradient of acetonitrile in water containing 0.01 M ammonium acetate neutralized to pH 7.4. The resulting pure peptide solution was analyzed (UHPLC, LC–MS), quantified using a chemiluminescent nitrogen-specific HPLC detector (Antek 8060 CLND), and freeze-dried in aliquoted portions to give the peptide ammonium salt as a white solid. Typical yields were 10–30%. Purities of synthesized peptides described here were confirmed to be of >95% by UHPLC, except compounds **14**, **30**, **39**, and **43** which were isolated with purities between 90 and 95% by UHPLC.

In Vitro Binding Assays. Homogenous binding competition assays were performed on plasma membranes prepared from 1321-N1 cells stably expressing the CCK-1R or the CCK-2R (PerkinElmer, ES-530-A and ES-531-A). ^{125}I-CCK-8 was used as a radio ligand (PerkinElmer, NEX203) in both assays. CCK receptor binding assays were performed in 96-well OptiPlates (PerkinElmer, 6005290) in a total volume of 200 μL per well. Reference and test compounds were serially diluted 5-fold in the assay buffer (50 mM Tris/HCl, 4.5 mM $MgCl_2$, 0.02% Tween 20, 0.25% ovalbumin, 0.1% Pluronic F-68, pH 7.4) to final assay concentrations ranging from 10^{-5} to 10^{-12} M. Wheat germ agglutinin-coated polyvinyltoluene SPA beads (PerkinElmer, RPNQ 0001) were reconstituted in assay buffer and mixed with the radio ligand and membrane preparation to give a final assay concentration of 0.5 mg/well SPA beads and approximately 50 pM of the radio ligand (corresponding to approximately 50.000 dpm/well).

Reference or test compounds (50 μL) were added to the OptiPlate, followed by addition of 150 μL of the mixture of the SPA bead, radio ligand, and plasma membrane. The plates were sealed and incubated in the dark at 22 °C for 2 h under shaking and thereafter centrifuged at 1500 rpm for 10 min. The SPA bead light emission was measured in TopCount NXT (PerkinElmer) for 2 min/well after a delay of 60 min.

Data were imported into GraphPad Prism (Prism 7.04, GraphPad Software, San Diego, CA, USA) and analyzed to determine the pIC_{50} ($-\log IC_{50}$) value by nonlinear curve fitting of a three-parameter logistic function with top and bottom constrained to same values for all datasets in an experiment. All binding studies were performed in duplicate and repeated independently at least three times. Mean pIC_{50} with lower and upper 95% confidence intervals (CIs) was calculated as a measure of compound affinity and assay variability, respectively. Mean pIC_{50} values below 5 are extrapolated values.

In Vitro Functional Assays. Accumulation of IP1 following stimulation of the receptors was measured using the IP-One HTRF (HTRF = homogeneous time-resolved fluorescence) assay kit, which is commercially available from CisBio Bioassays, France (catalog no. 62IPAPEB). Frozen aliquots of 1321-N1 cells stably expressing the CCK-1R or the CCK-2R (PerkinElmer) were thawed, washed twice in cell plating medium [Dulbecco's modified Eagle medium (Gibco, 31966-021), with 10% fetal calf serum (Gibco, 16140-071), 1% sodium pyruvate, 1% penicillin–streptomycin (Gibco, 15140-122)] before being suspended in cell plating medium, and seeded at 2×10^4 cells/well (CCK-1R) or 1×10^4 cells/well (CCK-2R) in 40 μL in a 384-well assay plate precoated with poly-D-lysine (Greiner Bio-One, 356661781945). The assay plate was then incubated overnight in an incubator (95% relative humidity, 5% CO_2, 37 °C) in a CO_2-permeable plastic bag.

On the day of the experiment, the assay plate with the cells was washed three times with Dulbecco's phosphate-buffered saline without calcium or magnesium (BioWhittaker, BE-17-512F). Reference or test compounds (30 μL) in serial 10-fold dilutions from 10^{-6} to 10^{-12} M in assay buffer [IP-One stimulation buffer (part of IP-One HTRF assay kit, see above; consisting of 10 mM HEPES, 1 mM $CaCl_2$, 0.5 $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4) with 0.005% Tween 20 (Merck, 8.22184.1) and 0.1% Pluronic F-68] were added. The plate was incubated for 1 h at 37 °C in an incubator followed by addition of 6 μL each of Tb-cryptate-conjugated anti-IP1 antibody and d2-conjugated IP1 diluted in lysis buffer (lysis buffer, d2-conjugated IP1, and Tb-cryptate-conjugated anti-IP1 antibody were all included with the IP-One HTRF Assay kit referred to above). The plate was incubated for 1 h at rt before it was read on a Mithras LB 940 fluorescence plate reader (Berthold Technologies, Bad Wildbad, Germany) using filters for excitation at 320 nm and emissions at 665 and 620 nm. Increasing levels of endogenous IP1 were measured as a reduction in FRET between Tb-cryptate-conjugated anti-IP1 antibody and d2-conjugated IP1. Data from the fluorescence reader were given as the fluorescence ratio ($10^4 \times 665$ nm/620 nm).

Data were imported into GraphPad Prism (Prism 7.04, GraphPad Software, San Diego, CA, USA) and analyzed to determine the pEC_{50} ($-\log EC_{50}$) value by nonlinear curve fitting of a three-parameter logistic function with top and bottom constrained to same values for all datasets in an experiment. All functional studies were performed in duplicate and repeated independently at least three times. Mean pEC_{50} with lower and upper 95% CIs was calculated as a measure of compound potency and assay variability, respectively. Mean pEC_{50} values below 6 are extrapolated values.

Pharmacokinetic Evaluation in Minipigs. The studies were performed in female Göttingen minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark), approximately 23 months of age and about 20 kg of weight. The pigs (n = 2 or 3) were instrumented with permanent central-venous catheters 3 weeks before study start. Dosing solutions consisted of 200 nmol/mL test compound, 50 mM phosphate, 70 mM sodium chloride, and 0.05% polysorbate 80 with pH adjusted to 7.4. Dosing solution was given as iv injection (0.025 mL/kg to achieve a nominal dose of 5 nmol/kg) through the central catheter. The catheter was flushed with at least 10 mL of saline post administration. The blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes from the central-venous catheter at following time points relative to dosing: predose, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 48, 72, 96, 120, 168, 192, 216, 240, 264, and 288 h. The blood samples were kept on ice for maximum 30 min and centrifuged at 4 °C and 1942g for 10 min. The resulting plasma was stored at −20 °C until analysis for peptide plasma concentration as described below. Plasma concentration–time profiles were analyzed by non-compartmental analysis using Phoenix WinNonlin Professional 6.4 (Pharsight, Mountain View, CA, US). Data analysis was performed using individual concentration–time values. The given mean values for clearance and volume of distribution are geometric means and half-life is harmonic.

Bioanalysis of Plasma Samples from iv-Dosed Minipigs. Plasma samples (including standard curve samples used for quantitation of unknowns and prepared from blank plasma spiked with CCK compound at a concentration range of 0.5–1000 nM) were treated with three volumes of methanol, and the resulting precipitates were centrifuged off. The supernatants were analyzed by electrospray LC-MS. For more details, see Supporting Information.

*Detection of Metabolites of Peptide **9** in Plasma Samples from iv-Dosed Minipigs.* Plasma samples from Göttingen minipigs iv-dosed with 5 nmol/kg compound **9** were treated with three volumes of methanol, and the resulting precipitates were centrifuged off. The supernatants were analyzed by electrospray LC–MS. The standard curve samples used for identification and quantification of peptide **9**

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were prepared from blank plasma spiked with peptide **9** at a suitable concentration range. For more details, see Supporting Information.

Acute Effect on Food Intake in Lean LYD Pigs. The studies were performed in female lean LYD pigs (Gundsøgård, Roskilde, Denmark) approximately 3 months of age weighing approximately 30−35 kg. The animals were fed ad libitum with pig fodder and had free access to water. Daily food intake was monitored online by logging the weight of fodder every 15 min. Food intake was monitored for 3 days prior to compound administration and for 4 days after administration. Baseline over 24 h was calculated as the average of the 3 days prior to compound administration. Animals were allocated into groups of four animals based on baseline food intake and body weight. In the morning on day 0, the animals were given a sc dose of the test compound. Dosing solutions consisted of 1200 nmol/mL test compound, 50 mM phosphate, 70 mM sodium chloride, and 0.05% polysorbate 80 with pH adjusted to 7.4. The dosing volume used was 0.025 mL/kg to achieve a nominal dose of 30 nmol/kg. During the studies, animals were observed daily and any findings such as injection site reaction, increased skin or body temperature, colic symptoms, vomiting, decreased feces, or suppressed behavior were noted. In the end of each study, the animals were anaesthetized and a blood sample was taken from the saphenous vein, transferred to EDTA-coated tubes, and kept on ice until centrifugation for 10 min at 4 °C with 2000*g*. Plasma was pipetted on dry ice and stored at −20 °C until analysis for peptide plasma concentration as described above for minipig samples. Hereafter, the animals were euthanized with an overdose of pentobarbital. Statistical analyses were performed in GraphPad Prism (GraphPad Prism version 7.04 for Windows, GraphPad Software, San Diego, California, USA). Statistical comparison of daily accumulated food intake between baseline and 0−96 h post treatment was done using repeated measures two-way ANOVA (Analysis of variants) followed by the Dunnett's multiple comparisons test. *P*-values below 0.05 were considered statistically significant. Significance levels $p < 0.0001$ (****), 0.001 (***), 0.01 (**), and 0.05 (*) are indicated in relevant figures.

Plasma Protein Binding of Peptide 9. The equilibrium shift assay technology was used to investigate binding between peptide **9** and plasma proteins. A kinetic analysis was performed to determine the dissociation constant K_d and percentage fraction unbound (f_u) of peptide **9** in the plasma.

HSA was immobilized to Mini-Leak Sepharose beads according to the procedure described by Kurtzhals et al.[60] Afterward, the beads were washed three times with phosphate buffer pH 11 and three times with glycine buffer pH 3. The beads were stored in PBS buffer at pH 7.4 at a concentration of 200 mg beads per mL.

Peptide **9** at a final concentration of 500 nM was incubated with various concentrations of immobilized HSA and two different concentrations of plasma by shaking for 2 h. After incubation, the samples were centrifuged for 10 min at 750*g* to remove the beads. One volume of the resulting supernatant was mixed with three volumes of methanol including 20 nM compound **7** as an internal standard, resulting in protein precipitation. The samples were then centrifuged for 25 min at 4500*g*, and the supernatant was analyzed by LC−MS. For more details, see Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01558.

LC−MS and analytical UHPLC data of synthesized peptides; description of LC−MS and UHPLC methods; UHPLC data used for purity analysis of peptides; analytical UHPLC of compounds **8**, **9**, **14**, **19**, **25**, **27**, **30**, **39**, **40**, **43**, and **56**; bioanalysis of plasma samples from iv-dosed minipigs; detection of metabolites of peptide **9** in plasma samples from iv-dosed minipigs; and LC−MS method used for determination of plasma protein binding of compound **9** (PDF)

Molecular formula strings (CSV)

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The manuscript was written through contributions of all authors.

Notes

The authors declare the following competing financial interest(s): All authors are or were full time employees of Novo Nordisk A/S, and most are minor share-holders of Novo Nordisk A/S.

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■ ABBREVIATIONS

CCK-1R, cholecystokinin 1 receptor; CCK-2R, cholecystokinin 2 receptor; CLND, chemiluminescent nitrogen detector; DIC, *N*,*N*′-diisopropylcarbodiimide; dpm, decays per minute; IP1, inositol 1 phosphate; LYD, Landrace Yorkshire Duroc; Mtt, 4-methyltrityl; NA, not available; SPA, scintillation proximity assay; TCE, 2,2,2-trichloroethyl

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