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Articles

Synthesis of Human CCK_{26-33} and CCK-33 Related Analogues on 2,4-DMBHA and TMBHA†

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New analogues of human cholecystokinin in which the Tyr(SO_3H) has been replaced by Phe(p-CH_2SO_3Na), methionines by norleucines, and tryptophan by 2-naphthylalanine{[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33} and [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33} were synthesized by Fmoc solid phase methodology on two different resins (2,4-dimethoxybenzhydrylamine- and 4-(benzyloxy)-2′,4′-dimethoxybenzhydrylamine resins, 2,4-DMBHA and TMBHA resins, respectively). While the syntheses on the TMBHA appeared to be more sluggish than those carried out on the 2,4-DMBHA, both final crude products were of equivalent relative purity and after purification gave approximately the same final yields of analogues estimated to have a purity greater than 93% using RPHPLC and CZE. The peptides were further characterized by amino acid analysis and LSIMS. Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 was submitted to 33 Edman cycles and shown to be the desired product with less than 3% preview. Both analogues were tested for their ability to stimulate amylase release from isolated rat pancreatic acini. In this assay, [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33} and Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 were 10 and 30 times less potent than CCK-8, respectively.

Introduction

Cholecystokinin (CCK) is a hormone originally isolated from porcine intestinal mucosa by Ivy and Oldberg[1] and described as a linear 33-amino acid peptide containing a sulfated tyrosine essential for its biological activity.[2,3] It has been found in mammals in both the digestive tract and the central nervous system.[4,5] Among its multiple biological functions, this hormone stimulates pancreatic exocrine secretion, gallbladder contraction, and intestine motility[6] and also may act as a neurotransmitter/neuromodulator in the central nervous system (CNS).[7-10]

Several different molecular forms of cholecystokinin have been detected *in vivo* (58-, 39-, 12-, 8-mers),[11-15] but its C-terminal fragment CCK_{26-33} has been the most extensively studied. This can be easily understood because CCK-8 (as it is called) is a short peptide, exhibits the full potency of the native hormone,[16] and is described as the predominant molecular form of CCK in both CNS and gastrointestinal tract[17] of some mammals.

CCK-8 and a large number of its analogues (agonists and antagonists) have been synthesized during the last decade[18-22] in order to perform structure–activity studies

† The abbreviations for the amino acids are in accord with the rules of the IUPAC Commission on Biochemical Nomenclature. *J. Biol. Chem.* 1975, *247*, 977–983. Additional abbreviations: 2,4-DMBHA, 2,4-dimethoxybenzhydrylamine; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; But, *tert*-butyl ether; CZE, capillary zone electrophoresis; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMF, *N*,*N*′-dimethylformamide; EDT, ethanedithiol; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-OSu, 9-fluorenylmethyl succinimidylcarbonate; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; LSIMS, liquid secondary ion mass spectrometry; MBHA, methylbenzhydrylamine; MeCN, acetonitrile; MeOH, methanol; Mtr, (4-methoxy-2,3,6-trimethylphenyl)sulfonyl; OtBu, *tert*-butyl ester; Pmc, (2,2,5,7,8-pentamethylchroman-6-yl)sulfonyl; RP-HPLC, reversed-phase high-performance liquid chromatography; TEA, triethylamine; TEAP, triethylammonium phosphate buffer; TFA, trifluoroacetic acid; TMBHA, 4-(benzyloxy)-2′,4′-dimethoxybenzhydrylamine; Trt, trityl.

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that have helped to determine the various biological activities of this hormone associated to different sites of its action in the central and peripheral systems. Better understanding of the function and mechanism of action of these molecules has resulted from these studies.

However, no similar studies are reported for the larger molecular forms of CCK. In fact, the syntheses of CCK-58, CCK-39, and CCK-33 and analogues (Miranda et al., 1993, manuscript in preparation) have been always considered a very difficult task because of the chemical instability of some of the amino acids residues present in those molecules during the synthetic process.

Kurano et al. reported the first total synthesis of porcine CCK-33 using the classical solution methodology with maximum side-chain protection strategy in 1987.[23] In the same year, Penke et al. described its synthesis using the solid-phase approach.[24] More recently, Penke and Nyerges reported the synthesis of porcine CCK-33 by the Fmoc strategy using a new resin specially developed for this sequence.[25,26] The results of those works confirmed the expected difficulties associated with the chemical instability of some residues present in the molecule but also proved that it is possible to reproduce a fully active CCK-33 preparation in the laboratory. This fact gave a new perspective to the field.

At this stage, it seemed that the availability of the corresponding synthetic human hormone and analogues would be an additional major contribution. From the fact that porcine and human CCK-33 are approximately 90% homologous according to Tatemoto et al.[12] and Eysselein et al.,[13] it would seem reasonable to think that human CCK-33 could also be made.[27]

These facts combined with the biological results previously described for some CCK-8 analogues suggested further investigations such as that of more stable analogues of CCK_{26-33} and human CCK-33. In the present paper we describe the preparation of[Phe(p-CH_2SO_3Na)^{27},-Nle^{28,31},Nal^{30}]-CCK_{26-33} and [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},-Nal^{30}]-CCK-33 in which the Tyr(SO_3H) has been replaced by Phe(p-CH_2SO_3Na), methionines by norleucines, and tryptophan by 2-naphthylalanine in order to increase the chemical stability of the peptides during the synthesis, full deprotection/cleavage and purification steps. The selection of Nle substitution in positions 7, 28, and 31, of L-Phe(p-CH_2SO_3Na) in position 27, and of Nal in position 30 stemmed from earlier reports in the literature. Among the large number of CCK_{26-33} (CCK-8) analogues described, some have both Met^{28} and Met^{31} residues replaced by norleucine (Nle) and were reported to be as potent as the parent analogues with Met.[19] The amino acid Phe(p-CH_2SO_3Na)-OH was described to be a good substitute for Tyr(SO_3H) in CCK-8.[28-30] Finally, tryptophan is described in the literature as a very acid sensitive amino acid[31] that can be replaced by 2-naphthylalanine in position 33 of CCK-8.[19]

Although those modifications have been introduced individually or two at a time earlier in the CCK-8 molecule, they have never been used simultaneously to prepare any CCK analogue.

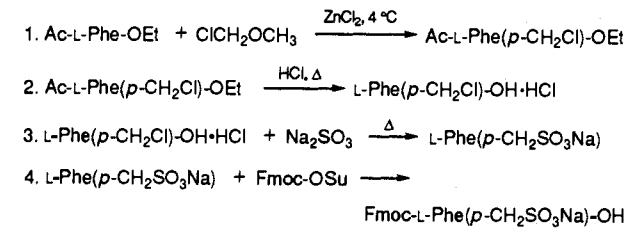
In order to achieve our goal we used the Fmoc solid-phase approach[32] and two different resins: 2,4-dimethoxybenzhydrylamine, 2,4-DMBHA,[33] and 4-(benzyloxy)-2',4'-dimethoxybenzhydrylamine resins, TMBHA (Penke et al., manuscript in preparation). We also prepared the Fmoc-(p-CH_2SO_3Na) using an alternative procedure to that described in the literature.[29]

Results and Discussion

Because of the challenge presented by the instability of Tyr(SO_3H) to acids and the presence of three methionines and of one tryptophan in its sequence, CCK-58 has been so far an elusive target for peptide chemists. We present here an Fmoc-based strategy that led to the successful synthesis of [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33} and [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 which opened the path to the synthesis of [Phe(p-CH_2SO_{3Na})^{52},-Nle^{32,53,56},Nal^{55}]-CCK_{58} (Miranda et al., 1993, manuscript in preparation). Two different resins (2,4-DMBHA and TMBHA) were evaluated. The attractiveness of these peptides (if potent enough) is their synthetic availability for the study of CCK's physiological role in vivo.

CCK-8 Analogues. Because of the commercial unavailability of Fmoc-L-Phe(p-CH_2SO_3Na)-OH, we investigated the possibility of obtaining large quantities of this protected amino acid following published procedures.[29] While this procedure was easy to reproduce, we found the resolution step to be limiting. We therefore investigated using a more direct approach described in Scheme I.

Scheme I. Synthetic Scheme of Fmoc-L-Phe(p-CH_2SO_3Na)-OH



Compound 1 was obtained as described by He et al.[34] Chloromethylation[35] yielded 2 which was carefully hydrolyzed to the corresponding unprotected amino acid. Without further purification, since the CH_2Cl is extremely reactive, 1 was sulfonated according to Gonzalez-Muniz et al.[29] to give the desired solid 3 mixed with salts. This mixture was converted to the Fmoc amino acid derivative 4 by reaction with Fmoc-OSu.[36,37] MS analysis data proved that 4 was the expected product. The identity of this amino acid was further confirmed by MS analysis of the synthetic CCK analogues.

Those results showed that it is possible to synthesize Fmoc-L-Phe(p-CH_2SO_3Na)-OH 4 following a shorter (four-step) procedure than that described by Gonzalez-Muniz et al.[29,38] which includes a cumbersome acylase-mediated resolution step.

Because of our significant experience with the MBHA resins with different levels of substitution (a parameter that was difficult to evaluate with the 2,4-DMBHA resin for which a limited number of batches were ever made in our laboratory[33,39]), and with the availability of Fmoc-L-Phe(p-CH_2SO_3Na)-OH, we first synthesized[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33}-MBHA using the Boc strategy (except at position 27 where we used the Fmoc protecting group). The same peptide was also synthesized using the Fmoc strategy with the 2,4-DMBHA. A comparison of the quality of the crude preparations obtained by the two t-Boc and Fmoc approaches based on an HPLC profile gave very similar results indicating that both strategies could be suitable for the synthesis of this

octapeptide. While crude peptides made by both strategies were of equivalent purity after eight cycles, by the time we had constructed the corresponding 14-mer (data not shown) the crude preparation synthesized using the Boc strategy and HF cleavage was considerably less pure than that synthesized by the Fmoc strategy with TFA cleavage and deprotection. From these results we concluded that the Fmoc strategy was most likely to yield the desired CCK-33 analogue.

The first step towards achieving the total synthesis of Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK-33 and shorter analogues was to identify the best resin to be employed.

Use of 2,4-DMBHA Resin. We first used the 2,4-DMBHA which had been successfully used in our laboratory[33,39,40] to synthesize[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31}]-CCK$_{26-33}$–2,4-DMBHA and[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},-Nal^{30}]-CCK$_{26-33}$–2,4-DMBHA. Different conditions were used for the TFA cleavage and deprotection. We found the mixture of TFA/thioanisole/water/phenol/EDT (83:5:5:2.5, reagent K[41] for 3 h at room temperature to be the most efficient on an analytical scale (10 mg of resin). Both crude peptides were analyzed by RP-HPLC showing profiles that suggested that[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},-Nal^{30}]-CCK$_{26-33}$ was less contaminated than[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31}]-CCK$_{26-33}$. This result suggested that the Nal containing peptide was more stable than the corresponding Trp containing analogue.

When cleaved by HF,[Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},-Nal^{30}]-CCK$_{26-33}$–2,4-DMBHA yielded the desired [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK$_{26-33}$ with a purity equivalent to that of the same peptide cleaved with TFA from the same resin. We concluded from this experiment that this nonapeptide was stable in both HF and TFA in the presence of the scavengers used. This is in good agreement with the observation of Gonzalez-Muniz et al.[29] that [Phe(p-CH_2SO_3Na)^{27}]-CCK$_{26-33}$ could be synthesized by the t-Boc strategy.

Use of TMBHA Resin. We also synthesized [Phe-(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK$_{26-33}$ on the TMBHA resin. This resin was developed by Penke et al. (Penke et al., manuscript in preparation). It is more labile to TFA than the 2,4-DMBHA and therefore compatible with the Pmc protecting group for Arg (Penke, personal communication). The synthesis of [Phe(p-CH_2SO_3Na)^{27},-Nle^{28,31},Nal^{30}]-CCK$_{26-33}$–TMBHA was done using the same Fmoc procedure employed for the synthesis on the 2,4-DMBHA resin. The final peptide-resin was cleaved with reagent K and the crude peptide analyzed by RP-HPLC. The profile thus obtained was essentially identical to that obtained with the 2,4-DMBHA resin.

Purification and Characterization of [Phe-(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK$_{26-33}$. Purification of the nonapeptide synthesized on the 2,4-DMBHA using semipreparative reverse-phase HPLC was straightforward. The analogue was characterized by HPLC in a system different from that used during purification and was found to be 94% pure. CZE could not be used because of solubility problems. Mass spectrometry and amino acid analysis gave the expected results (see the Experimental Section).

In summary, we have synthesized a CCK-8 analogue using three different resins and two general strategies (Boc and Fmoc). From these studies we concluded that the synthesis of a CCK-33 analogue would be most successful if attempted using the Fmoc strategy on either of the two resins 2,4-DMBHA or TMBHA.

CCK-33 Analogues. The synthesis of [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK-33 using the t-Boc strategy and the MBHA resin had to be aborted after the introduction of residue 14 as indicated earlier. The HPLC profiles showed that [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK$_{20-33}$ was significantly more contaminated than the corresponding analogue synthesized using the Fmoc strategy on either of the 2,4-DMBHA or TMBHA resins. We concluded that the Boc strategy was applicable for the synthesis of the CCK-8 analogue but not appropriate for the synthesis of much longer analogues.

We were further convinced of the difficulties of attempting the synthesis of even an acid stable CCK-33 analogue after the report of Penke and Nyerges who first described the synthesis of the natural CCK-33 by Fmoc chemistry. Those authors used 4-(succinylamido)-2,2′,4′-trimethoxybenzhydrylamine resin (SAMBHA) and chosen the Fmoc strategy because the overall yield obtained with the combination t-Boc chemistry/sulfation of the Tyr^{27} was relatively low (6%).[26] Two different syntheses of [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 with somewhat lower yields were ultimately achieved in our laboratories.

Use of the 2,4-DMBHA Resin. The peptide was built on the 2,4-DMBHA with interruptions of the synthetic process at positions 8, 14, 20, and 25 (Figure 1). At each

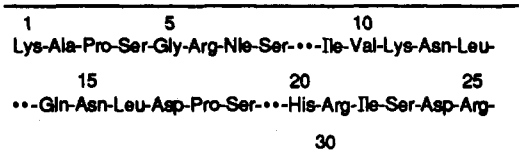


Figure 1. Strategy used for the synthesis of Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 on 2,4-DMBHA and TMBHA: (··) interruptions done during the elongation of the growing peptide.

interruption, small aliquots of the peptide resins were cleaved and deprotected using reagent K and different reaction times (see the Experimental Section). The crude preparation was analyzed by HPLC and the main component of the mixture was collected for MS analysis.

Figure 2 shows the profile of the crude peptide obtained from the preparative cleavage of the final peptide-resin with reagent K[32] for 8 h at room temperature. The analysis by LSIMS confirmed the identity of [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK-33 as the main component of the crude peptide. It is important to note that reagent K was used for cleavage and concomitant deprotection since it had been shown to be superior to other mixtures of scavengers. As a result of a kinetic study this step was extended to an 8-h period.

Use of the TMBHA Resin. We also synthesized [Phe-(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 on the TMBHA resin. The most important observation may be that while the substitution on both the 2,4-DMBHA resin and the TMBHA resin are relatively high (0.4 and 0.6 mmol/g), it soon became apparent that the introduction of the first residue (Fmoc-Phe) was never quantitative on the TMBHA resin despite repeated couplings. The TMBHA resin was therefore acetylated thus bringing back the substitution to a level probably comparable to that on the 2,4-DMBHA resin. Despite this lowering of substitution, we still observed that the couplings (under the conditions

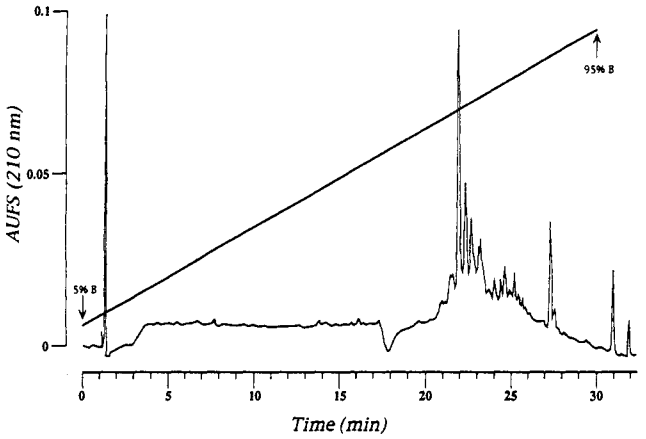


Figure 2. Analytical RP-HPLC elution profiles of the crude Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33. Experimental conditions: column, VYDAC-C_{18} (5 μm, 25 cm × 4.6 mm, pore size = 300 Å); solvent A, 0.1% TFA; solvent B, 0.1% TFA in 60% MeCN/H_2O; gradient 5–95% in 30 min; flow rate, 2 mL/min; detection, 210 nm).

described in the experimental section) of the first 15 residues were more sluggish than in the case of the 2,4-DMBHA. The final peptide-resin was cleaved with reagent K and the crude peptide analyzed by RP-HPLC. The profile thus obtained showed the same major component.

During those syntheses we have made some noteworthy observations. First, the synthesis of [Phe(p-CH_2SO_3-Na)^{27},Nle^{28,31},Nal^{30}]-CCK-33 on the 2,4-DMBHA resin went faster than that on the TMBHA resin since the couplings were easier to achieve. Second, acetylations seemed to influence the quality of the crude obtained: less acetylations were performed during the synthesis of the analogue on the 2,4-DMBHA resin. Yields of the highly purified peptides after extensive purification of the crudes obtained from independent manual syntheses can be considered to be equivalent independently of which of the two resins was used.

Purification of [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK-33. The purification of the crude CCK-33 analogue synthesized on the TMBHA resin was done by RP-HPLC (using TEAP[42] and TFA based buffers), but the final yield was extremely low (<1%). Our explanation for this result is that RP-HPLC is not efficient enough to separate the crude peptide from its impurities and as a consequence just a few fractions contain the highly purified peptide. In order to improve the overall yield of the purification step, we have used a cation-exchange (Mono-S) FPLC step followed by a desalting (0.1% TFA) RP-HPLC step using the same strategy as that followed by Miller et al. in the purification of synthetic rat histone H2A(1–53)-NH_2.[40] The ion-exchange step allowed the elimination of most of the hydrophobic contaminants which eluted first. The RP-HPLC step, probably because of an initial much lower load than when used with the crude peptide, gave better resolution and better yields. The final yields obtained after the two-step purification protocol were 3% for the crude made on the 2,4-DMBHA resin and 2% for the crude made on the TMBHA resin. These numbers are low and seem to be a reflection of the difficulty encountered by us and others to synthesize CCK analogues and CCK-33 analogues in particular.

Characterization of [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},-Nal^{30}]-CCK-33. The analogue was characterized in a highly resolutive HPLC system different from that used during purification (see the Experimental Section). We found that under the conditions used here, CZE (Buffer, 100 mM phosphoric acid (pH 2.50); voltage, 15 kV, current, 75 μA; capillary, 363 μm o.d. × 75-μm i.d. × 50-cm length; 30 °C) showing on major component (94%) and two or three minor, closely related impurities was less resolutive than RP-HPLC (89% pure using the TEAP buffer). Mass spectrometry and amino acid analyses gave the expected results (see the Experimental Section).

Sequence analysis using Edman degradation demonstrated that the expected sequence had been correctly assembled and that it contained, based on the very low level of preview (≤3%) which remained constant after residue 5, negligible amounts of deletion peptides. Repetitive yield was >91%.

In summary, we have synthesized two CCK-8 and CCK-33 analogues using two different resins and the Fmoc strategy. We found that constant monitoring of the synthetic process achieved by cleavage of small aliquots of the growing resin and analysis of the synthetic products by HPLC was particularly important in optimizing the synthetic protocols. This was found to be even more crucial during the synthesis of [Phe(p-CH_2SO_3Na)^{52},Nle^{32,53,56},-Nal^{55}]-CCK-58, a CCK-58 analogue with similar substitutions (Miranda et al., 1993, manuscript in preparation).

Biological Results. The relative biopotencies of CCK-8 and CCK-33 have been contested over the years and may have depended on the assays. While the results did not always agree and many investigators concluded that CCK-8 was 3–10 times more potent than CCK-33, others showed that those peptides had similar potencies.[3,16,43–48]

Based on those data,[49] we developed a protocol that evaluates those potencies more accurately. Briefly, the CCKs were first chromatographed (HPLC) to assess purity, the peptide content of the purified peptides was then determined by amino acid analysis, their relative immunoreactivities were compared using an antibody that recognized the common carboxyl terminus of these forms, and finally their relative potencies were measured by the comparison of their abilities to stimulate amylase release from isolated rat pancreatic acini. Using this procedure, we suggested that the differences in the biological potencies between CCK-8 and CCK-33 was not as great as previously described since CCK-8 was found to be only 36% more potent than CCK-33.

The potencies of [Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33} and Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 estimated by the method described above are expressed in Figure 3. The doses of each compound that produced half maximal amylase release were analyzed by the Wilcoxon rank sum test. Both[Phe(p-CH_2SO_3Na)^{27},-Nle^{28,31},Nal^{30}]-CCK_{26-33} and Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},-Nal^{30}]-CCK-33 were significantly less potent than CCK-8 ($P < 0.05$) by approximately 10- and 30-fold, respectively.

This significant difference between the relative potencies of the standard used and our CCK-8 analogue was not expected since none of the modifications introduced to increase chemical stability had been shown to be detrimental to potency when introduced either one or two at a time.[19,28,29] Our conclusion based on the fact that the octapeptide is 10 times less potent than the standard is that while each of the modifications was shown to generate an analogue approximately equipotent to CCK-8, in most cases the potency was slightly less than that of the standard. It is possible that cumulation of these marginally

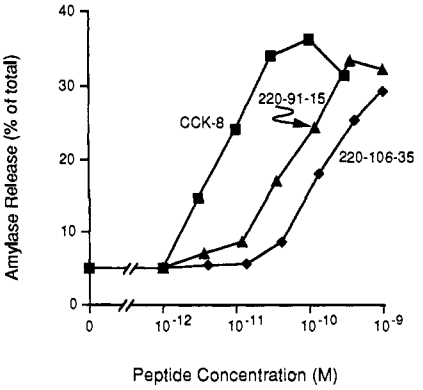


Figure 3. Effects of CCK-8,Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},-Nal^{30}]-CCK_{20-33} (compound 220-91-15) and Phe(p-CH_2SO_3Na)^{27},-Nle^{7,28,31},Nal^{30}]-CCK-33 (compound 220-106-35) on amylase release from rat pancreatic acini. The CCK-8 and the peptide aliquots containing the CCK analogues were diluted to appropriate concentrations and incubated with pancreatic acini. The amylase released into the media during a 30-min incubation was measured. Each value is mean ± SD of triplicate determinations.

detrimental substitutions ultimately led to this 10-fold loss of potency.

Considering the above and the fact that CCK-8 in a careful study of its biological potency relative to that of CCK-33[49] was found to be 36% more potent, we were not surprised to find out that Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},-Nal^{30}]-CCK-33 was approximately 30 times less potent than CCK-8. In vivo studies however should be more indicative of the stability of these analogues through exhibition of higher potency or longer duration of action.

Experimental Section

Reagents, Apparatus, and Methods. All solvents and reagents were purchased from Sigma, Mallinckrodt, Aldrich, and Fluka Chemika. They were reagent or analytical grade. TFA and TEA for chromatography were redistilled. The Boc- and Fmoc-L-amino acids and Ac-Phe-OEt were bought from Bachem Inc. (Torrance, CA). The MBHA and 2,4-DMBHA were prepared in our laboratory in accord to published procedures.[33,39,50] The TMBHA resin was a generous gift from Dr. B. Penke. BOP[51] was purchased from Richelieu Biotechnologies. CCK-8 was a gift from E. R. Squibb. RP-HPLC was performed on a Perkin-Elmer LC Series 410 apparatus equipped with a Rheodyne 7125 injector, a Linear Instruments Corp. recorder, and a Perkin-Elmer LCI-100 computer integrator using VYDAC-C_{18} or C_4 columns (5 μm, 25 cm × 4.6 mm), pore size = 300 Å, from Separation Group (Hisperia). The mobile phase employed 0.1% TFA as solvent A and 60% MeCN (Mallinckrodt) in A as solvent B (flow rate, 2 mL/min; UV detection, 210 nm).

Amino Acid Derivatives. Thin-layer chromatography was performed on precoated silica gel (DC-Alufolien-Kiesegel 60F-254, 0.2-mm thick, Merck) using the following solvent systems: (A) hexane/ethyl acetate/MeOH (6:4:0.4, v/v/v); (B) chloroform/MeOH/water/acetic acid (HAc) (5:5:1:0.5, v/v/v/v); (C) isopropyl alcohol/28% ammonium hydroxide (7:3, v/v); (D) chloroform/MeOH/HAc (65:5:5, v/v/v). Flash chromatography was done on a silica gel column (6 × 11 cm, silica gel 60, particle size 0.04-0.063 mm, Merck). Melting points were taken on an Unimelt apparatus (Arthur Thomas) and are uncorrected. Optical rotations were determined with a Perkin-Elmer Model 214 polarimeter. Electron impact (EI) mass spectra were measured with a JEOL JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan). The accelerating voltage for the EI mass spectra was +10 kV. A direct insertion probe heated up to 60 °C was used to help volatilize the sample which was bombarded with 70-eV electrons. Collision-induced dissociation (CID) occurring in the first field free region from a preselected precursor was measured with a product ion scan (B/E linked field scan). Helium gas was used to attenuate the precursor to 60% of the intensity prior to introduction of the collision gas. The ^{1}H-NMR spectrum was recorded in a Nickolet 360 MHz spectrometer.

Peptide Purification. The semipreparative purifications were carried out on the following systems: (1) A Waters apparatus consisting of two Waters 501 HPLC pumps, Waters Automated Gradient Controller, Waters 486 Tunable Absorbance Detector and Servocorder SR6253 by Datamark. The solvents used as eluents A and B were 0.1% TFA and 60% MeCN/H_2O, 0.1% TFA, respectively (flow rate, 3.5 mL/min; UV detection, 210 nm). The column was a VYDAC C_{18}, 5 μm, 25 × 1 cm, 300-Å pore size. (2) A Pharmacia (LKB Biotechnology) FPLC system using Mono S HR 10/10 (10 × 1.0-cm i.d.) and 16/10 (16 × 1.0-cm i.d.) columns prepacked with 10-μm Mono S resin (the charged group on the gel is $CH_2SO_3^-$). The buffers used were 50 mM sodium borate pH 8.7 or 50 mM sodium borate in 35% acetonitrile/65% water, pH 8.7 or pH 9.6, as solvent A; solvent B was solvent A containing sodium chloride (0.5 M) (flow rate, 4 or 6 mL/min; UV detection, 214 nm).

The preparative RP-HPLC purifications were carried out on a Waters Prep LC-500 liquid chromatograph equipped with a SPD-6A detector (Shimadzu), an OmniScribe register (Houston Instruments), a mixer, and a Chromat-A-Trol II programmer (Eldex Laboratories) using custom-made cartridges (4.7 × 30 cm, VYDAC C_{18} 15–20 μm, pore size = 300 Å). The peptides were eluted in linear gradients of TEAP pH 2.5 or 0.1% TFA (eluent A) and 60% MeCN/A (eluent B; flow rate, 90–100 mL/min; UV detection, 230 nm).

Peptide Characterization. The pure peptides were subjected to RP-HPLC, CZE, LSIMS, and amino acid analyzes.

CZE analyses were performed on a P/ACE System 2050 (Beckman) controlled by an IBM Personal System/2 Model 50Z using a ChromJet integrator (Spectra-Physics). The buffer used was 100 mM phosphoric acid adjusted to pH 2.50 by addition of 2 M NaOH; the field strength, 15 kV; the temperature, 30 °C; the capillary, Supelco ECelect P175 (363-μm o.d. × 75-μm i.d. × 50-cm length); the wavelength, 214 nm; the sample was applied by pressure injection.

Analytical RP-HPLC performed on a Hewlett-Packard apparatus consisted of a Series II 1090 liquid chromatograph with diode array detector, a Controller 362 and a Think Jet printer using a VYDAC-C_{18} column (5 μm, 15 cm × 2.1 mm, pore size = 300 Å). The mobile phase employed was TEAP, pH 2.25, as eluent A and 60% MeCN/A as eluent B; detection, 210 and 280 nm; temperature, 40 °C; flow rate, 0.2 mL/min.

Amino acid analyses were performed on a Perkin-Elmer Series 4 LC system comprising two Series 10 LC pumps, a ISS-100 injector, a RTC 1 column oven, a Kratos Spectroflow 980 fluorescence detector (Applied Biosystems), and a Chromatographics 3 computer after the products were hydrolyzed in 4 N methanesulfonic acid at 110 °C for 24 h. A Pierce AA511 ion-exchange column was maintained at 60 °C, and postcolumn derivatization with phthalaldehyde was performed at 52 °C. Samples containing the internal standard γ-aminobutyric acid were eluted (5 min after injection) with a gradient 0–100% B in 25 min and then 100% B for 15 min using a flow rate of 0.5 mL/min. The buffers A and B were Pierce Pico buffer pH 2.20 and Beckman Microcolumn sodium citrate buffer pH 4.95, respectively.

LSIMS mass spectra were measured with a JEOL JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of +10 kV and Cs^+ gun voltage of +30 kV were employed. The sample (ca. 20 μg; TFA salt; lyophilized) was added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. The spectra were calibrated using $Cs(CsI)_n$ cluster peaks. The spectra were measured at a nominal resolution of either 1000 or 3000 as determined by the mass and the intact $[M+H]^+$ peak intensity. For those spectra that were measured at 1000 resolution (CCK_{20-33}) the observed average m/z was compared with the calculated $[M+H]^+$ average mass. In those cases where the higher mass resolving power of the instrument was used (3000), the observed m/z of the monoisotopomer was compared with the calculated $[M+H]^+$ monoisotopic mass (CCK-33).

The sequence analysis was performed on an Applied Biosystems Model 470A gas phase automated protein sequencer. Peptides were immobilized on a glass fiber filter treated with

TFA, in the presence of 3 mg of polybrene and 0.2 mg of NaCl. PTH-amino acid derivatives were analyzed by RP-HPLC with an on-line Applied Biosystems Model 120A analyzer and identified by retention time.

Ac-L-Phe(*p*-CH_2Cl)-OEt (1). A mixture of Ac-L-Phe-OEt (20 g, 86 mmol), chloromethyl methyl ether (100 mL, 1.3 mol), and $ZnCl_2$ (44 g, 320 mmol) was stirred for 50 h at 0 °C, and the reaction was monitored by TLC (solvent A). After evaporation of the ether, the oily residue was completely dried and dissolved in ethyl acetate (100 mL) and water (200 mL). The product was extracted with ethyl acetate (3 × 100 mL) and washed with saturated NaCl (2 × 100 mL), saturated $NaHCO_3$ (2 × 100 mL), and saturated NaCl again (2 × 100 mL). The organic layer was dried over $MgSO_4$ and concentrated, giving crystalline needles. They were filtered, washed with ethyl acetate, and dried: yield 76% (18.5 g); mp 93–95 °C (lit.[34] mp 92–95 °C); R_{fA} 0.66; retention time (rt) 66.2% B (solvents A and B described above, linear gradient of 5–95% B in 30 min); α_D (c = 1, EtOH) +20.4 (lit.[24] +20.0); ^1H NMR (DMSO-d_6) 4.80 ppm (s, 2H, CH_2Cl; lit. 4.50 ppm).[34]

H-L-Phe(*p*-CH_2Cl)-OH·HCl (2). Optimized conditions for the hydrolysis of 1 consisted of refluxing 1 (6.0 g, 21 mmol) in 12 N HCl (60 mL) for 4 h with monitoring of the reaction by analytical HPLC (gradient = 5–95% B in 30 min). The reaction mixture was concentrated, and the resulting white precipitate was filtered, washed with cold 6 N HCl, and dried to give 3.80 g (72% yield): R_{fB} 0.60 (lit.[29] 0.57); rt 25.6% B (solvents A and B described above, linear gradient of 5–95% B in 30 min); mp 180 °C dec.

H-L-Phe(*p*-CH_2SO_3H)-OH (3). Compound **2** (3.5 g, 14 mmol) was refluxed for 3 h in water (77 mL) in the presence of Na_2SO_3 (11.5 g) following the procedure of Gonzalez-Muniz et al.[29] The water was evaporated, and the resulting solid was filtered, washed with ethanol, and dried, giving 13.5 g of desired product and salts (R_{fC} 0.40 (lit. 0.30); rt 17.4% B (solvents A and B described above, linear gradients of 5–95% B in 30 min). This product was not purified and was used directly in the following step.

Fmoc-L-Phe(*p*-CH_2SO_3Na)-OH (4). To a solution of compound 3 (6.0 g) in water (120 mL, pH 9) was added, at 0 °C, a solution of Fmoc-OSu (4.2 g; 12.5 mmol) in dioxane (120 mL).[37] The resulting mixture was stirred for 30 min at 0 °C and then at room temperature overnight (the pH was maintained at 9 during the entire process by an automatic titrator). The dioxane was evaporated, the excess Fmoc-OSu was extracted with diethyl ether (3×), and the aqueous solution was acidified to pH 5 with 1 N HCl and lyophilized giving the crude product (10 g). It was dissolved in methanol, the insoluble material removed by filtration, and the methanolic solution was evaporated to dryness. The residue dissolved in water was washed with ethyl acetate (3×) and lyophilized again, giving 5.3 g of product (yield 88%). This lyophilized foam (3.5 g) was chromatographed on silica gel[52] using solvent D as eluent to give 0.8 g of a slightly yellow secondary fraction and 0.7 g of pure compound (white foam; R_{fD} 0.40; rt 61.6% B (solvents A and B described above, linear gradient of 5–95% B in 30 min); mp 184 °C dec; MS m/z 480.1 ion corresponding to the $[M + H]^+$ species (calculated monoisotopic mass for $[M]^{\cdot+}$ = 481.120 Da) present in the EI mass spectrum was selected as the precursor for the CID product ion scan. The 20 most intense fragment ions had masses which could be correlated with structures generated by considering either one or two bond cleavages from the neutral precursor molecule.

Peptide Synthesis. Peptides were synthesized manually by the solid-phase approach.[32,53]

t-Boc Strategy. A 0.5-g portion of MBHA (0.76 mM/g) was placed in a vessel for manual synthesis and washed with DCM, MeOH, 10% TEA in DCM, MeOH, and DCM (2 times each). The Boc-L-amino acids (1.2–2.5-fold excess) were coupled in DCM or mixtures of DCM/DMF in the presence of DIC (1 M in DCM) for 1–2 h. The Fmoc-L-Phe(*p*-CH_2SO_3Na)-OH (1.2-fold excess) and Boc-L-His(Tos)·DCHA (2.5-fold excess) were coupled using BOP (1 equiv of amino acid/equiv of BOP) in DMF in the presence of DIPEA, pH 9.[51] Where needed, the recoupling step was accomplished using BOP reagent. Washes included 2-propanol (containing 1% EDT), DCM, and MeOH, and the ninhydrin test[54] was performed to check the coupling and recoupling reactions: when slightly positive the amino group not coupled was acetylated with acetic anhydride for 10 min. The t-Boc deprotections were done using TFA/DCM (50:50), and TEA (10% in DCM) was used for neutralization of the peptide resins after deprotection.

Fmoc Strategy. Fmoc-L-amino acids were protected as follows: OtBu for Glu and Asp, Boc for Lys, tBu for Ser, Tyr and Thr, Trt for His. The Arg was protected with Mtr in the syntheses using 2,4-DMBA and Pmc when the TMBHA-resin was used. The other amino acids side chains were unprotected. The 2,4 DMBHA- and TMBHA-resins were placed in vessel for manual synthesis and washed with 20% piperidine in DMF, MeOH/DCM, and DCM (2 times each). The Fmoc-amino acids (2.5-fold excess) were coupled in DCM or DCM/DMF (1:1) in the presence of 1 M DIC in DCM for 1–2 h. The couplings of Fmoc-Asn-OH and Fmoc-Gln-OH were performed in the same conditions, but in the presence of HOBt (1 equiv/1 equiv of amino acid). Fmoc-L-Phe(*p*-CH_2SO_3Na)-OH was coupled by BOP for 1.5–2 h using a 1.2-fold excess). The monitoring of the couplings was done by ninhydrin test.[54] When strongly positive, the recouplings were accomplished in DMF in the presence of BOP and DIPEA, pH 9 for 1–1.5 h; when weakly positive the recoupling was done using the same conditions described above for DIC couplings, and when very slightly positive the remaining amino group was acetylated by acetic anhydride for 10 min. The Fmoc was removed by treatment of the Fmoc-peptide-resin with piperidine 20% in DMF (1 × 1 min, 1 × 10 min). Washes included MeOH, DMF, and DCM (2 times each). BOP was used exclusively for residues 7–15 in the synthesis of [Phe(*p*-CH_2SO_3Na)^{27},-Nle^{7,28,31},Nal^{30}]-CCK-33 on either resin. The chain elongation processes were interrupted several times for cleavage and analysis of the peptides synthesized on the resins (Figure 1); after coupling and washing some resin was taken, deprotected, washed as described above, checked by ninhydrin test, washed 2 times with MeOH, and dried to be cleaved with TFA as described below. The main portion of the resin was kept in the refrigerator during these operations.

HF Cleavages. [Phe(*p*-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33}. The protected peptide-resin (32 mg) was cleaved with anhydrous HF (3–5 mL) containing 3% anisole at 0 °C for 90 min. The HF was then removed in vacuo. The peptide was precipitated with anhydrous diethyl ether, filtered, and extracted from the resin with 0.1% TFA in 60% MeCN/water and lyophilized giving 6.8 mg of crude peptide. The peptide was analyzed by analytical HPLC using a linear gradient from 5 to 95% B in 30 min.

[Phe(*p*-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{20-33}. The resin (72 mg) was cleaved as described above in anhydrous HF (10 mL). The crude product (17 mg) was analyzed by HPLC using a linear gradient from 5 to 95% B in 30 min.

[Phe(*p*-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33} Synthesized by Fmoc Strategy. The protected peptide-resin (300 mg) was cleaved with anhydrous HF (5–10 mL) in the presence of 3% anisole as described above to give 20 mg of crude peptide. It was purified on a semipreparative C_{18} column loading 4.5 mg at a time and using a gradient elution from 40% to 80% MeCN in 0.1% TFA in 40 min. The fractions were analyzed by HPLC under isocratic conditions, and the pure peptide fractions were pooled and lyophilized. The powder (1.7 mg) had its purity evaluated by RP-HPLC using a different solvent system and found to be 94%. The amino acid analyses gave the following results: Asp 2.05 (2), Gly 1.05 (1), Nle 2.00 (2), Phe 1.05 (1), Nal 0.61 (1), Phe(*p*-CH_2SO_3Na) was not detected in our present system; LSIMS calcd $[M + H]^+$ = 1116.47, obsd m/z 1116.5.

TFA Cleavages on an Analytical Scale. After the Fmoc deprotection step or when some resin was taken out for characterization during the process of chain elongation, the dry resin (50 mg) was treated with 0.5 mL of reagent K (TFA/thioanisole/water/phenol/ethanedithiol: (83/5/5/5/2.5)[41] for 30 min and 3 h (CCK_{26-33} fragments on TMBHA and 2,4-DMBHA, respectively), 2.5 and 4 h (CCK_{20-33} and CCK_{10-33} fragments on TMBHA, respectively), 6 h (CCK_{20-33} to CCK_{10-33} fragments on 2,4-DMBHA and CCK-33 analogue on TMBHA), and 8 h (CCK-33 analogue on 2,4-DMBHA) (Figure 1). When the reaction was completed, the peptide was precipitated with *tert*-butyl methyl ether (3 × 4 mL), and the mixture was centrifuged and decanted. The solid residue containing the

peptide and the resin was dried under N_2, the peptide was extracted with 0.5 mL of 0.1% TFA in 60% MeCN/water, and the solution was analyzed by RP-HPLC using a linear gradient from 5 to 95% B in 30 min. The main component was collected, lyophilized, and analyzed by MS to check if it corresponded to the desired peptide.

TFA Cleavages on a Semipreparative Scale and Purification of Human [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33. 2,4-DMBHA. The protected peptide-resin (450 mg) was cleaved in TFA (37.5 mL), thioanisole (2.3 mL), water (2.3 mL), liquefied phenol (2.3 mL), and EDT (1.15 mL) for 8 h at room temperature. The solution was split in three different centrifuge tubes (50 mL) and was precipitated with *tert*-butyl methyl ether (3 × 30 mL in each tube), and the suspension was centrifuged and decanted. The solid residues containing the peptide and the resin were suspended in 60% MeCN in 0.1% TFA/water and filtered. The solution was lyophilized to yield 86 mg of crude peptide. The purification was achieved in two steps:

1-FPLC Step. The crude peptide (two runs, 36 and 50 mg each) was dissolved in a mixture of 0.1% TFA in 60% MeCN/water and buffer A (pH 8.7) in 3 and 4 mL, respectively, and applied to a Mono HR S 10/10 column and eluted (4 mL/min) using a gradient of NaCl in 50 mM borate pH 8.7 from 0 to 0.125 M NaCl in 160 mL. The peptide eluted at an approximate concentration of 0.05 M NaCl. The fractions containing the desired peptide as determined by analytical HPLC under isocratic conditions (2 mL/min, 61% B, rt = 2.5 min) were pooled.

2-HPLC Step. The pool of fractions was loaded on a semipreparative C_{18} column and eluted with a gradient of 0.1% TFA in 60% acetonitrile/water from 20 to 80% in 40 min. The fractions containing the pure peptide and eluting at approximately 68% B were pooled after isocratic HPLC analysis and lyophilized giving 2.5 mg of powder purity {by RP-HPLC = 99%, (TEAP, 2.25, 40–70% B in 30 min, retention time 16.7 min at 2.0 mL/min); by CZE = 98%, 16.8-min migration time}. When this peptide was analyzed by LSIMS and amino acid analysis the following results were obtained: calcd $(M + H)^+$ = 3899.00, obsd m/z 3899.1; Asp 6.33 (6), Ser 3.94 (4), Glu 1.27 (1), Pro 2.03 (2), Gly 2.50 (2), Ala 1.03 (1), Val 0.62 (1), Ile 1.52 (2), Leu 2.00 (2), Nle 2.93 (3), Phe 1.04 (1), Lys 2.37 (2), His 1.06 (1), Arg 2.81 (3); Nal (1) and Phe(p-CH_2SO_3Na) (1) were not detected.

TMBHA. The protected peptide-resin (550 mg) was cleaved with 41.5 mL of TFA, 2.5 mL of thioanisole, 2.5 mL of water, 2.5 mL of liquefied phenol, and 1.25 mL of EDT for 7.5 h at room temperature. When the reaction was completed, it was worked up as described above; 129 mg of crude peptide was obtained. It was purified using the same procedure described above and used for the crude peptide cleaved from the 2,4-DMBHA: two FPLC runs (50 and 51 mg) were followed by two semipreparative HPLC desalting of the good fractions using the 0.1% TFA buffer (10–90% B in 30 min, elution time of ca. 22 min). The best fractions were pooled and lyophilized to give 2.8 mg of a white powder. Purity was 94% by CZE (migration time = 16.5 min) and 89% by RP-HPLC (TEAP 2.25, same gradient as above, rt = 17.7 min): LSIMS calcd $(M + H)^+$ = 3899.00, obsd = 3899.2; amino acid analysis Asp 5.09 (6), Ser 3.71 (4), Glu 1.00 (1), Pro 2.15 (2), Gly 2.13 (2), Ala 0.91 (1), Val 0.55 (1), Ile 1.52 (2), Leu 2.00 (2), Nle 3.00 (3), Phe 1.06 (1), Lys 2.16 (2), His 0.88 (1), Arg 2.83 (3) (1), Nal 0.72 (1); Phe(p-CH_2SO_3Na) was not detected in our present system. We also have tried to purify the crude preparation by preparative RP-HPLC (two runs in TEAP 2.25 followed by TFA desalting; see the Experimental Section). The purity of the final peptide was high (RP-HPLC, 98%; CZE, 98%), but the final amount of peptide was less than 1 mg.

Peptide Sequencing. A sequence analysis of [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 was performed at a level of 500 pmol. A preview of about 3% was seen starting at residue 5 of the sequencing and remained stable for the duration of the analysis which extended to residue 32 with a tentative assignment for Phe as residue 33. The repetitive yield was in the order of 91–92% throughout the analysis. The limit of detection was about 2 pmol. This analysis confirmed the fact that the desired sequence had been assembled correctly for at least the 32 last residues assembled on the resin. Phe(p-CH_2SO_3Na) and Nal at positions 27 and 30 could not be assigned.

In summary, all analytical data confirmed the identity and high degree of purity of this preparation of 1.

Peptide Aliquots. The pure and characterized peptides [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK_{26-33} (555 μg) and [Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33 (575 μg) were dissolved in 1 mL of water. To the solutions was added 2 mg of bovine serum albumin (BSA), and aliquots of 50 μL were taken in plastic tubes. Those were frozen, lyophilized, and stored in a freezer in the presence of dessicant.

Biological Assays. The bioactivities of the purified CCK_{26-33} and CCK-33 analogues were evaluated according to the method described by Liddle.[49] In this procedure, the biological activity of the CCK peptides are determined by their ability to stimulate amylase release from isolated rat pancreatic acini.[46]

Conclusions

The strategy we developed for the synthesis of Fmoc-Phe(p-CH_2SO_3Na)-OH is a shorter and alternative way to that described in the literature.[29] CCK_{26-33} and CCK-33 are new analogues containing this amino acid derivative. Nle and 2-Nal, are expected to be very stable in the conditions usually employed for the synthesis, final cleavage/full deprotection, and purification of peptides. Phe(p-CH_2SO_3Na)^{27},Nle^{28,31},Nal^{30}]-CCK_{26-33} can be easily synthesized by both Boc and Fmoc approaches and appeared to be stable when exposed to HF and the RP-HPLC purification conditions. For the synthesis of Phe-(p-CH_2SO_3Na)^{27},Nle^{7,28,31},Nal^{30}]-CCK-33, however, the Fmoc strategy seemed more applicable. The resins 2,4-DMBHA and TMBHA are both useful for the synthesis of this peptide despite the very low final yields obtained. The crude preparations obtained from the cleavage of the peptides built up on both resins required extensive ion exchange as well as reverse phase based purifications. The high degree of purity of the final products allows us to conclude that the scheme presented here has considerable promise. The use of LSIMS for the characterization of partially purified preparations during the optimization steps proved extremely valuable. The peptides were tested for their ability to stimulate amylase release from isolated rat pancreatic acini. In this assay, [Phe(p-CH_2SO_3Na)^{27},-Nle^{28,31},Nal^{30}]-CCK_{26-33} and Phe(p-CH_2SO_3Na)^{27},Nle^{7,28,31},-Nal^{30}]-CCK-33 were 10 and 30 times less potent than CCK-8, respectively. The data presented here were encouraging enough to warrant an attempt at the synthesis of the corresponding CCK_{20-58} and CCK-58 analogues (Miranda et al., 1993, manuscript in preparation).

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