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Synthesis and Characterization of a Sulfated and a Non-sulfated Cyclic CCK8 Analogue Functionalized with a Chelating Group for Metal Labelling

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Abstract: Two cyclic peptides, cyclo^{29,34}[Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 (**1**) and cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 (**2**), bearing the chelating moiety DTPA-Glu covalently bound to the Lys side chain have been synthesized by solid-phase methodology. The presence in compound **2** of many acidic functions characteristic of the chelating agent increases the lability of the sulfate group on the Tyr side chain. This finding suggests that prolonged acid treatments should be avoided during the preparation of such peptides. Sulfation of cyclo^{29,34}[Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 was performed using a pyridine–SO_3 complex as reagent. This reaction has been found to be the most suitable synthetic strategy for obtaining compound **2** in good yield. Cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 is a new promising CCK8 analogue, able to coordinate radioactive isotopes of metal ions such as ^{111}In(III), and to bind, in a selective way, the CCK_A-R receptor.

Keywords: CCK8 analogues; CCK8 chelating derivative; solid-phase synthesis; sulfated peptides

INTRODUCTION

Small radiolabelled compounds such as peptides are very attractive tools for the diagnosis of several different pathologies [1]. Among the possible biological targets for radiolabelled compounds, the cholecystokinin receptors CCK_A-R and CCK_B-R are very promising, due to their overexpression in many tumours: CCK_A-R is overexpressed in pancreatic cancer, while CCK_B-R is found in small cell lung cancer, colon and gastric cancers, medullary thyroid carcinomas, astrocytomas and stromal ovarian tumours [2].

These receptors belong to the GPCR superfamily and are localized in the cell membrane. Both CCK_A-R and CCK_B-R have been thoroughly investigated with the aim of characterizing the molecular basis of their interaction with the CCK peptide hormone [3–5]. Most of the studies focus on the binding mode of the *C*-terminal cholecystokinin octapeptide amide (CCK 26–33 or CCK8) that displays high affinity for both receptors, even if the sulfated form of CCK8 (with a sulfate group on Tyr27 sidechain) is 1000-fold more active than the non-sulfated CCK8 in binding to CCK_A-R. A detailed characterization of the interaction between CCK8 and the receptors is crucial for the development of CCK8 derivatives

Abbreviations: Boc, *tert*-butoxycarbonyl; CCK, cholecystokinin; CCK8, *C*-terminal octapeptide of cholecystokinin; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)-3-methyl-butyl; DIPEA, di-*iso*-propylethylamine; DMF, *N*,*N*-dimethylformamide; Dpr, α,β-diaminopropionic acid; EDT 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G-protein coupled receptor; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; PyBop, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium; tBu, *tert*-butyl; TFA, trifluoroacetic acid.

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bearing chelating agents able to coordinate, with high stability, radioactive metals for applications in cancer diagnosis by nuclear medicine techniques. The structural characterization of the bimolecular complex of CCK8 with the 47-residue *N*-terminal extracellular arm of CCK_A-R has recently been achieved by high-resolution NMR and computational refinement [6,7]. The NMR 3D-structure of the complex allowed us to design and synthesize the CCK8 cyclic analogue: cyclo^{29,34}[Dpr^{29}, Lys^{34}]-CCK8 and cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}]-CCK8. Preliminary biological studies confirmed the binding ability of the sulfated derivative toward the CCK_A receptor [8]. In these peptides there are two functional groups that are good candidates for binding a chelating agent, in order to obtain peptide conjugates able to coordinate radionuclides for diagnostic applications. Molecular modelling studies [8] indicate that the presence of a chelating agent on the N^{α}-terminal group or on the Lys side chain should not interfere in the peptide–receptor interaction. This paper describes the synthesis of the two cyclic peptides carrying the chelating moiety DTPA-Glu covalently bound to the Lys side chain (Figure 1). The DTPA-Glu chelating agent has been recently used to give *in vitro* and *in vivo* stable complexes with the radioactive isotope ^{111}In(III) [9]. The preparation of a peptide containing, within the

H-Asp^{26}-Tyr^{27}-Met^{28}-Gly^{29}-Trp^{30}-Met^{31}-Asp^{32}-Phe^{33}-NH_2

CCK8

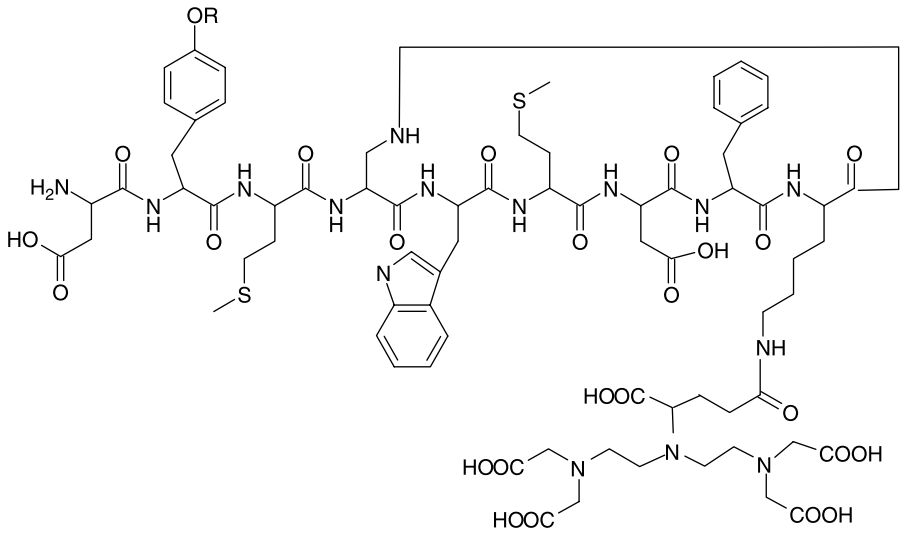
H-Asp-Tyr-Met-Dpr-Trp-Met-Asp-Phe-Lys(DTPA-Glu)

1 Cyclo^{29,34}[Dpr^{29},Lys^{34}(DTPA-Glu)]-CCK8

H-Asp-Tyr(SO_3H)-Met-Dpr-Trp-Met-Asp-Phe-Lys(DTPA-Glu)

2 Cyclo^{29,34}[Tyr^{27}(SO_3H),Dpr^{29},Lys^{34}(DTPA-Glu)]-CCK8

(a)



R = H **1**
R = SO_3H **2**

(b)

Figure 1 (a) Amino acid sequence of CCK8 and the cyclic analogues in their free (**1**) or sulfated (**2**) form containing the chelating agent DTPA-Glu on the Lys^{34} side-chain. The numbering scheme 26 → 33 follows that of the full 33-residue long CCK peptide. CCK8 is the *C*-terminal segment. (b) Chemical formulas of compounds **1** and **2**.

same molecule, the acid-labile sulfate group, the chelating moiety and the N ↔ C cyclization, is a difficult challenge in solid-phase peptide synthesis. Therefore, different synthetic procedures have been developed to obtain the desired molecule in good yield.

MATERIALS AND METHODS

PyBOP, HOBt, all Fmoc-amino acid derivatives and the 2-chlorotritylchloride resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). The DTPA-Glu pentaester, the chelating agent fully protected by tBu groups on its carboxyl functions with the exception of the carboxyl function on the Glu side chain, *N*,*N*-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]-amino]ethyl]-L-glutamic acid 1-(1,1-dimethylethyl)ester, was provided by Bracco Imaging SpA (Milan, Italy) and was used without further purification. For its synthesis the reader is referred to published methods [10]. All other chemicals were obtained by Aldrich (St Louis, MI), Fluka (Milwaukee, WI) or LabScan (Stillorgan, Dublin, Ireland) and were used without further purification, unless otherwise stated. Solid-phase peptide synthesis was performed on a fully automated Shimadzu (Kyoto, Japan) SPPS-8 synthesizer. Analytical RP-HPLC runs were carried out on a Shimadzu model 10A-LC apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6 × 250 mm. Preparative RP-HPLC was carried out on a Waters (Milford, MA) Delta Prep 4000 apparatus equipped with an UV lambda-Max 481 detector using a Vydac (Hesperia, CA) C18 column, 22 × 250 mm. Mass spectra were carried out on a MALDI-TOF Voyager-DE Perseptive Biosystem (Framingham, MA) apparatus using α-cyano-4-hydroxycinnamic acid as the matrix. ^{1}H-NMR experiments were carried out on a Varian (Palo Alto, CA) 600 MHz spectrometer in the H_2O/D_2O 5 : 95% mixture at 1.5 mM concentration. Chemical shifts were referenced to external TMS.

Cyclo^{29,34}(Dpr^{29}, Lys^{34} (DTPA-Glu))-CCK8 (1)

This compound was prepared by solid-phase methods using the standard Fmoc procedure. The first amino acid derivative, Dde-Lys(Fmoc)-OH was coupled to the 2-chlorotrityl chloride resin (1.04 mmol/g substitution; 0.100 g resin) under basic conditions (4 equiv. of DIPEA) in CH_2Cl_2. In order to estimate the resin loading obtained by attachment of the first amino acid derivative, an absorbance reading of the Fmoc protecting group was performed. The value found corresponded to 0.82 mmol/g loading (0.082 mmol scale). After removal of the Fmoc protecting group with a 20% solution of piperidine in DMF, the chelating agent (DTPA-Glu-pentaester) was linked, through its free carboxyl function, to the ε-NH_2 of the Lys residue. This coupling step was performed manually using 2.0 equiv. of DTPA-Glu-pentaester and HATU, and 4 equiv. of DIPEA. DMF was used as solvent. The coupling time, compared with the classical solid-phase peptide synthesis protocol, was increased to 2 h and completion of the reaction was checked by the ninhydrin test [11]. Successively, the substituted resin was transferred to the automatic synthesizer and the Dde protecting group onto the Lys α-NH_2 function was removed by a 2% solution of hydrazine in DMF. The peptide chain was elongated by sequential coupling and Fmoc deprotection of the following Fmoc-amino acid derivatives: Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Dpr(Mtt)-OH, Fmoc-Met-OH and Fmoc-Tyr(tBu)-OH. The amino acid derivative for the final coupling was Boc-Asp(OtBu)-OH. All couplings were performed twice for 2 h, by using an excess of 4 equiv. for each amino acid derivative. The α-amino acids were activated *in situ* by the standard HOBt/PyBOP/DIPEA procedure. DMF was used as a solvent. The linear peptide was cleaved from the solid support and the Dpr β-NH_2 function was simultaneously freed from its protecting group (Mtt) by suspending the resin in 1% solution of TFA in CH_2Cl_2 with stirring for 1 min. The resin was then filtered and the filtrate poured into a 10% solution of pyridine in CH_3OH. This procedure was repeated 10 times.

After the filtrate mixture was concentrated under reduced pressure, the crude product was isolated by precipitation into cold water. The precipitate was collected by centrifugation and dried *in vacuo* (over P_2O_5 pellets).

The protected conjugate was analysed by MALDI-TOF mass spectroscopy: $[M - H]^- = 2323$ (calcd = 2321). Cyclization, by amide bond formation between the *C*-terminal carboxylic group and the Dpr side-chain amino group, was obtained in CH_2Cl_2 by using PyBOP/DIPEA *C*-activation with stirring for 5 h. After removal of solvent under reduced pressure, the crude compound was suspended in TFA/H_2O/EDT (94 : 4 : 2) for 3 h. This procedure allowed both the chelating and the peptide moieties to be freed from all of the protecting groups. The

solution was then concentrated and the crude product isolated by precipitation into cold diethyl ether. The precipitate was collected by centrifugation and dried *in vacuo* (over KOH pellets).

The crude product was analysed and purified by using RP-HPLC. The system solvent used both for analytical and preparative HPLCs was: H_2O 0.1% TFA (A) and CH_3CN 0.1% TFA (B), linear gradient from 20% to 80% B in 20 min.

The main peak, eluted at R_t of 16.2 min of the analytical chromatogram, was confirmed by mass spectral analysis $[M - H]^- = 1651$ (calcd = 1651) to be the desired compound. After lyophilization 25 mg of 95% pure cyclo^{29,34}[Dpr^{29}, Lys^{34} (DTPA-Glu)]-CCK8, **1**, was isolated, corresponding to a final yield of 20%.

Cyclo^{29,34}(Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34} (Glu-DTPA))-CCK8 (2)

The same procedure described above for the synthesis of **1**, with only minor variations, was used to prepare the sulfated derivative. The synthesis was performed on 0.100 g of resin (1.04 mmol/g substitution). After coupling of the first amino acid derivative the resin loading, by Fmoc test estimation, turned out to be 0.82 mmol/g, corresponding to a 0.082 mmol scale. During peptide assembling on solid phase Fmoc-Tyr(SO_3H)-OH barium salt was incorporated into the peptide chain in order to obtain a sulfated Tyr-containing compound.

Analysis of the fully protected linear conjugate as well as the cyclic analogue, carried out by MALDI-TOF mass spectroscopy ($[M - H]^- = 2343$ and 2325, respectively; calcd = 2343 and 2325) confirmed the identity of the products. For the final deprotection step, the crude product was treated with TFA/H_2O/2-methylindole/*m*-cresol (87 : 10 : 2 : 1) by stirring the mixture overnight at 4 °C [12]. The crude product was analysed and purified using RP-HPLC. The solvent system used both for analytical and preparative HPLC was: H_2O 0.1 M $AcONH_4$ (A) and CH_3CN (B), linear gradient from 5% to 70% B in 30 min. Two products were isolated by HPLC: the compound eluted at 20.5 min was identified by MALDI-TOF analysis ($[M - H]^- = 1651$) as cyclo^{29,34}[Dpr^{29}, Lys^{34} (Glu-DTPA)]-CCK8. After lyophilization 10 mg of this compound was isolated corresponding to a 7% yield. The compound eluted at 19.8 min was identified by MALDI-TOF analysis ($[M - H]^- = 1731$; calcd = 1731) as the desired compound cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34} (Glu-DTPA)]-CCK8. After lyophilization less than 1 mg of this compound was isolated, which corresponded to a negligible yield of the desired product.

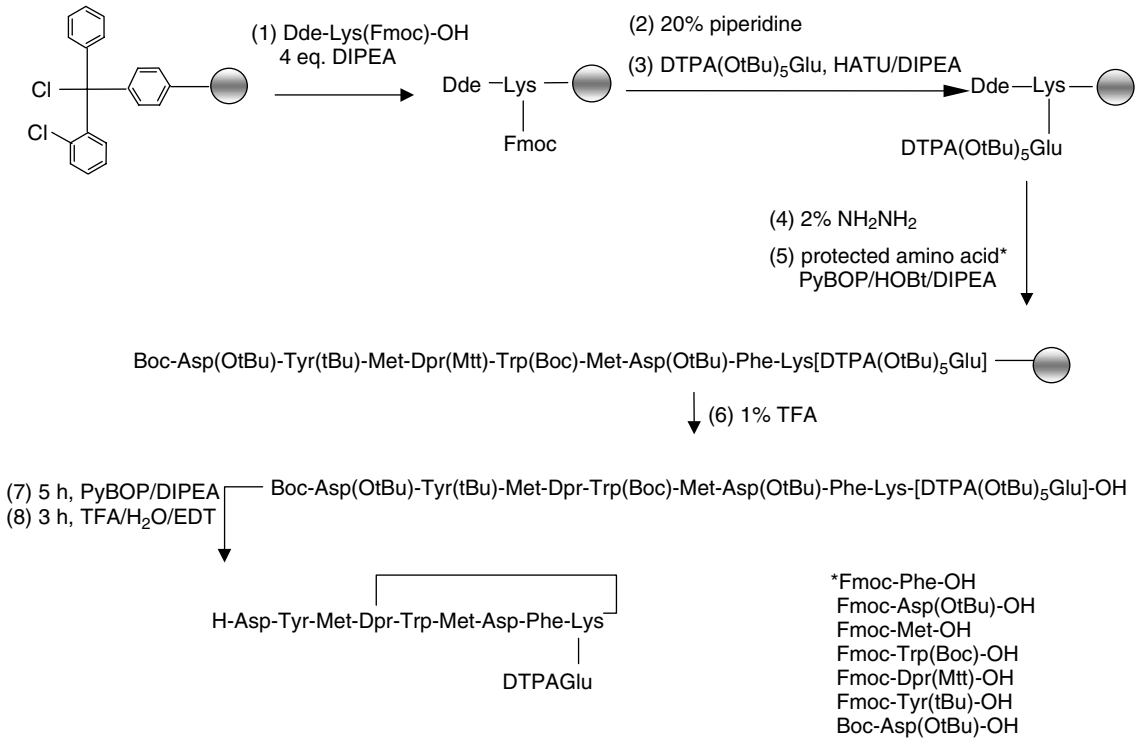
Cyclo^{29,34}(Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34} (Glu-DTPA))-CCK8 (2) by the Sulfation Reaction

The fully deprotected cyclic conjugate cyclo^{29,34}-[Tyr^{27}, Dpr^{29}, Lys^{34} (Glu-DTPA)]-CCK8, **1**, (10 mg) was suspended in a small amount (0.750 ml) of DMF/dry pyridine (2 : 1) and a large excess (39 mg, 40 equiv.) of pyridine–SO_3 complex was added. After stirring the mixture at room temperature overnight, the solvent was partially removed under reduced pressure, and a 2% aqueous solution of $NaHCO_3$ was added. Stirring was continued for 30 min in order to hydrolyse and neutralize the excess of the complex pyridine–SO_3 and to form the less sensitive sodium sulfate salt. Successively, the solution was acidified to pH 6.0 by addition of 1 M aqueous solution of $KHSO_4$, then analysed and purified using RP-HPLC. The solvent system used both for analytical and preparative HPLC was: H_2O 0.1M $AcONH_4$ (A) and CH_3CN (B), linear gradient from 5% to 70% B in 30 min. The main peak, eluted at R_t of 19.8 min of the analytical chromatogram, was confirmed by mass spectral analysis $[M - H]^- = 1731$ (calcd = 1731) to be the desired compound. After lyophilization 2.0 mg of 95% pure cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34} (Glu-DTPA)]-CCK8, **2**, was isolated, corresponding to a final yield of 20%.

RESULTS AND DISCUSSION

Synthesis of the Peptide Conjugates Cyclo^{29,34}(Dpr^{29}, Lys^{34} (DTPA-Glu))-CCK8 (1) and Cyclo^{29,34}(Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu))-CCK8 (2) by Solid-Phase Synthesis

The peptide conjugate cyclo^{29,34}[Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8, **1**, was synthesized on solid-phase using Fmoc chemistry and orthogonal protections on the two residues (Lys^{34} and Dpr^{29}) each containing two amino functions. The full synthetic strategy is reported in Scheme 1. The coupling of the DTPA-Glu pentaester, the chelating agent partially protected by *tert*-butyl groups, was very efficient: only 2 equiv of the DTPA-Glu-pentaester, activated by HATU, was used to obtain a quantitative Lys side-chain coupling. Then, the Dde protecting group onto the Lys α-NH_2 function was removed by a 2%



Scheme 1

solution of hydrazine in DMF and the peptide chain was elongated by sequential coupling and Fmoc deprotection. The amino acid derivative for the final coupling was Boc-Asp(OtBu)-OH.

After cleavage from the resin, the crude product was isolated by precipitation into cold water. The linear peptide bearing all the protecting groups on the amino acid side chains and the Boc group on the Asp ***N***-terminal group was analysed by mass spectrometry: the molecular weight corresponded to that expected. Cyclization between the carboxylic terminal function and the Dpr β-NH_2 group was obtained in DCM by using the PyBOP/DIPEA *C*-activation procedure. In the final step the cyclic peptide was suspended in TFA/H_2O/EDT in order to remove all the protecting groups both from the chelating carboxylic functions and from the peptide side-chain and *N*-terminal functions. The crude product was purified by RP-HPLC. After lyophilization 25 mg of 95% pure cyclo^{29,34}[Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8, **1**, was isolated, corresponding to a final yield of 20%.

The high yield obtained for the synthesis of cyclo^{29,34}[Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8, as well as the successful synthesis of cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}]-CCK8 previously obtained [8] by using the same synthetic scheme and Fmoc-Tyr(SO_3H)-OH instead of Fmoc-Tyr(OtBu)-OH, suggested to us that the peptide derivative Cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8, **2**, could be obtained by using the same synthetic strategy (Scheme 1) and incorporating the sulfated tyrosine derivative during the solid-phase peptide assembly. Unfortunately, at the end of the synthesis only a negligible yield of the desired product was obtained. The main HPLC product was identified as the cyclic peptide derivative without the acid-labile sulfate group on the Tyr side chain. By carefully checking all synthetic steps it was proven that the fully protected cyclic peptide derivative still maintained the sulfate group. Indeed, the mass spectrum of the fully protected cyclic peptide derivative showed, as the most abundant signal, the peak corresponding to the peptide derivative containing the sulfate group. Therefore, the sulfate group should be lost during the final deprotection step under acidic conditions. It has been reported that the acid-lability of the O-sulfated peptides is due to the proton-catalysed desulfation of the aryl monosulfate moiety [13]. It has also been predicted that the unstable amphoteric ion [ArO^+ (H)-SO_3^-], which rapidly decomposes to Ar-OH and SO_3, can be stabilized by

forming an acid-base pair both with intramolecular and intermolecular cationic functions [13].

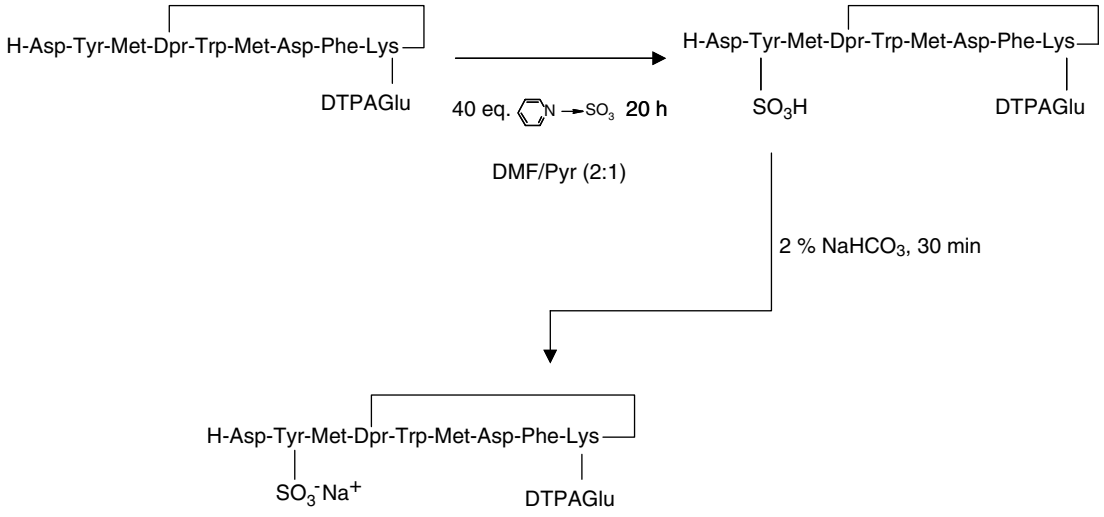
It is worth noting that the peptide chain in cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}]-CCK8 lacks basic functions except the α-amino group of the *N*-terminal Asp residue. Instead, in its structure there is a high number of acidic functions: five carboxylic groups on the DTPA-Glu moiety and two carboxylic groups on the side chain of Asp residues. Our hypothesis is that the high instability of the sulfated Tyr residue would be due to the additional number of carboxylic acid groups brought by the chelating agent. After the acid treatment for the final deprotection step, highly protonated molecules are expected that desulfate *via* the undesired but unavoidable amphoteric ion formation. This means the intramolecular carboxylic groups themselves catalyse the desulfation process. One way of overcoming this problem might be to use an ordinarily protected Tyr derivative as a building block during the peptide chain synthesis and then to perform the sulfation reaction on the fully deprotected cyclo^{29,34}[Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 product. By using this strategy one can avoid an acid treatment of the sulfated peptide derivative as the deprotection step has already been carried out.

Synthesis of Peptide Conjugate Cyclo^{29,34}(Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu))-CCK8 (2) by the Sulfation Reaction

The sulfation of the Tyr side chain in peptide **1** was obtained according to Scheme 2. The fully deprotected cyclic conjugate cyclo^{29,34}[Tyr^{27}, Dpr^{29}, Lys^{34}(Glu-DTPA)]-CCK8, suspended in a small amount of the mixed solvent DMF/dry pyridine (2 : 1), was treated with a large excess (40 equiv.) of the pyridine–SO_3 complex. The crude product was analysed and purified using RP-HPLC. The main peak, eluted at R_t of 19.8 min of the analytical chromatogram, was confirmed by mass spectral analysis to be the desired compound. MALDI-TOF spectra were carried out using the negative ionization method to minimize the loss of the sulfate group during the mass spectral analysis [13]. After lyophilization 95% pure cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 was obtained in a final yield of 20%. Even if the mass spectrum shows the presence of a signal corresponding to the unsulfated peptide ($[M - H]^- = 1651$), by comparing the HPLC chromatogram of the unsulfated (**1**) and sulfated (**2**) peptide derivatives it is concluded that the peak at MW = 1651 is due to the loss of the sulfate group in the gaseous phase during the mass spectral analysis.

This result confirms the feasibility of this strategy in order to obtain a peptide containing in the same molecule the acid-labile sulfate group, the chelating moiety, and the N $\leftrightarrow$ C cyclization. By using this strategy the acid treatment of the sulfated peptide derivative was eliminated. In this way the desired compound was recovered in high purity and in good yield.

Compound **2** was also characterized by ^{1}H-NMR, carried out in the H_2O/D_2O 5 : 95% mixture at 1.5 mM concentration. Proton assignments were



Scheme 2

Table 1 ^{1}H NMR Data for Cyclo^{29,34}[Tyr^{27}(SO_3), Dpr^{29}, Lys^{34} (Glu-DTPA)]-CCK8 (**2**) in the H_2O/D_2O 5 : 95% Mixture at 1.5 mM Concentration

Residue	^{1}H Atom	δ(ppm)[a]
Asp^{26}	Hα	4.14
	Others	2.77/2.66(Hβ)
Tyr^{27}	HN	8.88
	Hα	4.53
	Others	2.99(Hβ), 7.24 (Hδ), 7.10(Hε)
Met^{28}	HN	8.28
	Hα	4.31
	Others	1.82(Hβ), 2.35/2.30(Hγ), 1.94(Hε)
Dpr^{29}	HN	8.06
	Hα	4.39
	Others	3.56(Hβ), 7.84(Hγ)
Trp^{30}	NH	8.16
	Hα	4.66
	Others	3.26(Hβ), 7.44(Hδ1), 7.77(Hε3), 10.22(Hε1), 7.31(Hζ3), 7.68(Hζ2), 7.40(Hη2)
Met^{31}	NH	8.02
	Hα	4.17
	Others	1.82/1.68(Hβ), 1.99/1.91(Hγ), 2.00(Hε)
Asp^{32}	NH	8.18
	Hα	4.29
	Others	2.62/2.65(Hβ)
Phe^{33}	NH	8.21
	Hα	4.36
	Others	3.22/3.19(Hβ), 7.41(Hδ1), 7.36(Hε1), 7.52(Hζ)
Lys^{34}	NH	7.96
	Hα	4.22
	Others	1.81/1.66(Hβ), 1.15(Hγ), 1.58(Hδ), 2.93 (Hε), 7.82 (NHε)
DTPAGlu	$\underline{CH_2}$-COOH	4.01
	NCH_2CH_2N	3.50–3.40
	Others	4.05, 3.20–3.30, 3.00–3.10

[a] Chemical shifts are referenced to external TMS.

obtained by comparison with the results of the ^{1}H-NMR study on cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}]-CCK8 previously obtained [8]. Chemical shifts are reported in Table 1.

Stability Tests of the Sulfate Moiety in Cyclo^{29,34}(Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu))-CCK8

Considering the highly acidic properties of compound **2** and in order to investigate the stability of the sulfate group on the Tyr side chain in the peptide conjugate **2**, several solutions of cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 were prepared at different pH values and the HPLC profiles and the mass spectra were followed as a function of time. Significant results are summarized in Figure 2. In buffer at pH 7 the compound showed a good stability as it did not decompose even up to 14 h. In water at pH 5.5, measured after dissolving the compound in pure water at 10^{-4} M concentration, a partial desulfation was observed after 2 h, while the sulfate loss was almost complete after 14 h. At pH below 4.0, obtained by adding hydrochloric acid, a complete loss of the sulfate group was observed within 0.5 h.

All these experiments clearly revealed how the proton-donating effect of the intramolecular acidic groups in compound **2** might easily induce formation of the amphoteric ion, which is required for the desulfation process. In particular, the synthetic step in which TFA is used to remove the side-chain protecting groups is critical for preserving the sulfate group in this molecule, despite the use of appropriate scavengers and fixing the temperature at 4 °C. On the other hand, it is known that other Tyr-sulfate peptides, such as the sulfate derivatives of linear CCK8 and of the cyclic compound cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}]-CCK8, do not desulfate during prolonged TFA treatment either in presence of water, 2-methylindole or *m*-cresol at 4 °C [8].

All these results support the already mentioned hypothesis that the chelating agent, bearing acidic functions, is responsible for the peculiar instability shown by the sulfated Tyr derivative in cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8.

CONCLUSIONS

A synthetic strategy has been developed for obtaining an N $\leftrightarrow$ C cyclic peptide containing in the same molecule the acid-labile sulfate group on a Tyr residue and a highly acidic chelating DTPA derivative. The presence of many acidic functions increases the lability of the sulfate moiety on the Tyr

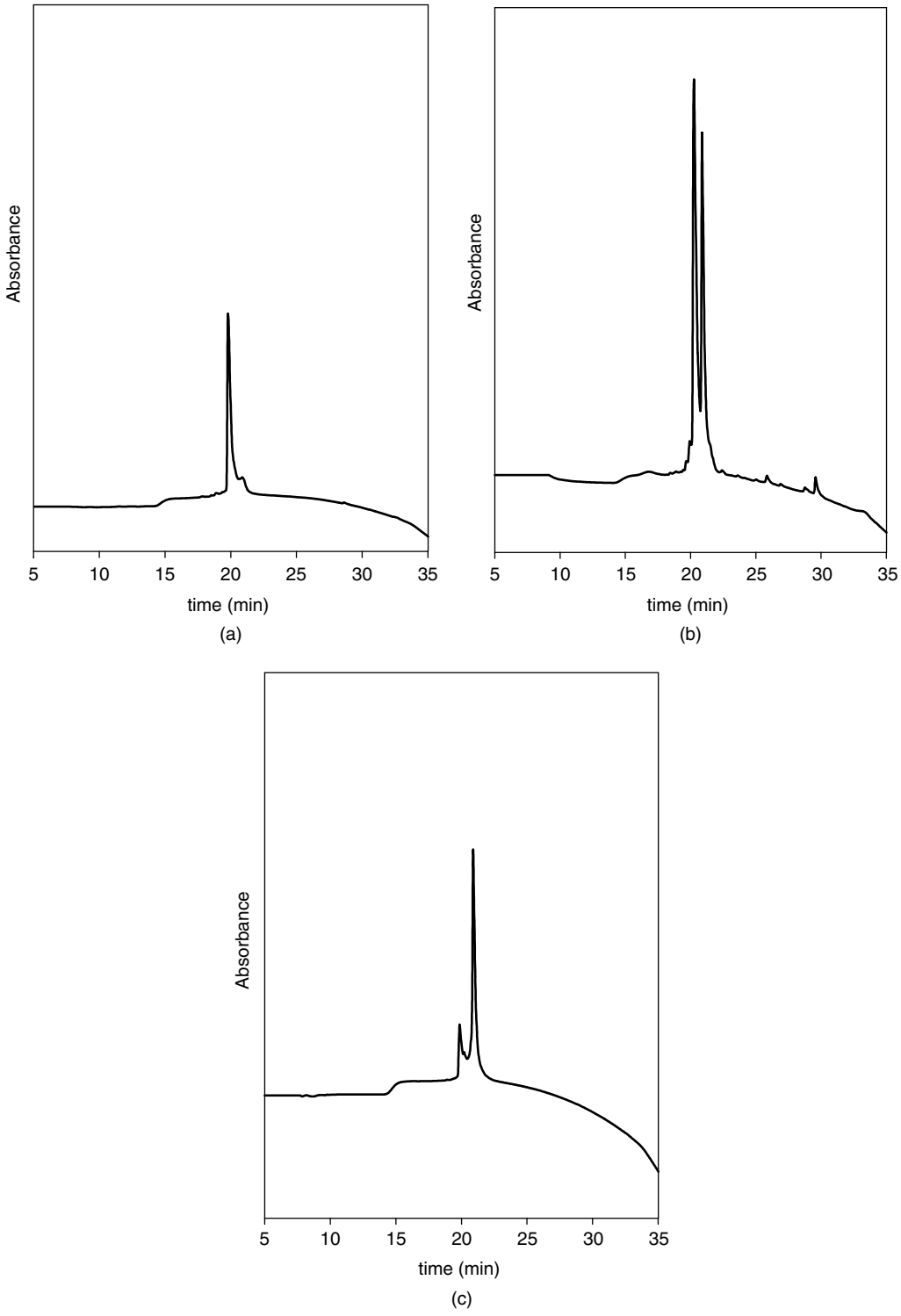


Figure 2 HPLC profiles of cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 in aqueous solutions at different pH values: (a) in buffer at pH = 7 after 14 h; (b) at pH = 5.5 after 2 h; (c) at pH = 5.5 after 14 h. The peak on the left (R_t = 19.8 min, mass value $[M - H]^-$ = 1731) corresponds to the sulfate derivative; the peak on the right (R_t = 20.5 min, mass value $[M - H]^-$ = 1651) corresponds to the unsulfated peptide. Solvent system: linear gradient of 0.1 M $AcONH_4$ (A) and CH_3CN (B), from 5% to 70% B in 30 min. Absorbance measured at 280 nm.

side chain suggesting that the best way to obtain such peptides in good yield is to avoid prolonged acid treatments. Therefore, the synthetic strategy, in which the Tyr side chain on the assembled peptide conjugate is sulfated by the pyridine–SO_3 complex, is the most suitable for the preparation of this new class of sulfated CCK8-analogue conjugates. Cyclo^{29,34}[Tyr^{27}(SO_3H), Dpr^{29}, Lys^{34}(DTPA-Glu)]-CCK8 is a new promising CCK8 analogue able to coordinate radioactive isotopes of metal ions such as ^{111}In(III) and to bind, in a selective way, the CCK_A-R receptor. Biological assays by nuclear medicine techniques to visualize cells overexpressing the CCK_A-R receptors are currently in progress.

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