SUMMARY

Use has been made of a modification of the method for the quantitative determination of cystine and cysteine in proteins with the aid of an amino acid analyzer after their oxidation to cysteic acid in the presence of 2% DMSO, which is faster and less laborious. The conversion of cystine and cysteine into cysteic acid under these conditions amounts to 97-100%. Because of the changes in some amino acids under the action of DMSO, the other amino acids must be determined from parallel samples obtained on hydrolysis in the absence of DMSO.

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SYNTHESIS OF THE HEXAPEPTIDE 11-16 OF THE NATURAL SEQUENCE OF HUMAN CALCITONIN

V. N. Karel'skii, E. P. Krysin
G. E. Rostovskaya, A. A. Antonov,
and M. B. Smirnov

UDC 547.953:665.37

A new variant of the preparative synthesis of hexapeptide 11-16 of the natural sequence of human calcitonin is described. In several of the stages 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline was used successfully as the condensing agent. The final and intermediate compounds were obtained with good yields in chromatographically homogeneous form. Their purity was checked by TLC and measurements of angles of optical rotation. The final product was identified additionally by ^{13}C NMR. Several physicochemical characteristics of the compounds synthesized (angles of optical rotation, chromatographic mobilities) are given.

Calcitonin is a 32-membered peptide which regulates the calcium and phosphorus metabolism in the human organism. Its synthesis is difficult in view of the fairly large set of amino acids, a number of which are polyfunctional. The synthesis is complicated in the choice of the tactically minimum protection of the amino acids. Nevertheless, such tactics permit a simplification of the scheme of synthesis and the use of cheap and more readily available compounds.

The aim of the present investigation was to synthesize the hexapeptide 11-16 of the natural sequence of human calcitonin in which the hydroxy groups of the hydroxyamino acids (threonine, tyrosine) are present in the unsubstituted state. The preparation of the fragment is usually performed from protected hydroxy amino acids [1] using dicyclohexylcarbodiimide (DCHC) as condensing agent or with the use of activated esters based on N-hydroxysuccinimide or p-nitrophenol. Rittel et al [2] have made wide use of the azide method of condensation. The process of obtaining the hexapeptide amounts to the successive growth of the peptide chain from the C-end beginning with phenylalanine or to the preparation of the tripeptide (11-13) and the dipeptide (15-16). Then glutamine tert-butoxycarbonylhydrazide is added

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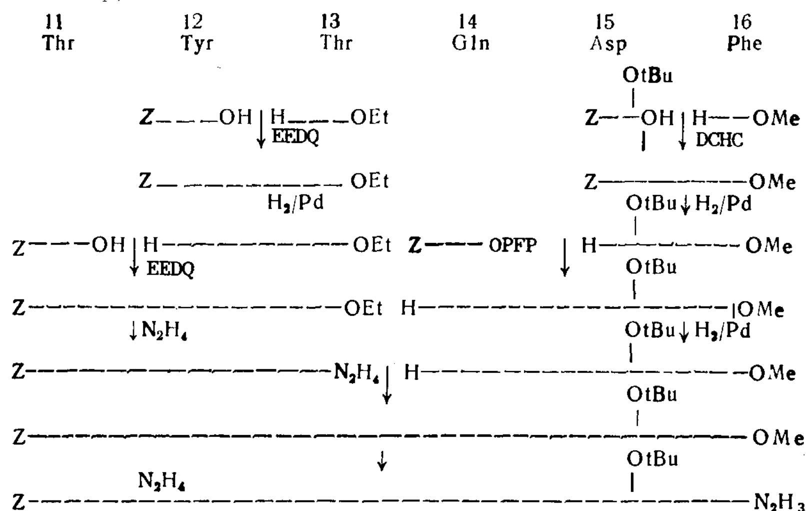
All-Union Scientific-Research Institute of the Technology of Blood Substitutes and Hormone Preparations, Moscow. Translated from Khimiya Prirodnykh Soedinenii. No. 4, pp. 555-558, July-August, 1985. Original article submitted October 29, 1984.

 0009-3130/85/2104-0520$09.50 © 1986 Plenum Publishing Corporation

by the azide method to the (11-13) fragment, and the dipeptide (15-16) to the (17-19) fragment, after which the tetrapeptide (11-14) is attached by azide condensation to the (15-19) fragment.

We have obtained this hexapeptide by the following scheme (Z represents the benzyloxycarbonyl group; OtBu the tert-butyl ester group; and OPFP the pentafluorophenyl ester group):



2-Ethoxy-N-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) is an extremely effective condensing agent in the case of such hydroxy acids as serine and tyrosine [3], and we therefore decided to use this compound in the preparation of the tripeptide (11-13) in place of DCHC and activated esters. The corresponding dipeptide (12-13) and tripeptide (11-13) were obtained in good yields and with a high degree of chromatographic purity. The dipeptide (15-16) was prepared in the usual way using DCHC. Glutamine was added to this with the aid of the pentafluorophenyl ester, instead of the p-nitrophenyl ester [1]. In the latter case, the reaction took place at a high rate, which is characteristic for the pentafluorophenyl esters, and the corresponding tripeptide (14-16) was isolated in the chromatographically homogenous state in fairly high yield. In parallel, an attempt was made to add N-benzyloxycarbonoylglutamine to the dipeptide (15-16) via the hydroxysuccinimide ester. However, the corresponding tripeptide was isolated in low yield (54%) and in poor quality (low chromatographic purity and 5° lower angle of optical rotation).

The tripeptide (11-13) was linked to the (14-16) fragment by azide condensation. It must be mentioned that the use of EEDQ and of DCHC at this stage did not give satisfactory results. Then the methyl ester obtained was converted by ordinary hydrazinolysis into the corresponidng hexapeptide hydrazide in high yield.

The products obtained were characterized by their angles of optical rotation and TLC behavior. The final hexapeptide was additionally analyzed by the ^{13}C NMR method.

EXPERIMENTAL

Angles of optical rotation were determined on a polarimeter, and chromatographic purities and mobilities were determined by the TLC method on silufol plates in the pyridine—acetic acid—water—ethyl acetate (2.5:0.7:1.4:30; ratio by volumes) system. The ^{13}C NMR spectra of solutions in hexadeuterodimethyl sulfoxide (c 100 mg/ml) were recorded on a WP-80DS spectrometer with a working frequency of 20.115 MHz. The recording conditions were similar to those given in [4]. The values of the chemical shifts of the signals from the C atoms of the amino acid residues in the spectra of solutions of the hexapeptide are given below (ppm relative to tetramethylsilane; pairs of signals marked a—a, b—b, are assigned alternatively):

The assignment of the signals was made on the basis of literature information [4, 6] and comparison with the spectra of the tripeptide ZThrTyrThrOEt. The positions of the signals of the C atoms of phenylalanine were confirmed by comparison with the spectrum of the tripeptide tritGlu(OtBu)His(trit)PheOEt:

Amino acid residue	C_0	C_α	C_β	C_γ	C_δ	C_ϵ	C_ξ
Thr-1	170,41	60,70	66,93	19,55			
Tyr	171,62	53,91	36,73 [b]	127,81	130,24	114,95	155,91
Thr-3	169,99 [a]	58 56	66,52	19,55			
Glu	171,07	52,37	27,72	31,50	174,21		
Asp	170,18 [a]	49,43	37,30 [b]	169,13			
Phe	171,62	54,28	36,50 [b]	137,00	128,42	129,13	126,69

All the amino acids used has the L configuration.

Amino acid residue	C_0	C_α	C_β	C_γ	C_δ	C_ϵ	C_ξ
Thr-1	170 60	60,58	66,96	19,54			
Tyr	171,78	53.86	36,75	127,83	130,38	114,97	155,98
Thr-2	169,99	58,14	66,91	20,18			

1. Preparation of ZtyrThroOEt. A solution of 12.1 g (66 mmole) of the hydrochloride of the ethyl ester of threonine in 100 ml of chloroform–methanol (3:1) system cooled to −10°C was treated with 10 ml (73 mmole) of triethylamine (TEA) and the mixture was allowed to stand for 20 min, after which 17.4 g (55 mmoles) of N-benzyloxycarbonyltyrosine in 20 ml of the system and 15 g (60 mmole) of EEDQ were added. The reaction mixture was stirred at −5°C for 1 h and was kept at 20-24°C for 24 h. The reaction products were washed with 1 N HCl, with H_2O, and with Na_2CO_3 (7%) and were dried and evaporated in vacuum. The dipeptide was recrystallized from methylene chloride. Yield 15.5 g (63% of theory). Chromatographically homogeneous; R 0,82—0,83; $[\alpha]_D^{20}$ −11,0° (c 1; MeOH).

2. Preparation of ZThrTyrThrOEt. With vigorous shaking, 10 g (24 mmole) of the ethyl ester of N-benzyloxycarbonyltyrosylthreonine in 160 ml of ethanol was reduced in a current of hydrogen at 18-20°C over palladium black (about 1 g) for 3 h (with monitoring by the TLC method). The catalyst was filtered off and the solvent was evaporated off in vacuum at a bath temperature not exceeding 40°C, after which the residue was dissolved in 90 ml of the chloroform–methanol (93:1) system and the dipeptide was condensed with N-benzyloxycarbonylthreonine (5.1 g, 20 mmole) in the presence of 5.4 g (22 mmole) of EEDQ by the method of paragraph 1 for 48 h. Yield 9 g (82% of theory); chromatographically homogeneous with R 0,66—0,69; $[\alpha]_D^{20}$ −25,0° (c 1; MeOH).

3. Preparation of ZThrTyrThrN_2H_3. The ethyl ester of N-benzyloxycarbonylthreonyltyrosylthreonine 8.9 g (16.3 mmole) in 150 ml of methanol − DMA (2:1) was treated with 9 ml of hydrazine hydrate. The mixture was kept at 18-20°C for 24 h and the hydrazide that had precipitated was washed with ether and dried in vacuum. Yield 7.5 g (84% of theory); chromatographically homogeneous, R_f 0.28-0.29 (with the addition of 1 ml of acetic acid to the system for better separation); $[\alpha]_D^{20}$ −6,0° (c 2 ; DMFA).

4. Preparation of ZAsp(OtBu)PheOMe. Using the method described by Rittel and by Nozaki [2], 10 g (19.8 mmole) of the DCHA salt of the β-tert-butyl ester of N-benzyloxycarbonyl-aspartic acid and 5.6 g (25.7 mmole) of the hydrochloride of the methyl ester of phenylalanine gave 8.8 g (92% of theory) of the product. Chromatographically homogeneous, R 0,88—0,89; $[\alpha]_D^{20}$ −15,0° (c 1; MeOH).

5. Preparation of ZglnAsp(OtBu)PheOMe. Using the method of paragraph 2, 4 g (8.2 mmole) of the β-tert-butyl ether of the benzyl ester of N-benzyloxycarbonylasparagylphenylalanine was reduced in solution in 60 ml of methanol with the addition of 1.7 ml of HCl in dioxane (0.19 g/ml). The solvent was driven off in vacuum, the residue was dissolved in a mixture of 5.5 g (12.4 mmole) of the pentafluorophenyl ester of N-benzyloxycarbonylglutamine [5] in 30 ml of of DMFA, the solution was cooled to 0°C, and 1.2 ml (9 mmole) of TEA was added and the reaction mixture was kept at 18-20°C for 24 h. The solvent was driven off with the aid of an oil pump and the residue was dissolved in the isopropanol–chloroform (1:2) system, the solution was then being washed with 1 N HCl, H_2O, and Na_2CO_3 solution. The organic layer was dried and the solvent was driven off in vacuum. The residue was triturated with diethyl ether. The product was recrystallized from methanol. Yield 3.8 g (76% of theory), chromatographically homogeneous, R 0,68 — 0,69; $[\alpha]_D^{20}$ −23,0° (c 1; DMFA).

6. Preparation of ZThrTyrThrGlnAsp(OtBu)PheOMe. Using the method of paragraph 2, 7.2 g (12 mmole) of the methyl ester of N-benzyloxycarbonyl-β-O-tert-butylasparaginylphenyl-

alanine was reduced in 100 ml of methanol in the presence of 1.7 ml of HCl in dioxane (0.27 g/ml). The solvent was driven off in vacuum and the residue was dissolved in 40 ml of DMFA. In parallel, 7.5 g (14 mmole) of the hydrazide of N-benzyloxycarbonylthreonyltyrosylthreonine was dissolved in 50 ml of DMFA and the solution was cooled to −2°C, and 5.7 ml in dioxane (0.27 g/ml) and 3.8 ml (28 mmole) of isoamyl nitrate were added successively and the mixture was stirred vigorously at −20 to −25°C for 15 min. Then, at −10°C, 9.6 ml of TEA and a cooled solution of the hydrochloride of the tripeptide (14-16) obtained above were added to the azide. The reaction was kept at 0°C for 96 h. The resulting precipitate was filtered off, and the solvent was driven off in vacuum. The residue was dissolved in 100 ml of the isopropanol–chloroform (1:1) system and the organic layer was washed successively with 1 N HCl, H_2O, Na_2CO_3 solution, and H_2O again. After drying, the solvent was driven off in vacuum and the residue was triturated with diethyl ether. The product was recrystallized from ethanol. Yield 7.7 g (66% of theory), chromatographically homogeneous, R_f 0.58-0.59 (with the addition of 1 ml of acetic acid to the system for better separation); $[\alpha]_D^{20}$ −11.0° (*c* 1; DMFA).

7. Preparation of $ZThrTyrThrGlnAsp(OtBu)PheN_2H_3$. A solution in 80 ml of DMFA – methanol (1:4) system of 7.9 g (8.1 mmole) of the peptide methyl ester obtained in paragraph 6 was added to 4 ml of hydrazine hydrate and the reaction mixture was stirred for 1 h and was then kept at 20-24°C for 10 h. The reaction products were washed on the filter with cold ethanol and diethyl ether and were dried in vacuum. Yield 7.4 g (94% of theory), chromatographically homogeneous R 0.43–0.44; $[\alpha]_D^{20}$ −17.0° (*c* ; DMFA), mp 196-198°C.

SUMMARY

The synthesis of the hexapeptide (11-16) of the natural sequence of human calcitonin has been performed using 2-ethoxy-N-ethoxycarbonyl-1,2-dihydroquinoline as the condensing agent in individual stages.

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