SYNTHESIS OF A DECAPEPTIDE WITH THE SEQUENCE 1–10 OF HUMAN CALCITONIN WITH MINIMUM PROTECTION OF THE AMINO ACIDS

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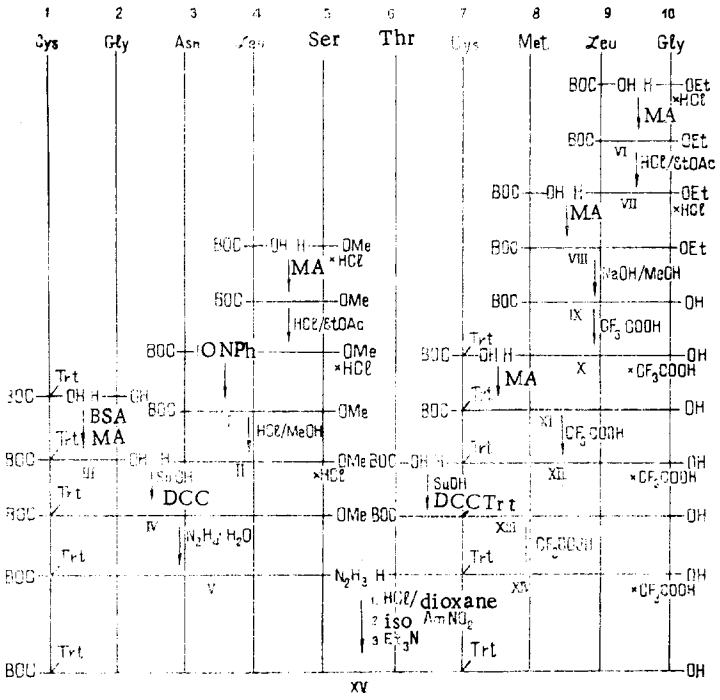
A decapeptide with sequence 1–10 of human calcitonin has been synthesized by a 5 + 5 scheme with the minimum protection of the side-chain functional groups of the amino acid.

Human calcitonin (CT) is a polypeptide consisting of 32 amino acid residues with a disulfide bond between the two cysteine residues in positions 1 and 7, and a prolinamide residue at the C-end [1]. In the preparation of CT as a medicament, a liquid-phase synthesis is used which permits the products to be purified at each stage. The synthesis of CT is carried out by the fragment condensation of appropriately protected {1–10} and {11–32} polypeptides of the human CT sequence by the carbodiimide method using 1-hydroxybenzotriazole [2] or by the activated ester method [3, 4], and also of {1–7} and {8–32} fragments by the azide method [5]. The decapeptide of the {1–10} sequence of CT is obtained both with protected [3, 4] and also with free [4] hydroxy groups of the serine and threonine residues by the condensation, using the azide method, of the {1–4} tetrapeptide with the {5–10} hexapeptide [3, 4] or of the {1–7} heptapeptide with the {8–10} tripeptide [2, 4, 6]. As a rule, schemes for obtaining this decapeptide include the synthesis of the dipeptide XSer(OR)-Thr(OR)OR', where X = a N-protecting group; R = H or tert-Bu; and R' = $PhCH_2$ or Me), the isolation of which is difficult because of its high solubility in water.



A scheme for obtaining the decapeptide with sequence 1–10 of human CT without the protection of the hydroxy groups of serine and threonine, consisting in the condensation of the pentapeptides BOCCys(Trt)-Gly-Asn-Leu-SerN_2H_3 (V) and HThr-Cys(Trt)-Met-Leu-GlyOH (XIV) by the azide method is shown above. The preparation of the polypeptide with the minimum protec-

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TABLE 1. Properties of the Peptides (I-XV) Obtained

Peptide	Yield, %	mp, °C	$[\alpha]_D^{20}$, deg. (c 1)	Solvent	R_f (system)
I	80	188—189	—40,5	DMFA	0,53 (3)*
II	98	164—168	—18	DMFA	0,3 (1)*
III	70,7	87—90	+14,5	MeOH	0,59 (2)
IV	59,8	149—150	—14	MeOH	0,35 (2), 0,5 (3)
V	82	197—198	—9	DMFA	0,6 (1), 0,11 (2), 0,3 (3)
VI	97	81—83	—24	MeOH	0,85 (3)
VII	95	—	+24	DMFA	0,42 (1)
VIII	92	102—105	—45	MeOH	0,78 (2), 0,63 (3)
IX	93,8	—	—45	MeOH	0,35 (2)
X	94	70—71	—11	MeOH	0,2 (1)
XI	61	133—136	—25,5	MeOH	0,51 (2)
XII	97,7	210—212 (decomp.)	±1	DMFA	0,06 (1)
XIII	92	203—205	—13	DMFA	0,4 (2)
XIV	93,5	>210 (decomp.)	—10	DMFA	0,35 (1)
XV	67,5	>205 (decomp.)	—11,5	DMFA	0,7 (1)

*For (I): mp 190°C $[\alpha]_D^{20}$ −24° (c 1; DMFA) [14]; for (II): mp 180°C $[\alpha]_D^{20}$ −23° (c 1; DMFA) [14].

tion of the side-chain functions of the amino acids makes it possible to use a simpler raw material; another important advantage of minimum protection consists in the easier elimination of a small amount of protective groupings in the production of the desired free peptide at the end of the synthesis. However, the preparation of the decpeptide CT_{1-10} with unprotected hydroxy groups of serine and threonine is complicated by the higher solubility of the intermediate peptides in water, which does not permit the reaction products to be separated from unchanged initial reactants. The use of the trityl group to protect the SH group of cysteine, which is eliminated by the action of I_2 in MeOH [7] raises the solubility of the intermediate products of the synthesis in organic solvents and makes it possible to perform purification by washings with acids and bases, while the free hydroxy groups of serine and threonone and also the free carboxy group of glycine substantially lower the solubility of the N-protected intermediates in ether, which permits the reaction products to be freed from unchanged BOCCys(Trt)OH by reprecipitation from MeOH into ether.

The tert-butoxycarbonyl group was used to protect the α-amino groups of cysteine, asparagine, leucine, threonine, and methionine and was eliminated by the action of CF_3COOH or HCl in EtOAc or MeOH. Methyl and ethyl ester groups, respectively, were used to protect the α-carboxy groups of serine and glycine, and these were subsequently hydrolyzed off with 3.7 N NaOH in MeOH at room temperature. Because of the fact that the dipeptide BOCCys(Trt)-Gly-OEt is not hydrolyzed under these conditions, apparently as the result of steric hindrance, for the temporary protection of the carboxy group we used N-bis(trimethylsilyl)acetamide [8]; the BOCCys(Trt)-GlyOSiMe$_3$ then formed underwent hydrolysis when the reaction mixture was washed with 0.1 N HCl.

In the preparation of the di-, tri-, and tetrapepetides we used the mixed-anhydride method with EtOCOCl (yield 60-95%) and in the preparation of the tri- and pentapeptides the activated (succinimide, p-nitrophenyl) ester method (yields 60-92%). The azide method was used for the condensation of the pentapeptides (V) and (XIV).

The peptides obtained were chromatographically homogeneous according to TLC on Silufol, and they were characterized by their melting points and angles of optical rotation (Table 1). The ^{13}C NMR method was used to identify the compounds obtained. The assignment of the signals in the spectra of the peptides was made on the basis of literature information [9-11] and in the light the spectra of a mixture of the deuterated and nondeuterated peptide (C=O signals of the C-terminal residues in the spectra of (I, XI, XIII, and XV) and of the nature of the splitting by spin—spin coupling in the spectra obtained in the gated decoupling regime (the glycine C=O signals in the spectra of (III, VIII, and X)). The values of the chemical shifts are given in Tables 2 and 3.

EXPERIMENTAL

The melting points of the peptides were determined in open capillaries without correction, and the angles of optical rotation on a polarimeter.

TABLE 2. Chemical Shifts in the ^{13}C Spectra of Peptides (I, III-V, and XV*)

Amino acid residue	Nucleus	I	III	IV	V	XV
Cys	C_0	—	170,33	170,52	170,42	170,50[b]
	C_α	—	53,47	53,57	53,53	53,53
	C_β	—	34,05	34,11	34,07	34,08
Gly	C_0	—	170,69	168,68	168,50	168,47
	C_α	—	40,87	42,28	42,17	†
Asn	C_0	172,64	—	170,91[a]	171,14	170,88
	C_α	51,57	—	49,93	49,91	49,85
	C_β	37,43	—	37,14	37,12	†
	C_γ	171,76	—	171,86	171,89	171,86
Leu	C_0	172,51	—	172,25	171,89	171,86
	C_α	51,10	—	50,98	51,66	51,36
	C_β	41,05	—	40,75	40,25	40,22
	C_γ	24,25	—	24,17	24,17	24,14
	C_{δ_1}	23,39	—	23,21	23,19	23,14
	C_{δ_2}	21,75	—	21,62	21,46	21,54
Ser	C_0	170,95	—	170,70[a]	169,13	170,28[b]
	C_α	55,03	—	55,05	54,41	55,56
	C_β	61,32	—	61,29	61,58	61,51
BOC	CH_3	28,27	28,19	28,22	28,20	28,19
	$-C-O$	79,04	78,57	78,76	78,70	78,67
	$C=O$	155,46	154,87	155,09	155,02	155,02
Trt	$-C-S$	—	65,97	66,11	66,04	66,10
			144,32	144,51	144,46	144,46
	C_{Ar}	—	129,08	129,24	129,20	129,19
			128,02	128,15	128,10	128,12
			126,72	126,92	126,85	126,84
Me	CH_3	51,94	—	51,89	—	—

*For peptide (XV) the assignment of the signals in the 1–5 fragment is given; for the assignment of the other signals, see Table 3; the groups of signals denoted by a–a, b–b, and c–c are assigned alternatively.
†Signal not observed because of overlapping with a signal from the solvent. The positions of a number of carbonyl group signals depend on the amount of water in the solvent.

The chromatographic purities and mobilities of the peptides obtained were determined by the TLC method on Silufol plates in the following systems (ratio by volume): 1) EtOAC–pyridine–AcOH–H_2O (15:5:1.5:2.75); 2) EtOAc–pyridine–AcOH–H_2O (15:1.24:0.37:0.68); and 3) $CHCl_3$–MeOH (8:2).

The ^{13}C NMR spectra of solutions of the peptides (c 100 mg/ml) in DMSO-d_6 were recorded on a WP-80 DS spectrometer (Bruker, GFR) with a working frequency of 2.115 MHz. Conditions for recording the spectra with broad-band suppression of proton coupling; memory volume for the accumulation of spectra 8 K and for reproduction 8 K with a computer resolution of 0.023 ppm; pulse length 3-6 μsec (25-50°C); time interval between scanning pulses 1.1 sec. Chemical shifts are given in the δ scale relative to tetramethylsilane (TMS). The values of the chemical shifts were determined relative to the signal of the solvent and recalculated to the δ scale based on TMS by means of the formula $\delta_{TMS} = \delta_{DMSO} + 39.6$ ppm. The replacement of the mobile hydrogen atom by deuterium in (I) was performed as described in [9].

The following amino acid derivatives were used in the investigation: BOCAsnONP, HCl·HSerOMe, BOCThrOH·DCHA, BOCMetOH·DCHA from Reanal; HCl·HGlyOEt, and BOCLeuOH·H_2O from

TABLE 3. Chemical Shifts in the ^{13}C NMR Spectra of Peptide (VIII, X, XI, XIII, and XV*)

Amino acid residue	Nucleus	VIII	X	XI	XIII	XV
Thr	C_0	—	—	—	170,36	169,74
	C_α	—	—	—	60,09	58,18
	C_β	—	—	—	66,92	66,51
	C_γ	—	—	—	19,56	19,13
Cys	C_0	—	—	169,93	169,47	169,49
	C_α	—	—	53,73	51,96	52,17
	C_β	—	—	33,55	33,40	33,36
Met	C_0	171 50	168,06	170,36	170,36	170,39[b]
	C_α	53,82	51,71[c]	51,68	51,97	52,17
	C_β	31,68	31,25	32,34	31,97	31,79
	C_γ	29,79	28,18	29,22	29,51	29,56
	C_ε	14 70	14,57	14,64	14,67	14,67
Leu	C_0	172,55	172,14	172,21	172,27	172,33
	C_α	50,77	51,34[c]	50,86	51,10	51,11
	C_β	40,66	40,78	40,66	40 66	†
	C_γ	24,06	24,17	24,12	24,14	24,14
	C_{δ_1}	23,00	23,10	22,99	23,02	23,05
	C_{δ_2}	21,69	21,64	21,55	21,61	21,54
Gly	C_0	169 65	171,09	171,00	171,06	171,12
BOC	C_α	41,28	40,92	40,94	†	†
	CH_3	28,15	—	28,13	28,19	—
	—C—O	78,36	—	78,70	78,53	—
	C=O	155,58	—	154,93	155,40	—
Trt	—C—S	—	—	66,02	65,99	66,04
	C_{Ar}	—	—	144,40 129,15 128,08 126,83	144,39 129,19 128,12 126,84	144,37 129,19 128,12 126,84
Et	CH_3	14,04	—	—	—	—
	CH_2	60,42	—	—	—	—

*For peptide (XV) the assignment of the signals in the 6–10 fragment is given. The assignment of the other signals is given in Table 2.
†See footnote to Table 2.

Reakhim; BOCCys(Trt)OH from Biokhimreaktiv; and the condensing agents: DCC from Ferak and EtOCOCl, pure, and also N-hydroxysuccinimide (SuOH) from Merck. The CF_3COOH was of pure grade. The N-bis(trimethylsilyl)acetamide (BSA) and the isoamyl nitrite were obtained as described in [12] and [13], respectively.

Preparation of BOCCys(Trt)-GlyOH (III). A suspension of 2.3 g ($3.06 \cdot 10^{-2}$ mole) of HGly-OH in 25 ml of absolute CH_2Cl_2 was treated with 16.5 ml ($6.74 \cdot 10^{-2}$ mole) of BSA, the mixture was stirred at 50°C for 6 h and was then added to the mixed anhydride (MA) obtained from 11.6 g ($2.5 \cdot 10^{-2}$ mole) of BOCCys(Trt)OH, 3.5 ml Et_3N, and 2.6 ml of EtOCOCl in 25 ml of CH_2Cl_2 at −20°C over 20 min. The reaction mixture was stirred at −5°C for 2 h and was left at the same temperature for 20 h. Then 50 ml of $CHCl_3$ was added to the reaction mixture and it was washed successively with 0.1 N HCl (6 × 20 ml) and saturated NaCl solution (2 × 20 ml). The organic solution was dried with anhydride Na_2SO_4 and the solvent was evaporated off at 15 mm Hg. the residue reprecipitated from EtOH into ether. The (III) was filtered off, washed with ether (2 × 15 ml), and dried at 40°C/0.1 mm Hg. This gave 9.2 g ($1.77 \cdot 10^{-2}$ mole) of (III) (Tables 1 and 2).

Preparation of BOCCys(Trt)-Gly-Asn-Leu-SerOMe (IV). At 7°C, with stirring, 3 g ($1.47 \cdot 10^{-2}$ mole) of DCC was added in small portions to a solution of 7 g ($1.34 \cdot 10^{-2}$ mole) of (III) and 1.7 g ($1.47 \cdot 10^{-2}$ mole) of SuOH in 30 ml of dioxane; the urea that deposited was filtered

off, the residue was washed with dioxane (2 × 10 ml), and the filtrate was evaporated at 15 mm Hg. The BOCCys(Trt)-GlyOSu obtained and 5.1 g ($1.34 \cdot 10^{-2}$ mole) of (11) were dissolved in 20 ml of DMFA and then 1.9 ml of Et_3N was added and the mixture was stirred at room temperature for 40 h; the DMFA was evaporated off with 0.1 mm Hg and the residue was dissolved in 100 ml of $CHCl_3$, after which the solution was washed successively with 0.1 N HCl (3 × 15 ml) and H_2O (2 × 20 ml). The organic solution was dried with anhydrous Na_2SO_4, the $CHCl_3$ was evaporated off at 15 mm Hg; the residue was treated with 150 ml of ether, and the product was filtered off, washed with ether (2 × 25 ml), and dried at 40°C/0.1 mm Hg. This gave 6.8 g ($8.01 \cdot 10^{-3}$ mole) of (IV) (Tables 1 and 2).

Preparation of BOCCys(Trt)-Gly-Asn-Leu-SerN_2H_3 (V). A solution of 5.5 g ($6.47 \cdot 10^{-3}$ mole) of (IV) in 30 ml of MeOH and 1 ml of DMFA was treated with 3.25 ml of hydrazine hydrate. The reaction mixture was stirred at room temperature for 24 h, and then 150 ml of ether was added, the precipitate was filtered off and was washed with ether (2 × 50 ml) and dried at 40°C/0.1 mm Hg. This gave 4.5 g ($5.30 \cdot 10^{-3}$ mole) of (V) (Tables 1 and 2).

Preparation of BOCMet-Leu-GlyOEt (VIII). Compound (VIII) was obtained by the successive growth of the chain from the C-end using the MA method (Tables 1 and 3).

Preparation of BOCMEt-Leu-GlyOH (IX). At 0°C, a solution of 8.9 g of NaOH in 20 ml of H_2O and 35 ml of MeOH was added to 20 g ($4.47 \cdot 10^{-2}$ mole) of (VIII) in 35 ml of MeOH, and the mixture was stirred at room temperature for 1.5 h; then 75 ml of H_2O was added and extraction was performed with ether (3 × 40 ml). With cooling, 90 ml of 3 N HCl was added to the aqueous solution to bring the pH to 2, and the oil that separated out was extracted with EtOAc (4 × 50 ml). The organic solution was dried with anhydrous Na_2SO_4, the solvent was evaporated off at 15 mm Hg, and the residue was dried at 40°C/0.1 mm Hg. This gave 17.6 g ($4.19 \cdot 10^{-2}$ mole) of (IX) (Table 1).

Preparation of HMet-Leu-GlyOH·CF_3COOH (X). At −20°C, 64 ml of CF_3COOH was added to 17.5 g ($4.17 \cdot 10^{-2}$ mole) of (IX), and the mixture was kept at room temperature for 1 h. Then the CF_3COOH was evaporated off at 15 mm Hg, and the residue was treated with 200 ml of ether. The ether was decanted off and the oil obtained was washed with ether (2 × 30 ml) and reprecipitated from MeOH into ether. The compound (X) was dried at 40°C/0.1 mm Hg over NaOH. This gave 17 g ($3.92 \cdot 10^{-2}$ μmole) of (X) (Tables 1 and 3).

Preparation of BOCCys(Trt)-Met-Leu-GlyOH (XI). A solution of 14.6 g ($3.15 \cdot 10^{-2}$ mole) of BOCCys(Trt)OH in 25 ml of EtOAc was treated with 4.4 ml of Et_3N and, at −25°C for 10 min, and then a suspension of $3.46 \cdot 10^{-2}$ mole of HMet-Leu-GlyOH in 80 ml of $CHCl_3$, obtained from 15 g ($3.46 \cdot 10^{-2}$ mole) of (X) and 3.8 ml of Et_3N in 20 ml of DMFA–$CHCl_3$ (1:1), was added. The reaction mixture was stirred at −5-0°C for 2 h and was kept at the same temperature for 14 h. Then 150 ml of $CHCl_3$ was added and it was washed successively with 0.1 N HCl (4 × 30 ml) and saturated NaCl solution (2 × 35 ml). The organic solution was dried with anhydrous Na_2SO_4, and the solvent was evaporated off at 15 mm Hg. The residue was treated with 200 ml of ether and the resulting precipitates was filtered off and washed with ether (2 × 50 ml). The compound (XI) was dried at 40°C/0.1 mm Hg. The yield of (XI) was 14.7 g ($1.92 \cdot 10^{-2}$ mole) (Tables 1 and 3).

Preparation of HCys(Trt)-Met-Leu-GlyOH·CF_3COOH (XII). Compound (XII) was obtained by treating (XI) with CF_3COOH as for the case of (X) (Table 1).

Preparation of BOCThr-Cys(Trt)-Met-Leu-GlyOH (XIII). A solution of 4.4 g (0.011 mole) of the BOCThrOH·DCHA salt in 100 ml of EtOAc was washed successively with 1 N H_2SO_4 (2 × 30 ml) and H_2O (2 × 30 ml) to pH 6-7; the EtOAc solution was dried with anhydrous Na_2SO_4, the solvent was evaporated off at 15 mm Hg, and the residue was dissolved in 20 ml of dioxane, and to this solution were added 1.4 g (0.012 mole) of glacial OH and then, at +5°C, in portions, 2.5 g (0.012 mole) of DCC. The reaction mixture was stirred at room temperature for 20 h, the urea that had deposited was filtered off and washed with dioxane (2 × 10 ml), and the filtrate was evaporated at 15 mm Hg. To the residue was added a solution of 7.8 g (0.01 mole) of (XII) in 25 ml of DMFA and then 1.4 ml of Et_3N, and the resulting mixture was stirred at room temperature for 24 h, and then 100 ml of $CHCl_3$ was added and the solution was washed successively with 0.1 N HCl (3 × 15 ml) and with saturated NaCl solution (3 × 20 ml). The organic solution was dried with anhydrous Na_2SO_4, the solvent was evaporated off at 15 mm Hg, and the residue was treated with 200 ml of ether. The resulting precipitate was filtered off and washed with ether (2 × 50 ml). The compound (XIII) so obtained was dried at 40°C/0.1 mm Hg. The yield of (XIII) was 8 g ($9.24 \cdot 10^{-3}$ mole) (Tables 1 and 3).

Preparation of HThr-Cys(Trt)-Met-Leu GlyOH·CF_3COOH (XIV). Compound (XIV) was obtained by treating (XIII) with CF_3COOH as for (X) (Table 1).

Preparation of BOCCys(Trt)-Gly-Asn-Leu-Ser-Thr-Cys(Trt)-Met-Leu-GlyOH (XV). At −30°C, 2.06 ml of 5 N HCl in dioxane and 2.74 ml of isoamyl nitrite were added to a solution of 3.5 g ($4.12 \cdot 10^{-3}$ mole) of (V) in 20 ml of DMFA and the mixture was stirred at −25°C for 25 min, after which 5.7 ml of Et_3N and 4 g ($4.53 \cdot 10^{-3}$ mole) of (XIV) were added. The reaction mixture was stirred at −5°C for 2 h and was kept at this temperature for 40 h. Then 200 ml of ether was added, and the precipitate was filtered off and was washed on the filter with hot MeOH (2 × 40 ml). The (XV) was dried at 40°C/0.1 mm Hg. The yield of (XV) was 4.4 g ($2.78 \cdot 10^{-3}$ mole (Tables 1-3).

SUMMARY

The decapeptide with sequence 1–10 of human calcitonin has been synthesized with the minimum protection of the side-chain functional groups of the amino acids by a 5 + 5 scheme.

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