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The peptides of α-aminosuberic acid II. Synthesis of deamino-dicarba-eel-calcitonin sequence 1–9

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Summary. The paper describes the synthesis of Asu[6]-octapeptide derivatives by condensing two alternative pentapeptide fragments with Asu-containing tripeptides. After partial deprotection these linear peptides compounds are subject to cyclization experiments aimed to give the N-terminal [1–9] sequence of deamino-dicarba-eel calcitonin. This is a key substance for the semi-synthesis of the respective analogues of eel calcitonin.

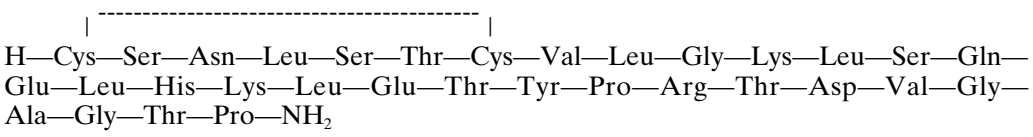
Keywords: α-Aminosuberic acid – Peptide synthesis – Synthetic intermediates – Cyclic peptides – Dicarba analogues – Calcitonin analogues

Abbreviations: Asu: L-α-aminosuberic acid, ASU: DL-α-aminosuberic acid, Z: benzyloxycarbonyl-, Boc: *tert*-butyloxycarbonyl-, Fmoc: 9-fluorenylmethyloxycarbonyl-, Me: methyl-, Bu^t: *tert*-butyl-, Ph: phenyl, Bzl: benzyl, Nb: 4-nitrobenzyl-, Su: succinimidyl-, DCHA: dicyclohexylammonium, TLC: thin layer chromatography

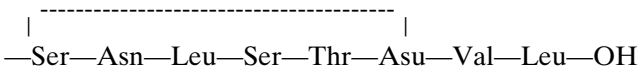
Introduction

Eel-Calcitonin was isolated in 1974 by Otani et al. (1974) and its primary structure was determined in by Noda and Narita (1976). The dotriacontapeptide amide has an analogous structure to the salmon hormone – only three residues are different – and identical biological activity.

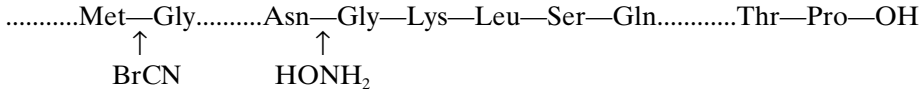
The syntheses of eel-calcitonins (Morikawa et al., 1976) and numerous analogues modified in the cyclic part of the molecule (Fujii et al., 1978), particularily of the deamino-dicarba-compound, called ELCATONIN (Morikawa et al., 1976; Sakakibara et al., 1978; Inove et al., 1992; Nishino et al., 1993), followed almost exclusively the scheme of condensation of the sequence 1–10 with the 11–32 amide in the last step, according to the procedure used in the synthesis of human calcitonin (Sieber et al., 1970). On



Eel calcitonin



Deamino–dicarba–eel calcitonin sequence 1–9



Model of the fusion protein

Scheme 1

the contrary semi-synthesis of ELCATONIN (Scheme 1) via condensation of a small synthetic fragment containing aminosuberic acid with the long tail sequence obtained by gene expression technology should respect the following considerations: The C-terminal part should start with the Gly residue (the sequence 10–32 in the case of Eel-calcitonin), because it is then possible to utilize the well established splitting points, either —Asn—Gly— (hydroxylamine) or —Met—Gly— (cyanogen bromide). These allow for a relatively easy cleavage of a sequence having the N-terminal Gly residue from the fusion protein. The incorporation of both the above splitting points (not too far from one another) in front of the needed 10–32 sequence enables a two-stage cleavage from the fusion protein via the intermediary "mini-fusion protein". In the latter molecule additional protection of ε-amino groups of lysines is possible. The second splitting of the Nα- and Nε- substituted "mini-fusion protein" results then in N_α-free, Nε-protected 10–32 sequence. The above considerations determine the condensation point between the 9th and 10th residue in the ELCATONIN sequence. The C-terminal Leu residue of the N-terminal fragment makes possible to use not only chemical but also enzymatic approach in the fragment condensation step, leaving the protection of lysine side chain amino groups unnecessary.

Results and discussion

Six protected octapeptide derivatives X–XV having the modified sequence 2–9 of the ELCATONIN hormone were synthesized in good yields by [5 + 3] fragment condensation of two respective N-terminal pentapeptides VIII–IX

Table 1. Intermediates in the synthesis of deamino-dicarba-octapeptides
—Ser—Asn—Leu—Ser—Thr—Asu/ASU—Val—Leu—R (R = OH, OMe, NHNHBoc,OBu^t)
| ---------- |

C-terminal Asu-containing tripeptides	
I	H—Asu(OBzl)—Val—Leu—OMe
II	H—ASU(OBzl)—Val—Leu—OMe
III	H—Asu—Val—Leu—OMe
IV	H—Asu(OBzl)—Val—Leu—NHNHBoc
V	H—Asu(OBzl)—Val—Leu—OBu^t
VI	H—ASU(NHNHZ)—Val—Leu—OBzl
VII	H—ASU(NHNHBoc)—Val—Leu—OMe
N-terminal pentapeptides	
VIII	Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—OH
IX	Boc—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—$NHNH_2$
N_α-protected Asu-containing octapeptides	
X	Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OBzl)—Val—Leu—OMe
XI	Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—OMe
XII	Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OBzl)—Val—Leu—NHNHBoc
XIII	Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OBzl)—Val—Leu—OBu^t
XIV	Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—ASU(NHNHZ)—Val—Leu—OBzl
XV	Boc—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—ASU(NHNHBoc)—Val—Leu—OMe
Nα-free Asu-containing octapeptides	
XVI	H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—OMe
XVII	H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—NHNHBoc
XVIII	H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—OBu^t
XIX	H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—ASU($NHNH_2$)—Val—Leu—OH
XX	H—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—ASU($NHNH_2$)—Val—Leu—OMe

with protected tripeptides of aminosuberic acid I–VII. The pentapeptides VIII–IX were prepared by classical methods of solution synthesis. These intermediates are listed in Table 1. After deprotecting the N-α and also the respective C-ω functionality the resulting linear octapeptide derivatives were subjected to cyclisation experiments. The cyclisation of precursors XVI–XVIII proceeded with excelent yields with HBTU in DMF at "high dilution conditions". On the contrary azide cyclisations of the C-ω hydrazides XIX–XX were not successful as of today.

Materials and methods

Melting points were determined on a Totolli's capillary melting point apparatus and are uncorrected. Optical rotations were measured in a jacketed 1 dm cell on Perkin Elmer polarimeter (model 241). Prior to elemental analyses the compounds were dried under vacuum. Thin layer chromatography was carried out on Merck silica-gel 60 plates using the following eluents: A: butanol-acetic acid-water (3:1:1); B: heptane-tert-butylalcohol-pyridine (3:1:1); C: heptane-butanol-acetic acid-water-pyridine (7.5:15:1.8:7.2:1); D: cyclohexane-chloroform-acetic acid (45:45:10). The peptidic compounds were visualized with chlorine/tolidine and ninhydrine reagents.

A. The fragment Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—OH

1. H—Ser(Bu^t)—Thr(Bu^t)—OH

H—Thr(Bu^t)—OH monohydrate (52.5 g) in 300 ml 1 M NaOH, 25 g $NaHCO_3$, 50 ml dioxane, and Z—Ser(Bu^t)—OSu (117 g, works better than Z—Ser(Bu^t)—ONp) were transformed and worked up in the usual manner (see Wünsch et al., 2000 for details of the procedure). It provided 124.5 g (91%) of the oily Z—Ser(Bu^t)—Thr(Bu^t)—OH. The oil has been catalytically hydrogenolyzed (Pd/C) in 1l 90% aqueous methanol with 1 ml acetic acid. After filtering off the catalyst, the solution was taken down and the residue crystallized from 2-propanol/diisopropylether. Yield 78 g (91%), $[\alpha]_D^{20} = +16.59°$, $[\alpha]_{546}^{20} = +19.10°$ (c = 1, methanol). For $C_{15}H_{30}N_2O_5$ (318.4) calculated 56.78% C, 9.50% H, 8.80% N; found 56.44% C, 9.58% H, 8.70% N.

2. Z—Leu—Ser(Bu^t)—Thr(Bu^t)—OH

H—Ser(Bu^t)—Thr(Bu^t)—OH (77 g) in 240 ml 1 M NaOH, 19 g $NaHCO_3$ in 100 ml water, 300 ml dioxane and Z—Leu—OSu (83 g) were transformed (TLC monitoring) and worked up in the usual manner (see Wünsch et al., 2000). The oily product crystallized from diisopropylether/light petroleum. Yield 107 g (82%), m.p. 69–71°C; $[\alpha]_D^{20} = +6.51°$, $[\alpha]_{546}^{20} = +7.70°$ (c = 1, methanol). For $C_{29}H_{47}N_3O_8$ (565.7) calculated 61.57% C, 8.37% H, 7.43% N; found 61.29% C, 8.52% H, 7.18% N.

3. H—Leu—Ser(Bu^t)—Thr(Bu^t)—OH

The Z-Tripeptide (103 g) in 1l 90% aqueous methanol was hydrogenolyzed and the product isolated in the usual manner. The oily product crystallized from methanol/diethylether with 1.5 mol water. Yield 75 g (69%), $[\alpha]_D^{20} = +23.54°$, $[\alpha]_{546}^{20} = +27.82°$ (c = 1, methanol). For $C_{21}H_{41}N_3O_6 + 1.5\ H_2O$ (458.6) calculated 55.00% C, 9.67% H, 9.16% N; 54.71% C, 9.76% H, 9.17% N.

4. Z—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—OH

The tripeptide (3) (44.5 g) in 900 ml DMF was reacted with Z—Asn—ONp (38.7 g) first for 2 h at 0° and then for 24 h at room temperature. Evaporation of DMF yielded the oily residue which crystallized upon dissolving in hot ethylacetate and cooling hereafter. Yield 55 g (81%), m.p. 155°C; $[\alpha]_D^{20} = -10.21°$, $[\alpha]_{546}^{20} = -12.36°$ (c = 1, methanol). For $C_{33}H_{53}N_5O_{10}$ (679.8) calculated 58.31% C, 7.86% H, 10.30% N; found 58.16% C, 7.69% H, 10.28% N.

5. H—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—OH

The Z-tetrapeptide (4) (54 g) was hydrogenolysed (Pd/C) in 500 ml of 90% aqueous methanol. After filtering off the catalyst the solution was taken down and the residue dissolved in hot 2-propanol. The product crystallized after careful addition of diisopropylether and light petroleum. Yield 42 g (97%), $[\alpha]_D^{20} = -5.47°$, $[\alpha]_{546}^{20} = -6.94°$ (c = 1, methanol). For $C_{25}H_{47}N_5O_8$ (545.7) calculated 55.03% C, 8.68% H, 12.83% N; found 58.16% C, 8.72% H, 12.68% N.

6. Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—OH

The tetrapeptide (5) (54.6 g) in 300 ml DMF was coupled with Z—Ser(Bu^t)—OSu (39.2 g, prepared from the equivalent of Z—Ser(Bu^t)—OH) first for 2 h at 0° and then for 24 h at room temperature (the reaction proceeds as well in aqueous NaOH – 1 equivalent in the presence of 8 g $NaHCO_3$ and 50 ml dioxane). After the usual work-up procedure

the product was crystallized from 2-propanol/water and washed with diethylether. Yield 74 g (90%), m.p. 185–186°C; $[\alpha]_D^{20} = +4.20°$, $[\alpha]_{546}^{20} = +4.68°$ (c = 1, DMF). For $C_{40}H_{66}N_6O_{12}$ (823.0) calculated 58.38% C, 8.08% H, 10.21% N; found 58.10% C, 8.08% H, 10.15% N.

B. The fragment Boc—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—$NHNH_2$

7. Boc—Ser(Bzl)—Thr(Bzl)—OBzl

H—Thr(Bzl)—OBzl hemioxalate (18 g) was treated with aqueous solution of sodium carbonate and diethylether. The ether phase was dried over sodium sulphate and evaporated to dryness. The resulting oil of the free amino ester was dissolved in DMF (150 ml) and reacted at −10°C with Boc—Ser(Bzl)—OH (14.8 g) and DCCI (10.5 g). The usual work up procedure yielded 22.6 g of the oily product (78.5%)

8. H—Ser(Bzl)—Thr(Bzl)—OBzl. HCl

The raw dipeptidester (7) (22.6 g) was overlaid with 250 ml of ice cold 4 M HCl in dioxane. Agitation of the mixture afforded a clear solution soon. After 2 h the mixture was concentrated in vacuum to a small volume and diluted with absolute diethylether. Trituration started the crystallization process which was completed on standing in the refrigerator. The product was filtered, washed with absolute diethylether and dried. Yield 17.6 g (70%, both steps), m.p. 134–135°C. For $C_{28}H_{32}N_3O_5$ (573.0) calculated 65.55% C, 6.48% H, 5.46% N; found 65.42% C, 6.52% H, 5.40% N.

9. Boc—Asn—Leu—OBu^t

Boc—Asn—OH (34.5 g) in 1 l DMF was mixed with triethylamine (21 ml), HOBt (20.2 g) and H—Leu—OBu^t. HCl (33.5 g). The reaction with DCCI (31 g) was started at −10°C. After 24 h (TLC monitoring) the solvent was evaporated in vacuum and the residue extracted with ethylacetate and water. Usual work up of the ethylacetate layer afforded the product which crystallized from ethylacetate/light petroleum. Yield 55 g (91%), m.p. 138–139°C, $[\alpha]_D^{20} = -38.44°$, $[\alpha]_{546}^{20} = -45.66°$ (c = 1, methanol). For $C_{19}H_{35}N_3O_6$ (401.6) calculated 56.84% C, 8.79% H, 10.47% N; found 56.86% C, 8.90% H, 10.48% N.

10. H—Asn—Leu—OH. HCl

The Boc-dipeptide (9) (53 g) was deacylated with 250 ml of ice cold 1 M HCl in acetic acid. When the evolution of CO_2 ceased the mixture was evaporated in vacuum. The product separated after the addition of excess absolute diethylether. The precipitate was isolated by suction filtering and dried in high vacuum. Yield 35 g (91%) of hygroscopic powder, $[\alpha]_D^{20} = -10.78°$, $[\alpha]_{546}^{20} = -12.32°$ (c = 1, 80% acetic acid). For $C_{10}H_{20}N_3O_4Cl$ (281.7) calculated 42.63% C, 7.16% H, 14.31% N, 12.58% Cl; found 42.51% C, 7.28% H, 14.82% N, 12.41% Cl.

11. Boc—Ser(Bzl)—Asn—Leu—OH

The dipeptide hydrochloride (10) (8.45 g) in DMF (100 ml) was mixed with triethylamine (4.6 ml) and reacted with Boc—Ser(Bzl)—OSu (12.9 g, prepared from 10 g of Boc—Ser(Bzl)—OH, 4 g N-hydroxysuccinimide and 7 g DCCI in dioxane). Most of the solvent has been evaporated in vacuum and the residue was mixed in highly diluted HCl, which induced crystallization. The precipitate was filtered, washed with water and dried. Yield

12.6 g (80%), m.p. 185–187°C; $[\alpha]_D^{20} = -7.12°$, $[\alpha]_{546}^{20} = -8.52°$ (c = 1, DMF). For $C_{25}H_{38}N_4O_8$ (522.7) calculated 57.46% C, 7.33% H, 10.72% N; found 57.40% C, 7.26% H, 10.70% N.

12. Boc—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—OBzl

The Boc-tripeptide (11) (10.5 g) and dipeptide ester hydrochloride (8) (10.3 g) in 150 ml DMF were condensed upon the addition of triethylamine (2.8 ml), N-hydroxysuccinimide (2.5 g) and DCCI (4.2 g) at 0°C. The usual work up procedure yielded the raw product which crystallized on cooling from solution in the boiling acetonitrile. Yield 15.1 g (77%), m.p. 160–162°C; $[\alpha]_D^{20} = -9.07°$, $[\alpha]_{546}^{20} = -11.04°$ (c = 1, DMF). For $C_{53}H_{68}N_6O_{12}$ (981.2) calculated 64.88% C, 6.99% H, 8.56% N; found 64.74% C, 6.98% H, 8.70% N.

13. Boc—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—$NHNH_2$

The Boc-pentapeptide ester (12) (10.8 g) in methanol (150 ml) and DMF (50 ml, the compound dissolved after slight warming) was cooled in ice cold water and stirred with hydrazine hydrate (24 ml, 98%) for 24 h at room temperature. The precipitated product was washed several times with methanol and water (after the addition of a small amount of acetic acid) and dried in high vacuum. The material thus obtained was digested with acetonitrile (Fraction 1). The methanolic mother liquors were evaporated to a small volume; on addition of water precipitated another portion which was filtered, dried and digested with diethylether (Fraction 2). The joint fractions were dried in high vacuum. Yield 8.2 g (82%), m.p. 223°C, $[\alpha]_D^{20} = -6.2°$, $[\alpha]_{546}^{20} = -7.6°$, (c = 1, DMF). For $C_{46}H_{64}N_8O_{12}$ (905.07) calculated 61.05% C, 7.13% H, 12.38% N; found 60.79% C, 7.14% H, 12.35% N.

C. Fragment condensations

14. Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OBzl)—Val—Leu—OMe

The Z-pentapeptide (6) (8.2 g) and tripeptide methylester hydrochloride I (5.4 g, see Wünsch et al., 2000) in DMFA (200 ml) were mixed with 1.1 g of N-hydroxysuccinimide, 1.6 g hydroxyoxobenzotriazine and 1.4 ml of triethylamine. The mixture was cooled to −10°C and 2 g of ethyl-dimethylaminopropylcarbodiimide hydrochloride were added. After mixing for 48 h at room temperature the mixture was concentrated in vacuo to about half of its volume and poured into a large quantity of water. The precipitated product was isolated by filtration and dried in vacuo. Yield 10.6 g (81%), m.p. 239°C (dec.), $[\alpha]_D^{20} = -8.3°$C, $[\alpha]_{546}^{20} = -10.1°$, (c = 1, DMF). For $C_{67}H_{107}N_9O_{17}$ (1310.65) calculated 61.40% C, 8.23% H, 9.62% N; found 61.25% C, 8.40% H, 9.56% N.

15. Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OH)—Val—Leu—OMe

The Z-pentapeptide (6) (2.4 g) and N-hydroxysuccinimide (0.35 g) in DMFA (50 ml) were cooled to −10°C and DCCI (0.62 g) was added. The batch was stirred first for 2 h at −5–0°C and then for 24 h at room temperature. Afterwards it was again cooled to 0°C and dicyclohexylurea was filtered off. The filtrate was added to a cooled (−10°C) suspension of H—Asu(OH)—Val—Leu—OMe (1.25 g) in DMFA (40 ml) and stirred first for 2 h at −5 −0°C, then for 48 h and after the additon of further 10 ml DMFA again for 36 h at room temperature (the suspension persisted). Afterwards about 20 ml DMFA was distilled off and the residue poured into about 700 ml of water and ice. The precipitate was filtered off, washed with water and dried. Yield 3.2 g (87%). $[\alpha]_D^{20} = -17.9°$, (c = 1, DMFA).

16. H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OH)—Val—Leu—OMe.HCl

The above Z-octapeptide methylester (14) (10 g) in methanol (250 ml, partial suspension) was catalytically hydrogenolyzed by a continuous stream of hydrogen on a Pd/C catalyst while titrating continuously the freeing amino group with 1 M HCl/MeOH (pH~4, overall consumption 7.7 ml, the suspension dissolved). The catalyst was filtered off and the filtrate was concentrated in vacuo. The product, which precipitated in absolute ethylacetate, was filtered off and dried in vacuo. Yield 8.2 g (93%, calculated for the dihydrate), m.p. 236°C (dec.), $[\alpha]_D^{20} = -10.5°$, $[\alpha]_{546}^{20} = -12.7°$, (c = 1, DMFA). For $C_{52}H_{96}N_9O_{15}Cl.\ 2H_2O$ (1158.98) calculated 53.89% C, 8.70% H, 10.88% N; found 53.86% C, 8.78% H, 11.01% N.

The octapeptide (15), treated in the same way, provided the octapeptide methylester (16) (having identical analytical data) with the analogous yield.

17. Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OBzl)—Val—Leu—NHNHBoc

The raw H—Asu(OBzl)—Val—Leu—NHNHBoc (8 g, see Wünsch et al., 2000), HOSU (1.04 g), HOBt (1.5 g) and Z-pentapeptide (6) (7.45 g) dissolved in DMFA (200 ml) was cooled to −10°C and ethyl-diaminopropylcarbodiimide (1.75 g) was added. The mixture was stirred for 48 h at room temperature while at adopted a gel-like consistency. It was liquified by further addition of DMFA (50 ml) and poured under vigorous stirring into 1.5 l of ice cold water containing 2 g of citric acid. The precipitate was filtered off, washed with water, dried, taken up in DMFA (50 ml) and again poured into ice cold water containing 2 ml of pyridine. The precipitate was suction filtered, washed with water and 50% 2-propanol and dried in vacuo. Yield 10.5 g (83%), m.p. 255°C, $[\alpha]_D^{20} = -12.3°$, (c = 1, DMFA). For $C_{71}H_{115}N_{11}O_{18}$ (1410.77) calculated 60.45% C, 8.22% H, 10.92% N; found 60.31% C, 8.40% H, 10.90% N.

18. H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—NHNHBoc. HCl

Z-octapeptide—NHNHBoc (17) in 95% methanol (300 ml) was catalytically hydrogenolyzed by a continuous stream of hydrogen on a Pd/C catalyst while titrating continuously the freeing amino group with 1 M HCl/MeOH (pH~5). The filtrate from the catalyst was evaporated in vacuo and the residue triturated with diethylether. The solid material was filtered off, washed with ether and dried. Yield 6.9 g (94%), m.p. 234°C (dec.), $[\alpha]_D^{20} = -28.2°$C, $[\alpha]_{546}^{20} = -34.1°$, (c = 1, acetic acid). For $C_{56}H_{104}N_{11}O_{16}$ (1222.97) calculated 55.00% C, 8.57% H, 12.60% N; found 54.84% C, 8.72% H, 12.42% N.

19. Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(OBzl)—Val—Leu—OBu^t

The raw H—Asu(OBzl)—Val—Leu—OBu^t (6.4 g, see Wünsch et al., 2000). HOSU (1.15 g), HOBt (1.63 g) and Z-pentapeptide (6) (6.58 g) was dissolved in DMFA (150 ml) and ethyl-dimethylaminopropylcarbodiimide (1.53 g) was added at −10°C. After stirring for 24 h at room temperature, the gel-like batch was liquified by the addition of further 50 ml DMFA and then it was poured into 1.5 l of ice cold water containing 2 ml 1 M sulfuric acid. The precipitate was filtered off, washed with water and dried. The product thus obtained was further dissolved in DMFA (50 ml) and the solution poured, with vigorous stirring, into 1.5 l of ice cold water containing 2 g sodium carbonate. The precipitate was filtered off, washed with water and 50% 2-propanol and dried in vacuo. Yield 7.9 g (72%), m.p. 243°C (dec.), $[\alpha]_D^{20} = -28.7°$, $[\alpha]_{546}^{20} = -34.7°$, (c = 1, acetic acid). For $C_{70}H_{119}N_9O_{17}$ (1352.73) calculated 62.15% C, 8.42% H, 9.32% N; found 61.88% C, 8.51% H, 9.42% N.

20. H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—OBu^t. HCl

Z-octapeptide *tert*-butylester (19) (7 g) in methanol (250 ml) was catalytically hydrogenolyzed while titrating continuously the freeing amino group with 1 M HCl/MeOH (pH~5, to the end to about 4.5). The filtrate from the catalyst was evaporated in vacuo and the residue treated with diethylether, filtered off and dried in vacuo. Yield 5.4 g (92%), m.p. 230°C, $[\alpha]_D^{20} = -24.6°$, $[\alpha]_{546}^{20} = -29.7°$, (c = 1, acetic acid). For $C_{55}H_{102}N_9O_{15}Cl$ (1164.93) calculated 56.71% C, 8.83% H, 10.82% N; found 56.52% C, 8.89% H, 10.70 % N.

21. Z—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(NHNHZ)—Val—Leu—OBzl

The Z-pentapeptide (6) (8.2 g), H—Asu(NHNHZ)—Val—Leu—OBzl. HCl (6.7 g, see Wünsch et al., 2000), HOSU (1.1 g), HOBt (1.6 g) and triethylamine (1.4 ml) was dissolved in DMFA (150 ml), cooled to −10°C and DCCI (2.1 g) was added. The mixture was stirred for 24 h at room temperature, concentrated to a small volume in vacuo and mixed into water. The precipitate was filtered off and washed with ether and ethylacetate. The material contained traces of dicyclohexylurea, these could have been removed only with big effort and didn't interfere with the next steps. Yield 13.6 g (94%), m.p. > 220°C (dec.), $[\alpha]_D^{20} = -6.3°$, $[\alpha]_{546}^{20} = -7.7°$, (c = 1, DMFA). For $C_{74}H_{113}N_{11}O_{18}$ (1444.79) calculated 61.52% C, 7.88% H, 10.66% N; found 61.70% C, 8.00% H, 10.80% N.

22. H—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu(NHNH$_2$)—Val—Leu—OH

The Z-octapeptide benzylester (21) (12 g) in DMFA (500 ml) was catalytically hydrogenolyzed (Pd/C). The gradually building gel-like substance was brought in solution by the addition of acetic acid. After the hydrogenation was finished (TLC monitoring) the filtrate from the catalyst was evaporated in vacuo and the oily residue treated with large amount of diethyl ether. The precipitate was filtered off and dried in vacuo (brownish in color due to traces of colloidal palladium). Yield 8.1 g (81%, diacetate). For $C_{51}H_{95}N_{14} + C_4H_8O_4$ (1206.50) calculated 54.75% C, 8.61% H, 12.77% N; found 54.73% C, 8.74% H, 12.47% N.

23. Boc—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—ASU(NHNHBoc)—Val—Leu—OMe

The Boc-pentapeptide hydrazide derivative (13) (9.05 g) was dissolved in DMFA (75 ml) and at −10°C mixed with 18.7 ml 3 M HCl/dioxane. Isoamylnitrite (2.1 ml) was added at −10°C and the solution was stirred for 20 min. After cooling to −30°C H—ASU(NHNHBoc)—Val—Leu—OMe. HCl (5.7 g) and triethylamine (9.25 ml) in 20 ml DMFA was added. The mixture was stirred for 24 h in ice bath and further 24 h at room temperature (further portions of DMFA were added if precipitation occured). The reaction mixture was concentrated in vacuo to about half of its volume and mixed into the excess amount of ice cold 0.1 M HCl. The precipitate was filtered off, washed with water, dried, digested with methanol, filtered and dried. Yield 11.5 g (82%), m.p. 223°C (dec.), $[\alpha]_D^{20} = -10.1°$, $[\alpha]_{546}^{20} = -12.26°$, (c = 1, DMFA). For $C_{71}H_{107}N_{11}O_{18}$ (1402.7) calculated 60.82% C, 7.69% H, 10.38% N; found 60.62% C, 7.63% H, 11.14% N.

24. H—Ser(Bzl)—Asn—Leu—Ser(Bzl)—Thr(Bzl)—ASU(NHNH$_2$)—Val—Leu—OMe + 2 Tfac—OH

Boc-octapeptide methylester derivative (23) (10.1 g) was added to a mixture of trifluoroacetic acid (25 ml) and dichloromethane (25 ml). The mixture was allowed to stand for 3 h at room temperature. After evaporating dichloromethane in vacuo the residue was treated with absolute ether. The precipitate was filtered off, washed with ether and dried in vacuo. Yield 10.3 g (nearly quantitative). For $C_{65}H_{93}N_{11}O_{20}F_6$ (1403.6) calculated 54.57% C, 6.55% H, 10.77% N; found 54.33% C, 6.53% H, 10.81% N.

25. Cyclo[1–6]—Ser(Bu^t)—Asn—Leu—Ser(Bu^t)—Thr(Bu^t)—Asu—Val—Leu—OMe

The octapeptide methylester hydrochloride (16) (3.3 g), HOBt (0.59 g) and triethylamine (1.05 g) in DMFA (300 ml) was slowly added dropwise into 2.5l DMFA containing 1.7 g benzotriazolyl-tetramethyluronium hexafluorophosphate (HBTU). The batch was further stirred for 72 h at room temperature, concentrated in vacuo to about 100 ml volume and stirred into 400 ml of ice cold water containing 1.26 ml triethylamine. The precipitate was filtered off, washed with water and diethylether and dried in vacuo. Yield 2.46 g (77%), m.p. 257°C, $[\alpha]_D^{20} = -43.7°$, $[\alpha]_{546}^{20} = -52.7°$, (c = 1, acetic acid). For $C_{52}H_{93}N_9O_{14}$ (1068.37) calculated 58.46% C, 8.77% H, 11.80% N; found 58.24% C, 8.81% H, 11.68% N.

References

Otani M, Noda T, Yamauchi H, Watanabe S, Matsuda T, Orimo H, Narita K (1974) Isolation, chemical structure, and biological properties of ultimobranchial calcitonin of the eel. In: Talmage R (ed) Proceedings of the 5th Parathyroid Conference, Oxford 1974. [Excerpta Medica (1974), pp 111–115]

Noda T, Narita K (1976) Amino acid sequence of eel calcitonin. J Biochem Tokyo 75: 353–359

Morikawa T, Munekata E, Sakakibara S, Noda T, Otani M (1976) Synthesis of eel-calcitonin and [Asu^{1,7}]-eel-calcitonin: contribution of the disulfide bond to the hormonal activity. Experientia 32: 1104–1106

Fujii S, Yakamoto Y, Shimizu F, Inai M, Hirohashi M (1978) Polypeptide derivatives. Jap. Pat. 47322, 106452, 190159, 248478, 317932

Sakakibara S, Noda T, Morikawa T, Munekata E, Kimora T, Nakagawa Y (1978) Polypeptides and process for producing the same. Jap. Pat. 50-52064, D. Pat. 2616399 C2, U.S. Pat. 4,086,221

Inove T, Kishida S, Ohsaki M, Kimura H (1992) Cyclization of N-terminal fragments of calcitonin. Bull Chem Soc Japan 65: 1728–1730

Nishino N, Mihara H, Izumi N, Makinose Y, Fujimoto T (1993) Synthesis of calcitonins by oxime resin solid phase synthesis and segment condensation. In: Okada Y (ed) Peptide chemistry 1993. Proceedings of the 31th symposium on peptide chemistry 1993. Protein Research Foundation, Osaka, pp 13–16

Sieber P, Riniker B, Brugger M, Kamber B, Rittel W (1970) Menschliches Calcitonin VI. Die Synthese von Calcitonin M. Helv Chim Acta 53: 2135–2150

Wünsch E, Stocker H, Maloň P, Gut V, Čeřovský V, Žertová M, Laffan D (2000) The peptides of α-aminosuberic acid I. New intermediates for synthetic studies. Amino Acids 18: 219–227

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