Synthesis of [2-Serine, 8-Valine]-Human Calcitonin[1,2]

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The [2-serine, 8-valine]-analog of human calcitonin (**1**) was synthesized by the liquid phase method. Analog **1** was built up from five fragments with minimized protection for the side functional groups. These fragments were linked stepwise to the C-terminal fragments by the azide method. A gel filtration was effectively applied for the purification of the resulting peptide in each fragment condensation. Analog **1** had about the same hypocalcemic activity as that of human calcitonin. The result showed that the high activity of eel or salmon calcitonin 1 or 2 is not ascribable only to the amino acid sequence of their *N*-terminal decapeptide.

Among calcitonins isolated from various origins, salmon[3] and eel hormones[4] exhibit conspicuously high activity, about 20—50 times the hypocalcemic potency of any other calcitonin from mammalian species.[5-8] All the calcitonins so far isolated are composed of 32 amino acids with a disulfide bridge between two cysteinyl residues in positions 1 and 7. The structural homology of these calcitonins is found in the C-terminal prolinamide, glycine in position 28, and the region around the disulfide bridge; amino acids in positions 1, 3, 4, 5, 6, 7, and 9 are common to all the hormones. Salmon calcitonin 3 with methionine in position 8 exhibits the hormonal activity of one third of salmon calcitonin 2,[9] which has the same amino acid sequence but valine at this position. The oxidation of methionine in the same position as that of human calcitonin remarkably reduces the activity of the hormone,[7] but not the oxidation of the amino acid in position 25 of the porcine hormone.[5] Similarity in the N-terminal structure of calcitonins and the remarkable decrease of hormonal activity induced from the oxidation of methionine in position 8 suggests the presence of an "active site" around the disulfide bridge of the hormone.

Several analogs of human calcitonin have been synthesized but the structure-activity relationship of calcitonin has not been explained clearly.[10] To investigate the effect of variation of the primary structure around the disulfide bridge of the human hormone on its hypocalcemic activity, we synthesized [2-serine, 8-valine]-human calcitonin, **1** (Fig. 1), in which glycine in position 2 and methionine in position 8 of the original hormone are replaced by serine and valine, respectively. Analog **1** is a hybrid peptide consisting of the N-terminal decapeptide of eel calcitonin or salmon calcitonin (1 or 2), the hormones with the highest hypocalcemic potency, and the C-terminal decosapeptide amide of human calcitonin, the lowest potent hormone.

Results and Discussion

Analog **1** was built up from five subfragments prepared in the liquid phase method according to the routes illustrated in Schemes 1—5. The protection of the side functional groups of fragments was minimized in order to facilitate the application of various kinds of chromatography to the purification of elongated peptides. The subfragments were linked stepwise to the C-terminal fragments by Rudinger's modification of the azide method[11] as shown in Scheme 6. Each product of the azide coupling was purified after removal of its N-terminal protecting group. The restriction of the molecular size of the N-terminal fragment used in every coupling reaction to be sufficiently smaller than that of the resulting peptide made the gel filtration efficient in the purification of the product. When necessary, partition chromatography on Sephadex[12] was further applied to the purification of deprotected peptides. The yield and properties of N-protected intermediates are

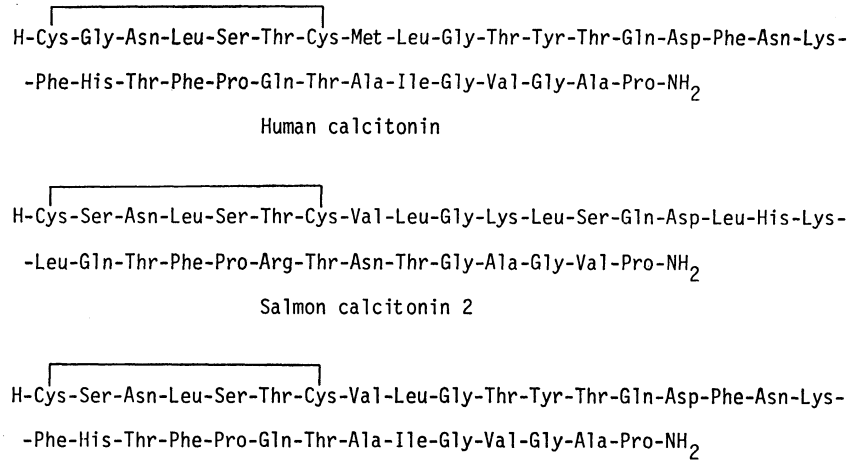


Fig. 1.

Table 1. Physical properties of N-protected peptides

Compd	Yield (%)	Mp (°C)	$[\alpha]_D$	Molecular formula	Elemental analysis (%) Found C	H	N	Calcd C	H	N
2[a]	94	oil	—		—	—	—	—	—	—
3[a]	86	168—169[b]	−89.1° (*c* 1.0, MeOH)[c]	$C_{16}H_{21}O_4N_3$	60.47	6.87	13.32	60.17	6.63	13.16
11	82	198—199	−9.6° (*c* 2.0, DMF)[d]	$C_{21}H_{31}N_3O_6$	60.08	7.66	10.11	59.84	7.41	9.97
13	74	224—226	−8.3° (*c* 1.0, DMF)[e]	$C_{35}H_{38}O_8N_4$	57.29	7.95	10.73	57.46	7.33	10.72
15	89	250	−4.9° (*c* 1.0, HMPA)[f]	$C_{30}H_{46}O_{10}N_6\cdot 1/2H_2O$	54.27	7.14	12.80	54.61	7.19	12.74
17	88	243	−31.8° (*c* 1.0, HMPA)[g]	$C_{35}H_{53}O_{11}N_7\cdot 1/2H_2O$	55.33	7.27	12.83	55.54	7.19	12.95
18	81	246—247	−13.6° (*c* 0.5, HMPA)[g]	$C_{33}H_{51}O_{11}N_9$	53.81	7.12	16.86	54.01	7.01	17.18
20[a]	82	251—252	−35.2° (*c* 1.0, DMF)[h]	$C_{48}H_{74}O_{14}N_{12}$	55.53	7.14	16.11	55.27	7.15	15.48
21	64	109—111	−3.3° (*c* 1.0, EtOH)[i]	$C_{23}H_{28}O_6N_2$	64.49	6.60	6.61	64.47	6.59	6.54
23	73	108—117	−2.8° (*c* 1.0, DMF)[h]	$C_{27}H_{35}O_7N_5$	61.55	6.41	12.43	61.58	6.24	12.38
24	70	204—205	−15.1° (*c* 1.0, DMF)[g]	$C_{27}H_{33}O_6N_7\cdot H_2O$	57.00	6.18	17.27	56.93	6.19	17.21
27	82	120—123	−15.3° (*c* 1.0, MeOH)[e]	$C_{30}H_{41}O_7N_3$	64.44	7.50	7.57	64.85	7.44	7.56
29	42	176—177	−17.2° (*c* 1.0, DMF)[e]	$C_{30}H_{43}O_7N_9$	61.34	7.07	10.95	60.97	7.07	10.45
30	96	199—201	−36.0° (*c* 1.0, DMF)[g]	$C_{32}H_{45}O_8N_7$	58.39	6.87	14.79	58.61	6.92	14.95
34	80	169—171	−11.1° (*c* 1.0, DMF)[g]	$C_{23}H_{35}O_6N_3$	61.34	8.06	9.81	61.45	7.85	9.35
35	90	190—191	−52.2° (*c* 1.1, EtOH)[h]	$C_{21}H_{31}O_6N_3$	59.47	7.69	9.53	59.84	7.41	9.97
36	51	195—196	−16.5° (*c* 1.0, DMF)[g]	$C_{27}H_{34}O_8N_4$	59.62	6.46	10.00	59.77	6.32	10.33
37	83	152—154	−8.9° (*c* 1.0, DMF)[f]	$C_{30}H_{34}O_7N_2$	67.76	6.58	5.80	67.40	6.41	5.24
39	63	174—177	−5.1° (*c* 1.0, DMF)[j]	$C_{27}H_{35}O_9N_3$	59.29	6.44	7.66	59.44	6.47	7.70
41[a]	95	203—206	−14.1° (*c* 1.0, DMF)[j]	$C_{40}H_{58}O_{12}N_6\cdot H_2O$	57.99	7.13	10.11	57.68	7.26	10.09
42	95	213—214	−12.6° (*c* 1.0, DMF)[j]	$C_{38}H_{56}O_{11}N_8$	57.29	7.33	13.82	56.99	7.05	13.99
43	99	75—81	−21.7° (*c* 1.0, DMF)[g]	$C_{33}H_{38}O_8N_4\cdot 1/2H_2O$	63.42	6.26	8.22	63.14	6.26	8.93
45	77	176—178	−29.5° (*c* 1.0, DMF)[g]	$C_{31}H_{40}O_{10}N_6\cdot 1/2H_2O$	55.65	6.03	12.39	55.93	6.21	12.62
47[a]	91	208—210	−17.7° (*c* 1.0, DMF)[g]	$C_{61}H_{86}O_{19}N_{12}\cdot 2H_2O$	54.60	6.62	13.18	55.19	6.83	12.66
48[a]	58	195—199	−14.8° (*c* 0.5, DMF)[j]	$C_{56}H_{79}O_{17}N_{12}Cl\cdot 3H_2O$	52.30	6.53	12.77	52.47	6.68	13.11
50	61	91—96	−22.8° (*c* 1.0, EtOH)[e]	$C_{20}H_{30}O_6N_2S$	56.68	7.07	6.77	56.32	7.09	6.57
51	90	163—166	−13.9° (*c* 1.0, DMF)[g]	$C_{18}H_{28}O_5N_4S\cdot 1/2H_2O$	51.36	6.62	13.76	51.29	6.93	13.29
52	70	117—119	−15.3° (*c* 1.0, DMF)[h]	$C_{19}H_{28}O_6N_2$	59.72	7.54	7.47	59.98	7.42	7.36
54	77	202—204	−24.7° (*c* 1.0, DMF)[g]	$C_{23}H_{34}O_8N_4$	55.71	7.06	12.07	55.86	6.93	11.33
56	70	212	−18.9° (*c* 1.0, DMF)[h]	$C_{33}H_{52}O_{11}N_6S\cdot 1/2H_2O$	51.92	7.08	11.20	52.23	7.09	11.08
57	75	204—205	−16.9° (*c* 1.0, DMF)[g]	$C_{31}H_{50}O_{10}N_8S\cdot H_2O$	50.33	6.96	14.86	49.99	7.04	15.04

Table 1. (Continued)

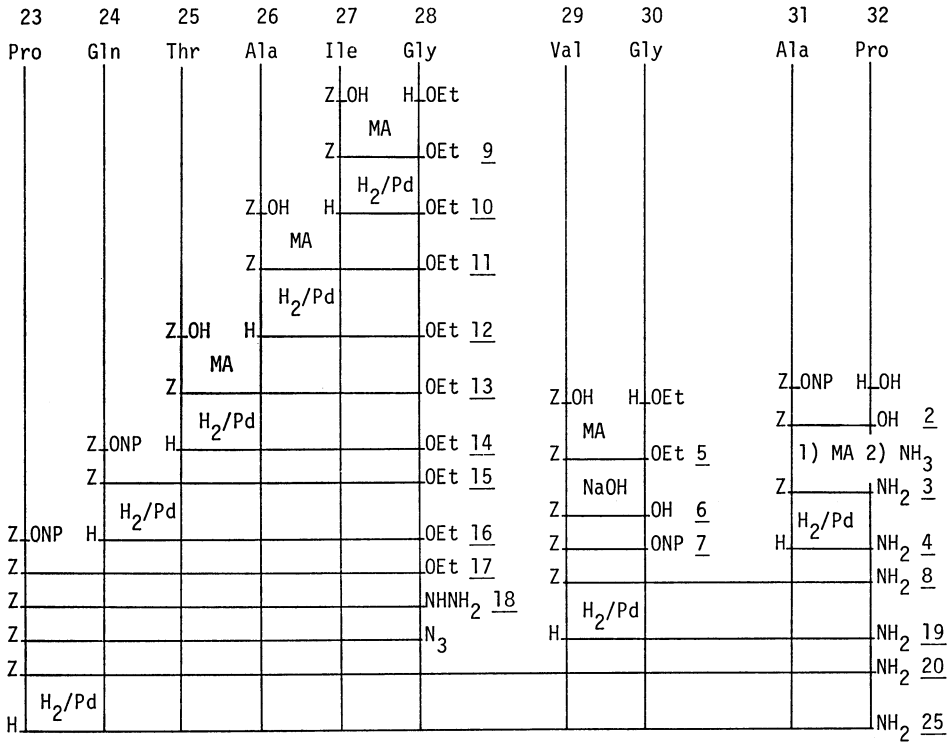
Compd	Yield (%)	Mp (°C)	$[\alpha]_D$	Molecular formula	Elemental analysis (%) Found C	H	N	Calcd C	H	N
58	44	139—141	−27.7° (*c* 1.0, DMF)[h]	$C_{24}H_{30}O_6N_2S$	60.50	6.12	5.84	60.73	6.37	5.90
60[a]	67	202	−23.2° (*c* 1.0, DMF)[j]	$C_{47}H_{70}O_{14}N_8S_2$	54.12	6.78	10.76	54.53	6.82	10.82
61	89	201—203	−21.0° (*c* 1.0, DMF)[g]	$C_{45}H_{68}O_{13}N_{10}S_2 \cdot 2H_2O$	51.42	6.75	12.88	51.12	6.68	13.25
62[a]	66	198	−25.2° (*c* 0.5, DMF)[g]	$C_{31}H_{54}O_{13}N_{10}S_2 \cdot CH_3COOH \cdot H_2O$	43.40	6.52	14.55	43.22	6.60	15.27

a) For details see Experimental. b) Lit,[14] mp 168—169°C. c) At 26 °C. d) At 23 °C. e) At 22 °C. f) At 18 °C. g) At 20 °C. h) At 21 °C. i) At 25 °C. j) At 24 °C.

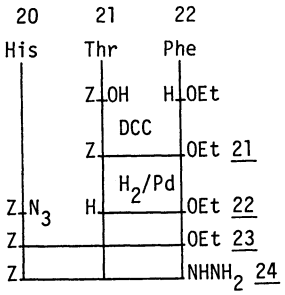
given in Table 1.

Positions 23—32. Acylation of free proline with benzyloxycarbonylalanine *p*-nitrophenyl ester followed by a gel filtration on Sephadex LH-20 yielded benzyloxycarbonylalanylproline (**2**) nearly in a theoretical yield. The mixed anhydride prepared from **2** and isobutyl chloroformate was ammonolyzed in tetrahydrofuran to give benzyloxycarbonylalanylprolinamide (**3**) in a 86% yield. This route is applicable to the preparation of a dipeptide containing prolinamide, since it is much more convenient for preparation of **3** than other ones starting with prolinamide or *via* ammonolysis of benzyloxycarbonylalanylproline ethyl ester. The azide formation from benzyloxycarbonylprolylglutaminylthreonylalnanylisoleucylglycine hydrazide (**18**) was slow. The amine component, valylglycylalanylprolinamide (**19**), should be added to the azide solution after the hydrazide is consumed completely, in order to avoid serious difficulties in the separation of the desired decapeptide amide (**20**) from the remaining hydrazide.

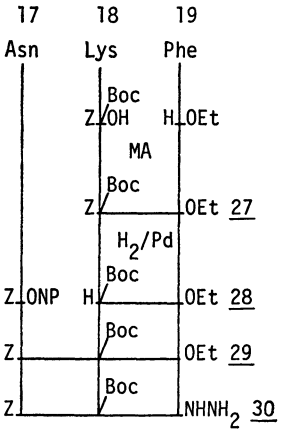
Positions 20—32. Benzyloxycarbonylhistidylthreonylphenylalanine hydrazide (**24**) was converted into the azide, which was allowed to couple with the C-terminal decapeptide amide (**25**). The product was hydrogenated and applied to a gel filtration. Further purification was performed by means of partition chromatography on Sephadex with the system of 1-butanol–acetic acid–water. The eluate was monitored at 254 nm, three compounds (peptide A, B, and C) being obtained after removal of the solvent. The amino acid compositions in acid hydrolysates of those peptides agreed with the theoretical value for peptide **26**. By aminopeptidase M digestion, however, peptide A left undigested core of threonylphenylalanylprolylglutaminylthreonylalanine,



Scheme 1. Preparation of fragment positions at 17—32.



Scheme 2. Preparation of fragment positions at 20—22.

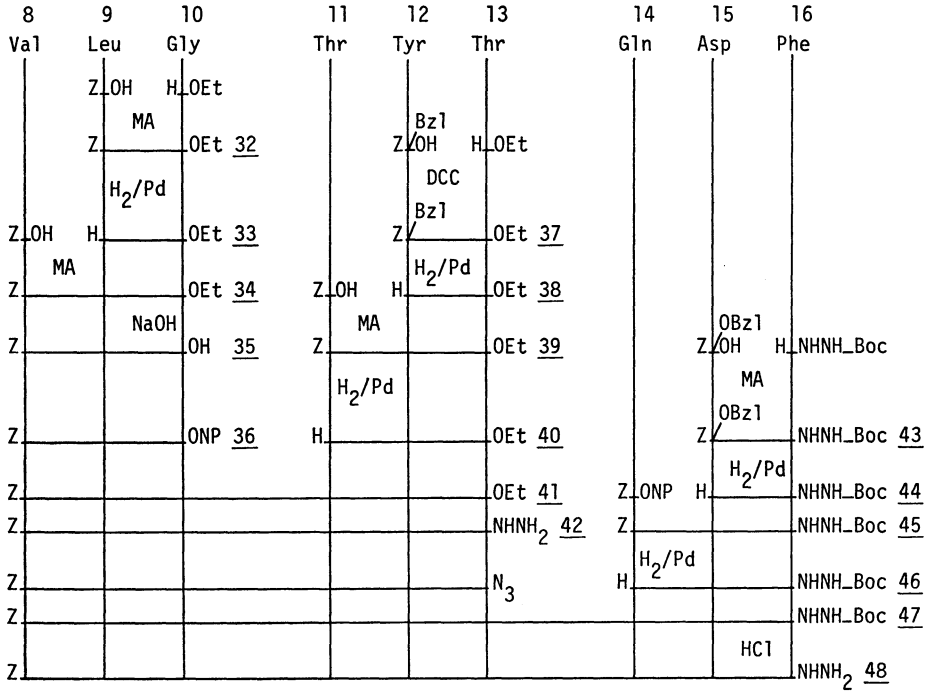


Scheme 3. Preparation of fragment positions at 17—19.

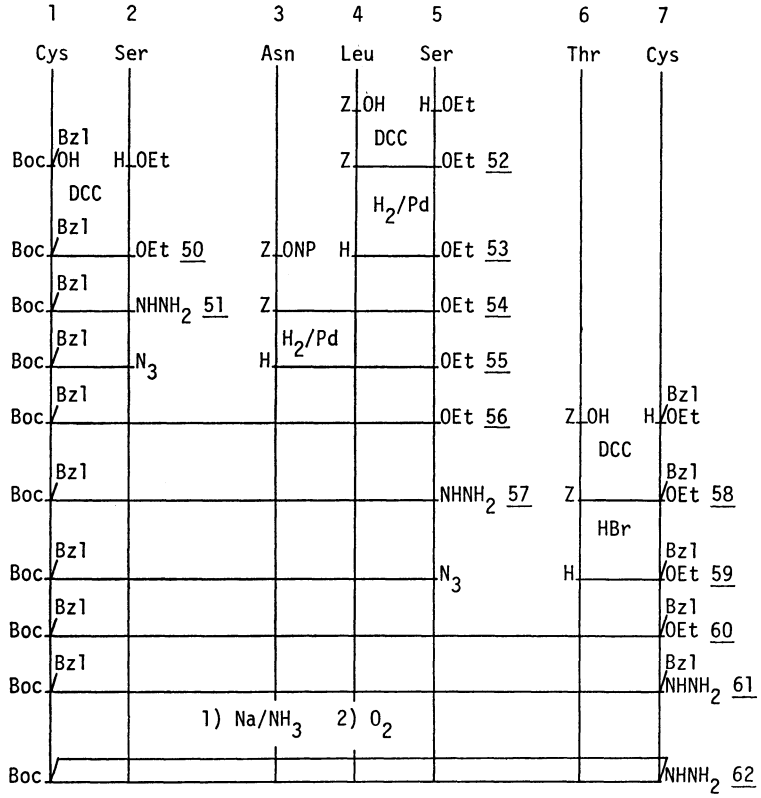
while peptides B and C gave the expected amino acid ratios. The results and other data[13] on aminopeptidase M from our laboratory suggest the presence of D-phenylalanine in peptide A. No difference was observed between peptide B and C on TLC with several solvent systems and between the results of Edman degradation. Peptide C was positive on Beilstein test, while B was negative. Thus, the two peptides, B and C, were thought to be the acetate and the hydrochloride, respectively, of the desired peptide (**26**) and were used for further elongation.

Positions 17—32. Rapid formation of cyclo-N^{ε}-*t*-butoxycarbonyllysylphenylalanyl occurred during the hydrogenolysis of N^{α}-benzyloxycarbonyl-N^{ε}-*t*-butoxycarbonyllysylphenylalanine ethyl ester (**27**) even in the presence of an equivalent amount of acetic acid. N^{ε}-*t*-butoxycarbonyllysylphenylalanine ethyl ester (**28**), the desired product, thus was obtained by use of excess acetic acid. The tridecapeptide (**26**) was acylated by the azide derived from benzyloxycarbonylasparaginyl-N^{ε}-*t*-butoxycarbonyllysylphenylalanine hydrazide (**30**). A gel filtration of the product after hydrogenolysis gave the hexadecapeptide amide (**31**).

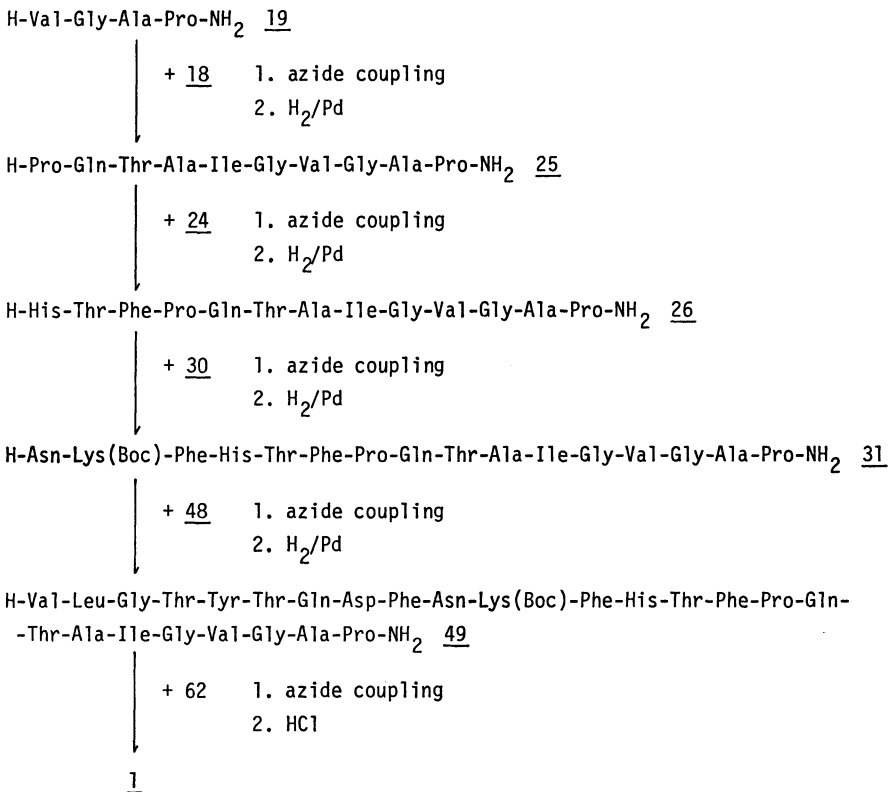
Positions 8—32. Tyrosine was introduced to the peptide chain as *N*-benzyloxycarbonyl-*O*-benzyl ester. The protection of the side functional group was removed together with the N-protecting group prior to the further elongation. Benzyloxycarbonylvalylleucylglycine (**35**) was coupled with threonyltyrosylthreonine ethyl ester (**40**) by the two procedures. By the dicyclohexylcarbodiimide/*N*-hydroxysuccinimide method, the yield of the hexapeptide (**41**) was 69%, while the use of benzyloxycarbonylvalylleucylglycine *p*-nitrophenyl ester (**36**) improved the yield to 95%. In our preliminary experiments, benzyloxycarbonylvalylleucylglycylthreonyltyrosylthreonylglutaminyl-β-*t*-butylaspartylphenylalanine ethyl ester could not be converted into the hydrazide under mild conditions. The use of the protected hydrazide at the C-terminal of the nonapeptide made it difficult to find a suitable blocking group for β-carboxyl function of the aspartyl residue. The presence of the free β-carboxyl group in aspartylphenylalanine



Scheme 4. Preparation of fragment positions at 8—16.



Scheme 5. Preparation of fragment positions at 1—7.



Scheme 6. Preparation of [2-serine, 8-valine]-human calcitonin (**1**) by stepwise fragment condensation.

t-butoxyhydrazide (**44**), however, caused no difficulty for the construction of tripeptide (**45**) and the longer peptides. In the hydrogenolysis of the *t*-butoxycarbonylhydrazides, **43** and **45**, some by-products formed, especially after contact with hydrogen for a long time, but their amount was negligibly small when the hydrogenolysis was performed in a short period. The coupling of glutaminylaspartylphenylalanine *t*-butoxycarbonylhydrazide (**46**) with the azide derived from benzyloxycarbonylvalylleucylglycylthreonyltyrosylthreonine hydrazide (**42**) yielded protected nonapeptide (**47**). The *t*-butoxycarbonyl group of **47** was removed by concentrated hydrochloric acid. The use of trifluoroacetic acid for the deprotection unexpectedly gave a mixture showing multiple spots on TLC. Acylation of the hexadecapeptide amide (**31**) with the azide derived from **48** produced the pentacosapeptide amide, which was purified after removal of the N-protecting group by a gel filtration and succeeding partition chromatography on Sephadex to give **49**.

Positions 1—7. The azide prepared from *N-t*-butoxycarbonyl-*S*-benzylcysteinylserylasparaginylleucylserine hydrazide (**57**) was allowed to couple with threonyl-*S*-benzylcysteine ethyl ester (**59**) to yield the heptapeptide ester (**60**), which was led to the hydrazide (**61**) prior to the formation of disulfide bridge, lest the ester group should be ammonolysed during the course of sodium/ammonia treatment. After the removal of *S*-benzyl group, the thiols of cysteinyl residues were oxidized in a dilute solution by air to disulfide in a slightly basic region. In neutral or lower pH region, the thiol was not oxidized completely even after contact with air for a long time. Gel filtration on Sephadex LH-20 using DMF was successful for the purification of the product (**62**).

Positions 1—32. Two equivalent amount of the azide prepared from **62** was used for the acylation of pentacosapeptide amide (**49**). The *t*-butoxycarbonyl groups of the crude product were removed by concentrated hydrochloric acid treatment at −5 °C for 10 min to give **1**. Analog **1** was purified by gel filtration and carboxymethyl cellulose chromatography. The homogeneity of **1** was confirmed by tlc in several solvent systems and amino acid analyses of the acid hydrolysates, as well as of the aminopeptidase M digests.

Analog **1** exhibited hypocalcemic activity of 80—120 MRC units/mg. The potency is similar to or a little higher than that of human calcitonin but much lower than that of eel or salmon calcitonins. This suggests that the high activity of salmon or eel hormone is not ascribable only to the amino acid sequence of their N-terminal decapeptide and that the presence of methionine in position 8 of human calcitonin is not responsible by itself for the low potency of the hormone.

Experimental

Melting points are uncorrected. Amino acid analyses were carried out on a JLC-6AS automatic analyzer (JEOL) after acid hydrolysis with HCl of constant bp (110 °C, 16—20 h), or aminopeptidase M (Röhn and Haas Co.) digestion. The theoretical values of amino acid ratios are shown in parentheses after each result. Edman degradation was carried out with a JAC-47K automatic sequencer (JEOL). Biological activities were measured according to the method of Cooper *et al.*[15] The N-protected dipeptides, **5**,[16] **9**,[17] **21**, **27**, **32**,[18] **37**, **43**, **50**, **52**, and **58**, were prepared by the usual methods as shown in the Schemes. The longer peptides, **11**, **13**, **15**, **17**, **23**, **29**, **34**, **39**, **45**, and **54**, were synthesized by the stepwise elongation method. Compounds **8**[19] and **56** were prepared from the subfragments by the active ester method and the azide method, respectively. Hydrazides, **18**, **24**, **30**, **42**, **51**, **57**, and **61**, were obtained from the corresponding ethyl esters. The active esters, **7**[20] and **36**, were prepared from **6**[21] and **35** by the use of DDC. Compounds **6** and **35** were prepared from **5** and **34**, respectively, by saponification. The N-terminal free peptides, **10**, **12**, **14**, **16**, **19**, **22**, **33**, **38**, **40**, **44**, **46**, **53**, **55**, and **59**, were obtained by the usual catalytic hydrogenolysis of the corresponding benzyloxycarbonyl derivates. In TLC, silica gel G (Merck and Co.) plate (Sh), silica gel-precoated plate (Merck and Co.) (Sp) and cellulose-precoated plate (Avicel)(C) were used with the following solvent systems: chloroform–methanol, 95/15 (1a), 95/5 (1b); chloroform–methanol–acetic acid, 95/5/3 (2a), 36/4/1 (2b); 1-butanol–acetic acid–water, 4/1/1 (3a), 3/1/1 (3b), 10/1/1 (3c); 1-butanol–1 M acetic acid, 2/1 upper phase (4a), 1/1 upper phase (4b); 2-propanol–water, 4/1 (5); 1-butanol–pyridine–acetic acid–water, 16/3/1/4 (6); 2-butanol–15 M ammonia–water, 24/1/5 (7).

Cbz-Ala-Pro-OH (2). Cbz-Ala-ONP (6.50 g) was added to a suspension of proline (2.30 g) and triethylamine (2.78 ml) in DMF (50 ml). The mixture was stirred at room temperature for 4 days and the solvent was removed. The residue was dissolved in ethyl acetate and washed with 1 M HCl and water. An oil obtained after removal of solvent of the organic layer was dissolved in a small amount of ethanol. About half of the solution was applied onto a Sephadex LH-20 column (2.5×76 cm) and the product was eluted with ethanol. The colorless fractions containing the peptide were evaporated to give an oil. The rest of the ethanol solution was treated in the same way. The combined product was dried in reduced pressure; 5.70 g. R_f (Sh, 1b) 0.33.

Cbz-Ala-Pro-NH$_2$ (3). BCC (2.24 ml) was added with stirring to a solution of **2** (5.35 g) and *N*-methylmorpholine (1.69 g) in THF (200 ml) at −15 °C. After 5 min, dried ammonia was passed into the mixture at −15 °C for 20 min and then at ice-bath temperature for 1.5 h. The mixture was stirred for 2 h in the ice-bath and at room temperature for 2 days. The residue obtained after removal of the solvent was dissolved in chloroform–water (100 ml and 20 ml each), and the aqueous phase was extracted with chloroform (70 ml×2). The organic phases were combined and washed with water (10 ml×3). After removal of the chloroform the residue was recrystallized from ethyl acetate; 4.60 g. R_f (Sh, 2a) 0.67.

Cbz-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH$_2$ (20). HCl/THF (3.08 M, 1.95 ml) was added dropwise to a suspension of **18** (1.47 g) in DMF (20 ml) at −40 °C. 1-Butyl nitrite (0.25 ml) was added to the suspension. The mixture was stirred for 40 min at −20 °C, and then cooled at −40 °C. Triethylamine (0.83 ml) was added to the azide solution followed by **19** (2 mM) and triethylamine (0.28 ml) in DMF (10 ml). The mixture was stirred at ice-bath temperature for 3 days. After removal of the solvent, the residue was triturated with water and the solid collected was washed with 1 M citric acid and water. The purified decapeptide amide was obtained by reprecipitation from DMF–ether; 1.69 g. R_f (Sh, 3a) 0.41, (Sh, 5) 0.52. Amino acid ratios in acid hydrolysate: Thr 0.88 (1), Glu 1.08 (1), Pro 2.05 (2), Gly 2.00 (2), Ala 2.08 (2), Val 1.01 (1), Ile 0.99 (1).

H-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2 (25). The protected decapeptide amide, **20** (2.09 g), was hydrogenated in the presence of Pd black in DMF (200 ml) and water (40 ml) at 40—50 °C for 3 h. The mixture was diluted with additional water (40 ml) and the hydrogenolysis was continued at 40—50 °C for 3 h and at 70—80 °C for 1 h. After removal of the catalyst the solvent was evaporated to give a solid in a quantitative yield. R_f (Sh, 3b) 0.20.

H-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2 Hydrochloride (26). HCl/THF (1.70 M, 5.56 ml) was added to a solution of **24** (2.40 mM, 1.32 g) in DMF (8 ml) at −40 °C. 1-Butyl nitrite (0.288 ml) was added dropwise and the mixture was stirred at −25 °C for 20 min and then cooled at −40 °C. To the azide solution thus obtained were added triethylamine (1.34 ml) and **25** (2.00 mM) in HMPA (10 ml) and DMF (6 ml). Stirring was continued at ice-bath temperature for 3 days and the solvent was removed. The residue was dissolved in water-saturated 1-butanol and washed with 5% $NaHCO_3$ and water. A syrup which remained after removal of the solvent was solidified by addition of ether. The solid (2.68 g) was hydrogenated in ethanol (100 ml) and water (20 ml) containing HCl. After removal of the catalyst and the solvent, the product was applied to a Sephadex G-10 column (3×32.5 cm) and eluted with water. Fractions of 5 ml each were collected and the desired peptide was obtained from fractions 13—20. The material from fractions 21—25 was rechromatographed on the same column, and an additional crop was obtained. Colorless powder (2.33 g), R_f (C, 4a) 0.18 and 0.14. A part of the product (0.996 g) was dissolved in a small amount of the upper phase of 1-butanol–acetic acid–water (5/1/5) and this was applied to a Sephadex G-25 column (3.5×42.5 cm) which had been equilibrated with the lower phase of the solvent system and then washed with the upper phase. The elution was carried out with the upper phase and the optical density of the eluate was observed at 254 nm to show three major peaks. Peptide A (0.119 g), B (0.079 g), and C (0.305 g) were obtained respectively from the fractions corresponding to the peaks. Peptide A: R_f (C, 4a) 0.18. Amino acid ratios in acid hydrolysate; His 1.17 (1), Thr 1.91 (2), Glu 1.02 (1), Pro 1.96 (2), Gly 1.99 (2), Ala 1.91 (2), Val 1.02 (1), Ile 1.01 (1), Phe 0.98 (1). Amino acid ratios in AP-M digest; His 1.00 (1), Thr 0.34 (2), Gln 0.15 (1), Gly 1.68 (2), Ala 0.48 (1), Val 0.90 (1), Ile 1.00 (1). Peptide B: R_f (C, 4a) 0.14. Amino acid ratios in acid hydrolysate; His 1.00 (1), Thr 1.89 (2), Glu 1.15 (1), Pro 2.17 (2), Gly 1.99 (2), Ala 1.98 (2), Val 0.93 (1), Ile 0.98 (1), Phe 0.91 (1). Amino acid ratios in AP-M digest; His 1.03 (1), Thr 1.88 (2), Gln 1.07 (1), Gly 2.08 (2), Ala 1.07 (1), Val 0.96 (1), Ile 0.91 (1). Peptide C: R_f (C, 4a) 0.14. Amino acid ratios in acid hydrolysate; His 1.07 (1), Thr 1.96 (2), Glu 1.14 (1), Pro 2.13 (2), Gly 1.93 (2), Ala 1.90 (2), Val 0.97 (1), Ile 0.96 (1), Phe 0.92 (1). Amino acid ratios in AP-M digest; His 1.03 (1), Thr 1.91 (2), Gln 1.12 (1), Gly 1.96 (2), Ala 1.05 (1), Val 0.96 (1), Ile 0.98 (1).

Peptide B (0.689 g) obtained by repeating a similar procedure was dissolved in 0.05 M HCl (24 ml). After removal of insoluble materials, the solvent was evaporated to give the hydrochloride, peptide C, as solid (0.644 g). Found: C, 47.96; H, 6.84; N, 15.94; Cl, 6.58%. Calcd for $C_{62}H_{93}O_{17}N_{18}\cdot 3HCl\cdot 5H_2O$: C, 47.46; H, 6.95; N, 15.95; Cl; 7.13%.

H-Lys(Boc)-Phe-OEt Acetate (28). The protected peptide (**27**) (7.50 g) was hydrogenated in ethanol (250 ml) and acetic acid (3.75 ml) to give the desired product. R_f (Sh, 3a) 0.75. However, when the hydrogenolysis was carried out with an equivalent amount of acetic acid, a product having high melting point and poor solubility was obtained. This was recrystallized from DMF and supposed to be cyclo-Lys(Boc)-Phe. R_f (Sh, 1a) 0.41. mp 246 °C. $[\alpha]_D^{20}$ −24.7° (*c* 0.6, DMF). (lit,[22]) mp 224—226 °C, $[\alpha]_D^{20}$ −24.7° (*c* 0.5, DMF). Found: C, 64.50; H, 7.90; N, 11.31%. Calcd for $C_{20}H_{28}O_4N_2$: C, 63.99; H, 7.78; N, 11.19%.

H-Asn-Lys(Boc)-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2 Acetate (31). To the azide solution, prepared from **30** (0.36 mM, 0.237 g) in DMF (1 ml) as described for Z-His-Thr-Phe-N_3 in the preparation of **26**, was added a solution of **26** (0.30 mM, 0.410 g) and triethylamine (0.083 ml) in HMPA (3 ml) and DMF (3.5 ml). The mixture was stirred at ice-bath temperature for 4 days and the solvent was removed. The residue was washed and then solidified as in the preparation of **26**. The crude product (0.620 g) was hydrogenated in ethanol (50 ml) and water (10 ml) containing acetic acid (0.12 ml). After removal of the catalyst and the solvent, the product was applied to a Sephadex G-10 column (3×32.5 cm) and eluted with water. Fractions of 5 ml each were collected and the desired peptide was obtained from fractions 11—18. 0.375 g (57%). R_f (Sp, 6) 0.20 with a trace spot at 0.24, (Sp, 3a) 0.31 with a trace spot at 0.38. Amino acid ratios in acid hydrolysate: Asp 1.06 (1), Thr 1.94 (2), Glu 0.98 (1), Pro 1.94 (2), Gly 1.98 (2), Ala 1.97 (2), Val 1.01 (1), Ile 1.00 (1), Phe 2.01 (2), Lys 1.09 (1), His 1.09 (1).

Cbz-Val-Leu-Gly-Thr-Tyr-Thr-OEt (41). a) Triethylamine (0.695 ml) was added dropwise to an ice-cold solution of **36** (2.32 g) and **40** (5.0 mM) in DMF (20 ml). The mixture was stirred at room temperature for 2 days. After removal of the solvent the residue was dissolved in water-saturated 1-butanol and washed with 5% citric acid and water. The solvent was evaporated to give a solid, which was recrystallized from DMF–ether. 3.67 g. R_f (Sp, 1a) 0.27, (Sp, 2a) 0.08. b) DCC (0.825 g) in DMF (4 ml) was added in an ice-cold solution of **35** (1.69 g), **40** (3.76 mM) and HOSu (0.460 g) with triethylamine (0.523 ml) in DMF (25 ml). After 2.5 h, HOSu (0.460 g) was added and the mixture was stirred at room temperature overnight. The insoluble substances were removed and the filtrate was evaporated to give a solid, which was washed and reprecipitated from DMF–ether; 2.10 g (69%).

Cbz-Val-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-NHNH-Boc (47). To an azide solution prepared from **42** (2.00 g) in DMF (8 ml) as in the preparation of **26** was added a solution of **46** (1.54 g) and triethylamine (0.417 ml) in DMF (12 ml). The mixture was stirred at ice-bath temperature for 4 days and the solvent was removed. After the usual treatment, the desired peptide was recrystallized from DMF–ether; 2.94 g. R_f (Sp, 3a) 0.69 (Sp, 6) 0.65. Amino acid ratios in acid hydrolysate: Asp 0.93 (1), Thr 2.05 (2), Glu 0.85 (1), Gly 0.95 (1), Val 0.99 (1), Leu 1.06 (1), Tyr 1.12 (1), Phe 1.06 (1).

Cbz-Val-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-$NHNH_2$ Hydrochloride (48). The Boc-hydrazide (**47**) (0.30 g) was dissolved in ice-cold 12 M HCl. After 5 min, the solution was poured into NaCl-saturated water (30 ml) at ice-bath temperature. The resulting precipitate was collected and washed with saturated NaCl and with a small amount of water. The solid was dried *in vacuo* over NaOH pellets and recrystallized from DMF–ethyl acetate; 0.17 g. R_f (Sp, 3a) 0.51.

H-Val-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys(Boc)-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2 (49). To the azide solution prepared from **48** (0.200 mM, 0.242 g) in DMF (0.8 ml) in the usual way was added a solution of **31** (0.173 mM, 0.320 g) and triethylamine (10% in DMF, 0.481 ml) in DMF (2.1 ml). The mixture was stirred at ice-bath temperature for 4 days and the solvent was

removed. The residue was dissolved in water-saturated 1-butanol and washed with water. The butanol solution was concentrated into a syrup, to which was added ether and the resulting precipitate was collected. 0.538 g. A part of the crude product (0.240 g) was hydrogenated in ethanol (50 ml) and water (10 ml) in the presence of acetic acid (0.09 ml). After removal of the catalyst and the solvent, the aqueous solution of the residue was applied to a Sephadex G-25 column (3×22 cm), and eluted with 0.05 M ammonium acetate. Fractions of 3 ml each were collected and the desired peptide was obtained from fractions 16—22. R_f (Sp, 3a) 0.47 with minor spots at 0.45 and 0.50. The peptide was further applied to a Sephadex G-25 column (3.5×41 cm), which had been equilibrated with 1-butanol—0.5 M acetic acid (1/1) in a similar way to that described in the preparation of **26**, and was eluted with the upper phase of the solvent system. Fractions of 3 ml each were collected and the purified peptide was obtained from fractions 52—68. 0.090 g. R_f (Sp, 6) 0.04, (Sp, 5) 0.74, (Sp, 3a) 0.43, (C, 4a) 0.05. Amino acid ratios in acid hydrolysate: Asp 1.96 (2), Thr 3.82 (4), Glu 1.91 (2), Pro 2.07 (2), Gly 3.18 (3), Ala 2.13 (2), Val 1.90 (2), Ile 1.04 (1), Leu 0.87 (1), Tyr 0.97 (1), Phe 3.01 (3), Lys 1.04 (1), His 1.11 (1).

*Boc-Cys(Bzl)-Ser-Asn-Leu-Ser-Thr-Cys(Bzl)-OEt (**60**).* To an azide solution prepared as usual from **57** (6.30 g) in DMF (50 ml) and THF (10 ml) was added a solution of **59** (9.25 mM) and triethylamine (1.29 ml) in DMF (50 ml). The mixture was stirred at ice-bath temperature for 3 days. Cold water (200 ml) was poured into the mixture and the resulting precipitate was collected and washed as usual; 6.00 g. R_f (Sh, 2b) 0.30 with a trace spot at 0.62. A part of the product (3.00 g) was applied to a silica gel column (3×38 cm) and eluted with chloroform-methanol (94/4). The purified peptide was obtained in a considerably low yield because of the strong adsorption of the peptide to the gel; 1.10 g. R_f (Sh, 2b) 0.30, (Sh, 3c) 0.75.

*Boc-Cys-Ser-Asn-Leu-Ser-Thr-Cys-$NHNH_2$ (**62**).* The heptapeptide hydrazide, **61** (0.256 g), was dissolved in liquid ammonia (about 50 ml). To the solution was added sodium in a small portions until the blue color of the solution remained for 10 min (0.087 g of sodium was required for a period of 25 min). NH_4Cl (0.282 g) was added to the mixture and the ammonia was evaporated under reduced pressure. The residue was dissolved in water (200 ml) and the solution (pH 8.05) was stirred vigorously in a stream of air for 3 h. The desired peptide was extracted with 1-butanol (40 ml×4, 20 ml×5). The combined organic phase was washed with water (20 ml) and acidified with acetic acid, and then the solvent was evaporated. The residue was applied to a Sephadex LH-20 column (3×32 cm) and eluted with DMF. Fractions of 3 ml each were collected and the desired peptide was located in fractions 21—24. The fractions were combined and concentrated to a small volume and ether was added. The resulting precipitate was collected and washed with ether; 0.139 g. R_f (Sp, 3a) 0.46 with a trace spot at 0.57. Amino acid ratios in the acid hydrolysate: Asp 1.14 (1), Thr 0.94 (1), Ser 1.94 (2), Cys_2 0.93 (1), Leu 1.11 (1).

*H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2 (**1**).* To the azide solution prepared from **62** (0.050 mM, 42.1 mg) in DMF (0.8 ml) was added a solution of **49** (0.025 mM, 71.7 mg) and triethylamine (10% in DMF, 0.07 ml) in DMF (0.6 ml). The mixture was stirred at ice-bath temperature for 4 days and the solvent was removed. The residue was solidified by the addition of ether. The solid was dissolved in 12 M HCl (1 ml) at −5 °C. After 10 min, ammonium hydrogencarbonate (950 mg) was added to the solution. The solution was diluted with water (about 12 ml), adjusted to pH 3.5 with formic acid and concentrated to about 5 ml volume. The resulting precipitate was separated (ppt fraction). The solution was applied to a Sephadex G-25 column (2.5×23 cm) and eluted with 0.05 M ammonium formate (pH 3.5). Fractions of 4 ml each were collected and the eluate in fractions 13—21 was lyophilized to give a solid. The crude product was further applied to a CM-cellulose column (Serva, 1.2×28 cm) and eluted with a linear gradient of ammonium formate buffer (0.01 M, pH 4.7—0.1 M, pH 5.2, 220 ml each). Fractions of 4 ml each were collected and the eluate in fractions 53—64 was lyophilized. The solid obtained was again desalted with a Sephadex G-10 column (1.5×14 cm) to give the desired peptide **1**; 2.27 mg. R_f (Sp, 3a) 0.45, (Sp, 6) 0.21, (Sp, 7) 0.17, (C, 4b) 0.21. $[\alpha]_{231}^{24}$ −1633° (*c* 0.2, 1 M acetic acid). Amino acid ratios in acid hydrolysate: His 0.98 (1), Lys 1.09 (1), Asp 3.16 (3), Thr 4.82 (5), Ser 2.19 (2), Glu 2.07 (2), Pro 2.24 (2). Gly 3.11 (3), Ala 2.22 (2), Cys_2 0.60 (1), Val 1.82 (2), Ile 1.11 (1), Leu 1.87 (2), Tyr 0.82 (1), Phe 2.96 (3). Average recovery of the amino acids was 90%. Amino acid ratios in AP-M digest: Asp 1.00 (1), Asn 1.99 (2), Thr 4.94 (5), Ser 2.13 (2), Gln 1.92 (2), Pro 0.11 (2), Gly 3.28 (3), Ala 1.53 (2), Cys_2 0.70 (1), Val 1.92 (2), Ile 1.36 (1), Leu 1.87 (2), Tyr 0.93 (1), Phe 2.35 (3), Lys 1.02 (1), His 1.12 (1). AP-M used in this analysis cleaved the peptide bond X-Pro (X=amino acid) very slowly. The low recoveries of Phe, Pro, and Ala are due to this. Hypocalcemic activity: 80—120 MRC units/mg. The product (18.96 mg), which still contained the amine component, positions 8—32, was recovered from the "ppt fraction" and the void volume fractions of CM-cellulose chromatography.

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2) Amino acids used are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature. Other abbreviations are as follows; MRC=Medical Research Council, AP-M=aminopeptidase M, Cbz or Z=benzyloxycarbonyl, Boc=*t*-butoxycarbonyl, Bzl=benzyl, OBzl=benzyloxy, ONP=*p*-nitrophenyl, BCC=isobutyl chloroformate, DCC=dicyclohexylcarbodiimide, HOSu=*N*-hydroxysuccinimide, HMPA=hexamethylphosphoric triamide.

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