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Studies on Peptides. CXV.[1,2] Synthesis of Hylambatin, a New Frog Skin Peptide of the Kassinin Family

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Hylambatin, a dodecapeptide amide isolated from the skin of an African rhacopharid frog (*Hylambates maculatus*), was synthesized by two routes, by successive condensations of four fragments, *i.e.*, (1—3), (4—5), (6—10), and (11—12) in one case and (1—3), (4—7), (8—10), and (11—12) in the other, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The *S*-alkylation observable during N^{α}-deprotection of Met-containing peptides with trifluoroacetic acid was found to be suppressed more effectively by the use of 3,5-dimethylanisole containing 2% ethanedithiol as a scavenger system than by the use of anisole containing 2% ethanedithiol.

The contractile potency of synthetic hylambatin in isolated guinea-pig duodenum was 0.42 times that of synthetic kassinin.

Keywords——hylambatin; frog skin peptide; tachykinin; N^{G}-4-methoxy-2,6-dimethylbenzenesulfonylarginine; 3,5-dimethylanisole; trifluoromethanesulfonic acid deprotection; thioanisole-mediated deprotection; smooth muscle contractile activity

In 1981, Yasuhara *et al.*[3] elucidated the structure of a new tachykinin peptide, named hylambatin, isolated from the skin of an African rhacopharid frog (*Hylambates maculatus*). This dodecapeptide amide is homologous to kassinin,[4] another frog skin peptide of the tachykinin family, but has the characteristic dipeptide unit, Met–Met–NH_2, at the C-terminus instead of Leu–Met–NH_2 of the other tachykinins,[5] as shown in Fig. 1. This frog skin contains an additional dodecapeptide amide, named [Glu^2, Pro^5]-kassinin (hylambates-kassinin), synthesis of which was previously reported.[1]

In this paper, we wish to report the synthesis of hylambatin, which was performed in two ways without protection of the two Met-residues. The *S*-alkylation[6,7] observable during N^{α}-deprotection of Met-containing peptides with TFA was suppressed by the use of anisole

H–Asp–②–Pro–④–⑤–Asp–⑦–Phe–⑨–Gly–⑪–Met–NH_2

Positions	2	4	5	7	9	11
Hylambatin	Pro	Asp	Pro	Arg	Tyr	Met
Hylambates-kassinin	Glu	Lys	Pro	Gln	Val	Leu
Kassinin	Val	Lys	Ser	Gln	Val	Leu

Fig. 1. Structures of Hylambatin and Related Peptides

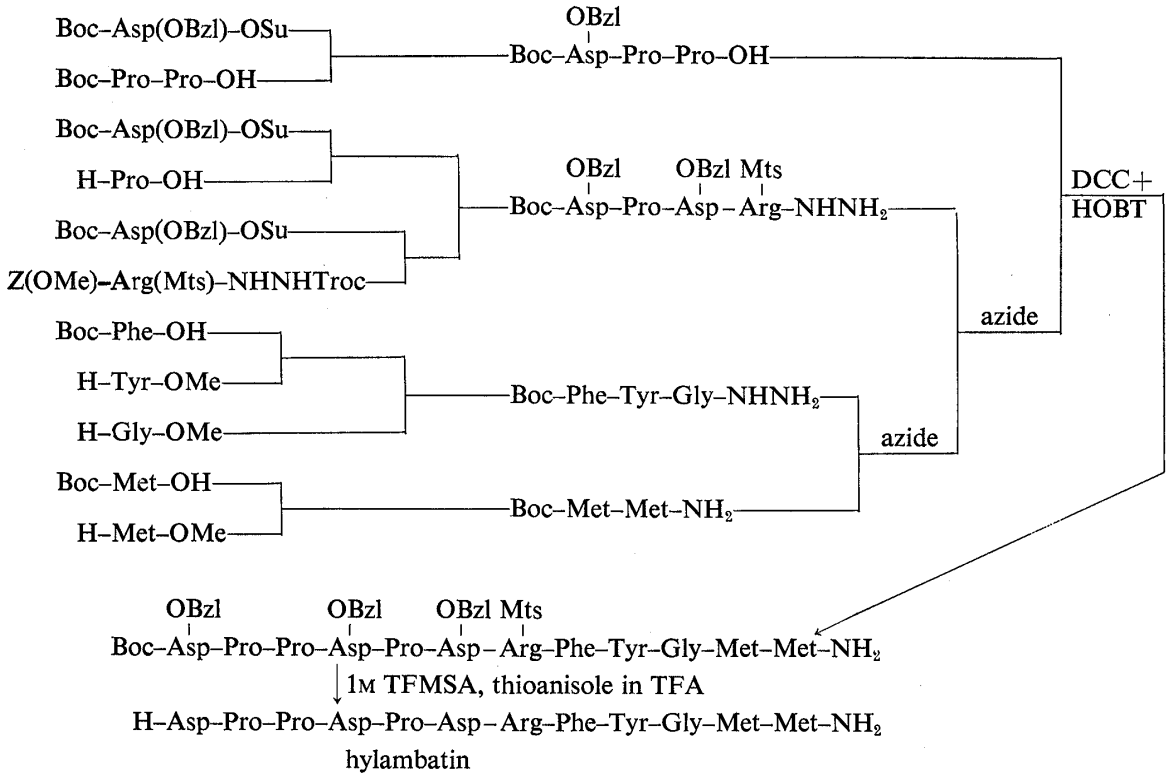

Fig. 2. Synthetic Scheme for Hylambatin

containing 2% EDT[6,8] in our initial synthesis of hylambatin, as had been done in the above-mentioned synthesis of [Glu^2, Pro^5]-kassinin, but it was found later that this side reaction could be suppressed more efficiently by 3,5-dimethylanisole than by the above scavenger system. Using this new scavenger, we repeated the synthesis of hylambatin.

Figure 2 shows our initial synthetic route to hylambatin. Starting with Boc–Met–Met–NH_2, three fragments were successively condensed by the azide[9] or the DCC+HOBT procedure[10] to construct the dodecapeptide chain of this new amphibian skin peptide. Of these fragments, Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–$NHNH_2$ was prepared with the aid of Troc–$NHNH_2$,[11] the protecting group of which could be removed easily by treatment with Zn in acetic acid.[12] As stated above, the Boc group was removed from intermediates by treatment with TFA in the presence of anisole containing EDT, prior to each condensation, in order to suppress partial *S*-alkylation at the Met residues. However, we encountered some technical difficulty, because of the presence of two Met residues, sensitive to such by-product formation. In the last step, all protecting groups employed, Boc, Bzl and Mts, were removed from the protected hylambatin by treatment with 1 M TFMSA–thioanisole in TFA[13] in the presence of *m*-cresol,[14] as reported in our previous synthesis of hylambates-kassinin. The deprotected peptide was incubated with DTT[15] and then purified by gel-filtration on Sephadex G-25, followed by partition chromatography on Sephadex G-25 using the solvent system of *n*-BuOH–AcOH–H_2O (4 : 1 : 5). Even after incubation with DTT, purification of Met-containing peptides always suffers some decrease in yield due to partial sulfoxide formation during manipulation. Partition chromatography as mentioned above was an effective tool for separating sulfoxide peptides of this size, as demonstrated in our previous synthesis of gastrin releasing peptide[16] which also contains two Met residues.

After this synthesis, we felt it necessary to find a more effective scavenger system than anisole–EDT during N^{α}-TFA deprotection in order to synthesize peptides containing a large number of Met residues without *S*-protection. Though by-products in the TFA deprotection,

TABLE I. Effect of Various Cation Scavengers during the TFA Treatment of Z(OMe)–Met–OH or Boc–Met–OH

Scavenger	By-product from Z(OMe)–Met–OH	By-product from Boc–Met–OH
None	71.2	37.1
Anisole	10.0	11.0
Anisole + 2% EDT	9.8	10.2
m-Cresol	4.9	8.7
Thioanisole	34.1	13.4
Skatole	6.4	8.4
3,5-Dimethylphenol	17.1	16.4
Veratrole	7.7	8.9
3,5-Dimethylanisole	7.3	7.7
3,5-Dimethylanisole + 2% EDT	3.9	2.9

Average values of three experiments (%).

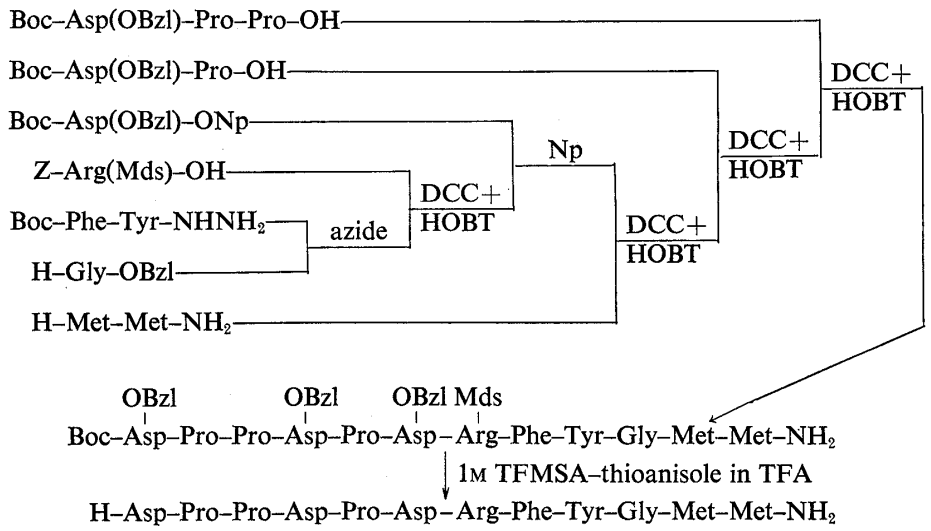

Fig. 3. Alternative Scheme for the Synthesis of Hylambatin

S-alkylated Met sulfonium salts, are easily reduced by thiols[15] or decomposed slowly to regenerate Met during manipulation, *e.g.* during treatment with methanol, formation of heterogeneous N^{α}-deprotected products cannot be fully suppressed if a large number of Met residues is present in a peptide. Z(OMe)–Met–OH or Boc–Met–OH was treated with TFA in the presence of various candidate scavengers and their abilities to trap the *p*-methoxybenzyl or the *tert*-butyl cation were examined by measurement of the amount of the by-product, *S-p*-methoxybenzyl-Met or *S-tert*-butyl-Met sulfonium salt,[6] using a Shimadzu dual-wavelength thin-layer chromatography (TLC) scanner. As shown in Table I, the amount of the by-product from the Boc group was judged to be slightly less than that from the Z(OMe) group. A substituted anisole, 3,5-dimethylanisole (DMA), was found to be more effective than anisole or anisole containing 2% EDT. Its effectiveness was further enhanced by addition of EDT.

Next, we decided to re-synthesize hylambatin using this new scavenger system, DMA–2% EDT. The alternative scheme employed for the synthesis of this Met-containing peptide is shown in Fig. 3, in which a new Arg derivative, Arg(Mds),[17] was adopted and two fragments, Boc–Asp(OBzl)–Pro–OH, and Boc–Asp(OBzl)–Arg(Mds)–Phe–Tyr-Gly–OH, were selected in order to construct the peptide backbone exclusively by the racemization-free DCC+

HOBT condensation procedure.[10]

The above pentapeptide was prepared as shown in Fig. 3. Boc–Phe–Tyr–Gly–OBzl, prepared by the azide condensation of Boc–Phe–Tyr–$NHNH_2$ with H–Gly–OBzl, was treated with TFA and the peptide chain of the resulting tripeptide was elongated by the DCC addition of Z–Arg(Mds)–OH, followed by the active ester addition[18] of Boc–Asp(OBzl)–OH. The benzyl ester group was removed by hydrogenation at the tetrapeptide stage.

On TLC, each N^{α}-deprotected peptide with TFA–DMA–2%EDT was judged to be homogeneous, and after condensation reactions, each product was easily purified by recrystallization from ethanol or methanol and ether. In the final step, all protecting groups, Boc, Bzl and Mds, were removed by treatment with 1 M TFMSA–thioanisole in TFA as described above and after gel-filtration on Sephadex G-15, the deprotected peptide was purified by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. In view of the possible sulfoxide formation during manipulation, the final product was incubated with 2-mercaptoethanol and isolated by gel-filtration on Sephadex G-10.

When the contractile potency in isolated guinea-pig duodenum was examined,[19] the relative potency of our synthetic hylambatin with respect to that of synthetic kassinin (taken as 1) was 0.42.

Experimental

General experimental methods employed in this paper are essentially the same as described in Part LXXXVIII of this series.[20] ^{1}H-Nuclear magnetic resonance (NMR) spectra were measured with a Varian CFT-20 (80 MHz) NMR spectrometer in TFA-d_1 solution with tetramethylsilane (TMS) as an internal standard. A Shimadzu dual-wavelength TLC chromatoscanner (Model CS-910) was used to measure the ninhydrin stained color intensity. TLC was performed on silica gel (Kieselgel G, Merck) routinely and on another gel (DC-Fertigplatten Kieselgel 60 F254, Merck) for scavenger investigation. *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (9 : 1), Rf_2 $CHCl_3$–MeOH–AcOH (9 : 1 : 0.5), Rf_3 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30 : 6 : 20 : 24), and Rf_6 benzene–MeOH–AcOH (7 : 2 : 1). High performance liquid chromatography (HPLC) was conducted with a Shimadzu LC-3A instrument, using a column of Cosmosil (5C18, 4.6 × 100 mm) and CH_3CN–0.02 N tetraethylammonium phosphate, pH 3.0 (20 : 80 v/v), as the eluant.

Boc–Met–Met–OMe——A mixed anhydride [prepared from 9.84 g (39.5 mmol) of Boc–Met–OH] in THF (100 ml) was added to an ice-chilled solution of H–Met–OMe [prepared from 7.90 g (39.5 mmol) of the hydrochloride] in DMF (80 ml) and the mixture was stirred in an ice-bath for 5 h. The solvent was removed by evaporation and the residue was dissolved in AcOEt. The organic phase was washed with 5% $NaHCO_3$, 5% citric acid and H_2O–NaCl, then dried over Na_2SO_4 and concentrated. Trituration of the residue with *n*-hexane afforded a powder, which was recrystallized from AcOEt and *n*-hexane; yield 12.83 g (82%), mp 61—64 °C, $[\alpha]_D^{16}$ −19.3° (*c* = 1.0, MeOH), Rf_3 0.74. *Anal.* Calcd for $C_{16}H_{30}N_2O_5S_2$: C, 48.70; H, 7.66; N, 7.10. Found: C, 48.55; H, 7.70; N, 7.12.

Boc–Met–Met–NH_2——In a sealed flask, Boc–Met–Met–OMe (12.80 g, 32.4 mmol) in MeOH (130 ml) was treated with liquid ammonia for 3 d. After evaporation of the solvent, the residue was recrystallized from MeOH and isopropyl ether; yield 9.73 g (79%), mp 138—140 °C, $[\alpha]_D^{16}$ −23.0° (*c* = 1.0, MeOH), Rf_3 0.64. *Anal.* Calcd for $C_{15}H_{29}N_3O_4S_2 \cdot 1/2H_2O$: C, 46.37; H, 7.78; N, 10.82. Found: C, 46.81; H, 7.72; N, 11.15.

Boc–Phe–Tyr–OMe——DCC (9.33 g, 45.2 mmol) was added to a mixture of Boc–Phe–OH (9.98 g, 37.6 mmol) and H–Tyr–OMe [prepared from 8.73 g (37.7 mmol) of the hydrochloride] in THF–DMF (100 ml–50 ml) and the mixture was stirred at room temperature overnight, then filtered. After evaporation of the solvent, the residue was dissolved in AcOEt. The organic phase was washed with base and acid as described above, dried over Na_2SO_4 and concentrated. Trituration of the residue with *n*-hexane afforded a powder, which was recrystallized from AcOEt and *n*-hexane; yield 14.26 g (92%), mp 144—145 °C, $[\alpha]_D^{16}$ −5.0° (*c* = 1.0, MeOH), Rf_3 0.74. *Anal.* Calcd for $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.00; H, 6.88; N, 6.42.

Boc–Phe–Tyr–$NHNH_2$——Boc–Phe–Tyr–OMe (14.25 g, 32.2 mmol) in MeOH (100 ml) was treated with hydrazine hydrate (9.66 ml, 5 eq) overnight, and the resulting solid was precipitated from DMF with MeOH; yield 11.79 g (83%), mp 213—215 °C, $[\alpha]_D^{16}$ +16.6° (*c* = 1.0, DMF), Rf_3 0.50. *Anal.* Calcd for $C_{23}H_{30}N_4O_5$: C, 62.42; H, 6.83; N, 12.66. Found: C, 62.16; H, 6.89; N, 12.67.

Boc–Phe–Tyr–Gly–OMe——The azide [prepared from 9.80 g (22.1 mmol) of Boc–Phe–Tyr–$NHNH_2$] in DMF (50 ml) and Et_3N (3.1 ml, 22.1 mmol) were added to an ice-chilled solution of H–Gly–OMe [prepared from 5.55 g (44.2 mmol) of the hydrochloride] in DMF (40 ml) and the mixture was stirred at 4 °C overnight. The solvent was removed by evaporation and the residue was treated with H_2O. The resulting powder was washed with 5% $NaHCO_3$,

5% citric acid and H_2O, and recrystallized from MeOH and ether; yield 8.35 g (76%); mp 182—185 °C, $[\alpha]_D^{16}$ −19.7° (c=1.0, MeOH), Rf_3 0.70. *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.51; H, 6.66; N, 8.41. Found: C, 61.96; H, 6.58; N, 8.33.

Boc–Phe–Tyr–Gly–NHNH$_2$——Boc–Phe–Tyr–Gly–OMe (8.34 g, 16.7 mmol) in DMF (70 ml) was treated with hydrazine hydrate (5.0 ml, 5 eq) overnight, then the solvent was removed by evaporation. Treatment of the residue with EtOH afforded a powder, which was precipitated from DMF with EtOH; yield 7.95 g (95%); mp 224—227 °C, $[\alpha]_D^{16}$ −11.7° (c=1.0, DMF), Rf_3 0.53. *Anal.* Calcd for $C_{25}H_{33}N_5O_6$: C, 60.10; H, 6.60; N, 14.02. Found: C, 59.87; H, 6.64; N, 13.79.

Z(OMe)–Arg(Mts)–NHNH–Troc——Troc–NHNH$_2$[11] (3.68 g, 17.7 mmol) in THF (20 ml) was added to a solution of a mixed anhydride [prepared from 8.57 g (16.1 mmol) of Z(OMe)–Arg(Mts)–OH] in THF (80 ml). After being stirred in an ice-bath for 4 h, the solution was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over Na_2SO_4 and concentrated. Trituration of the residue with isopropyl ether afforded a powder, which was recrystallized from AcOEt and isopropyl ether; yield 9.72 g (85%), mp 99—101 °C, $[\alpha]_D^{20}$ +7.1° (c=1.0, DMF), Rf_3 0.73. *Anal.* Calcd for $C_{27}H_{35}Cl_3N_6O_8S \cdot 1/2H_2O$: C, 45.10; H, 5.05; N, 11.69. Found: C, 44.97; H, 4.95; N, 11.75.

Boc–Asp(OBzl)–Arg(Mts)–NHNH–Troc——Z(OMe)–Arg(Mts)–NHNH–Troc (10.0 g, 14.1 mmol) was treated with TFA–anisole (30 ml–10 ml) in an ice-bath for 60 min, then *n*-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (80 ml), together with Et_3N (4.3 ml, 31.0 mmol) and Boc–Asp(OBzl)–OSu (8.89 g, 21.2 mmol). After being stirred at 4 °C overnight, the solution was neutralized with a few drops of AcOH and concentrated. The residue was dissolved in AcOEt. The organic phase was washed with 5% $NaHCO_3$, 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated. Trituration of the residue with isopropyl ether and *n*-hexane afforded a powder; yield 11.39 g (95%), mp 99—101 °C, $[\alpha]_D^{16}$ +4.0° (c=1.0, MeOH), Rf_3 0.66. *Anal.* Calcd for $C_{34}H_{46}Cl_3N_7O_{10}S$: C, 47.97; H, 5.45; N, 11.52. Found: C, 48.10; H, 5.36; N, 11.58.

Boc–Asp(OBzl)–Pro–OH——Boc–Asp(OBzl)–OSu (9.23 g, 21.9 mmol) in dioxane (50 ml) was mixed with a solution of H–Pro–OH (7.51 g, 65.8 mmol) and Et_3N (9.2 ml, 65.8 mmol) in H_2O (50 ml). After 24 h, the solvent was removed by evaporation and the residue was dissolved in H_2O. The aqueous phase was washed with ether and acidified with citric acid. The resulting precipitate was extracted with AcOEt and the extract was washed with 5% citric acid and H_2O–NaCl, then dried over Na_2SO_4 and concentrated to give an oily product; 8.21 g (90%), Rf_3 0.47. For characterization, a part of the sample was subjected to column chromatography on silica gel and eluted with $CHCl_3$–MeOH (20:1) to obtain an amorphous powder; $[\alpha]_D^{30}$ −53.9° (c=1.8, MeOH). *Anal.* Calcd for $C_{21}H_{28}N_2O_7 \cdot 1/2H_2O$: C, 58.73; H, 6.81; N, 6.52. Found: C, 58.76; H, 6.86; N, 6.56.

Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–NHNH–Troc——Boc–Asp(OBzl)–Arg(Mts)–NHNH–Troc (5.0 g, 5.9 mmol) was treated with TFA–anisole (15 ml–5 ml) as usual, then *n*-hexane was added. The resulting oily precipitate was dissolved in 4.45 N HCl–dioxane (13.1 ml, 10 eq), and after 10 min, *n*-hexane was added. The resulting hydrochloride was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (40 ml), together with Et_3N (0.9 ml, 6.5 mmol), Boc–Asp(OBzl)–Pro–OH (4.94 g, 11.7 mmol) and HOBT (1.59 g, 11.7 mmol). After addition of DCC (2.66 g, 12.9 mmol), the solution was stirred overnight, then filtered and concentrated. The residue was purified by the extraction procedure as described above and recrystallized from AcOEt and isopropyl ether; yield 4.80 g (71%), mp 93—95 °C, $[\alpha]_D^{16}$ −26.0° (c=1.0, MeOH), Rf_3 0.65. *Anal.* Calcd for $C_{50}H_{64}Cl_3N_9O_{14}S$: C, 52.06; H, 5.59; N, 10.93. Found: C, 52.08; H, 5.76; N, 10.67.

Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–NHNH$_2$——Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–NHNH–Troc (2.50 g, 2.2 mmol) dissolved in a mixture of MeOH (13 ml) and AcOH (2.5 ml) was treated with Zn dust (2.8 g, 20 eq) at room temperature for 3 h. The solution was filtered, the filtrate was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 3% ethylenediaminetetraacetic acid (EDTA) and H_2O–NaCl, dried over Na_2SO_4 and concentrated. Trituration of the residue with ether afforded a power, which was recrystallized from AcOEt and ether; yield 1.60 g (76%), mp 108—110 °C, $[\alpha]_D^{16}$ −24.6° (c=1.0, MeOH), Rf_3 0.50. Amino acid ratio in 6 N HCl hydrolysate: Asp 1.85, Pro 1.00, Arg 0.85 (recovery of Pro, 97%). *Anal.* Calcd for $C_{47}H_{63}N_9O_{12}S \cdot H_2O$: C, 56.67; H, 6.58; N, 12.66. Found: C, 56.74; H, 6.57; N, 12.46.

Boc–Asp(OBzl)–Pro–Pro–OH——Boc–Pro–Pro–OH[21] (4.22 g, 13.5 mmol) was treated with TFA–anisole (12 ml–4 ml) as usual, then *n*-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (60 ml), together with Et_3N (4.1 ml, 29.8 mmol) and Boc–Asp(OBzl)–OSu (3.78 g, 9.0 mmol). After being stirred at 4 °C overnight, the solution was concentrated and the residue was dissolved in H_2O. The aqueous phase was washed with ether and then acidified with citric acid. The resulting precipitate was extracted with AcOEt. The organic phase was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated to give an oily product; yield 3.44 g (74%), Rf_3 0.61. A part of the sample was subjected to column chromatography on silica gel and eluted with $CHCl_3$–MeOH–H_2O (90:15:5) to give an amorphous powder; $[\alpha]_D^{30}$ −86.9° (c=1.1, MeOH). Amino acid ratios in 6 N HCl hydrolysate; Asp 1.00, Pro 2.00 (recovery of Asp, 87%). *Anal.* Calcd for $C_{26}H_{35}N_3O_8 \cdot 1.5H_2O$: C, 57.34, H, 7.03; N, 7.72. Found: C, 57.79; H, 6.50; N, 7.65.

Boc–Phe–Tyr–Gly–Met–Met–NH$_2$——Boc–Met–Met–NH$_2$ (6.03 g, 15.9 mmol) was treated with TFA (18 ml) in the presence of anisole containing 2% EDT (6 ml) in an ice-bath for 60 min, then *n*-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (40 ml) containing Et_3N (2.3 ml,

17.5 mmol). The azide [prepared from 7.94 g (15.9 mmol) of Boc–Phe–Tyr–Gly–$NHNH_2$] in DMF (30 ml) and Et_3N (2.2 ml, 15.9 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was concentrated. Treatment of the residue with H_2O afforded a powder, which was washed with 5% citric acid and H_2O and precipitated from DMF with AcOEt; yield 8.50 g (72%), mp 214—218 °C, $[\alpha]_D^{16}$ −2.0° (c=1.0, DMF), Rf_3 0.65. Amino acid ratios in 6 N HCl hydrolysate: Phe 0.93, Tyr 1.02, Gly 1.00, Met 1.88 (recovery of Gly, 99%). *Anal.* Calcd for $C_{35}H_{50}N_6O_8S_2 \cdot H_2O$: C, 54.97; H, 6.85; N, 10.99. Found: C, 55.15; H, 6.63; N, 10.73.

Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–Phe–Tyr–Gly–Met–Met–NH_2——Boc–Phe–Tyr–Gly–Met–Met–NH_2 (1.0 g, 1.3 mmol) was treated with TFA–anisole containing 2% EDT (3 ml–1 ml) as described above, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (7 ml) containing Et_3N (0.2 ml, 1.5 mmol). The azide [prepared from 1.30 g (1.3 mmol) of Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–$NHNH_2$] in DMF (10 ml) and Et_3N (0.2 ml, 1.3 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was concentrated. Ttreatment of the residue with 5% citric acid afforded a powder, which was washed with 5% citric acid and H_2O and precipitated from DMF with AcOEt; yield 1.49 g (70%), mp 160—163 °C, $[\alpha]_D^{16}$ −31.1° (c=1.0, DMF), Rf_3 0.68. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.02, Pro 0.74, Arg 0.90, Phe 0.97, Tyr 0.98, Gly 1.00, Met 1.64, Met(O) N.D. (recovery of Gly, 74%). *Anal.* Calcd for $C_{77}H_{101}N_{13}O_{18} \cdot H_2O$: C, 57.41; H, 6.45; N, 11.30. Found: C, 57.37; H, 6.32; N, 11.33.

Boc–Asp(OBzl)–Pro–Pro–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–Phe–Tyr–Gly–Met–Met–NH_2 ——Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mts)–Phe–Tyr–Gly–Met–Met–NH_2 (1.0 g, 0.63 mmol) was treated with TFA–anisole containing EDT (3 ml–1 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (10 ml), together with Et_3N (96 μl, 0.7 mmol), Boc–Asp(OBzl)–Pro–Pro–OH (0.65 g, 1.3 mmol) and HOBT (0.17 g, 1.3 mmol). After addition of DCC (0.29 g, 1.4 mmol), the solution was stirred overnight, filtered and concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was washed with 5% citric acid and H_2O and precipitated from DMF with AcOEt; yield 0.68 g (54%), mp 142—145 °C, $[\alpha]_D^{16}$ −45.1° (c=1.0, DMF), Rf_3 0.68. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.71, Pro 2.53, Gly 1.00, Met 1.54, Tyr 0.96, Phe 0.88, Arg 0.88 (recovery of Gly, 73%). *Anal.* Calcd for $C_{98}H_{126}N_{16}O_{23}S_3 \cdot 3H_2O$: C, 57.52; H, 6.50; N, 10.95. Found: C, 57.49; H, 6.50; N, 11.26.

H–Asp–Pro–Pro–Asp–Pro–Asp–Arg–Phe–Tyr–Gly–Met–Met–NH_2——The above protected dodecapeptide amide (200 mg, 0.1 mmol) was treated with 1 M TFMSA–thioanisole in TFA (5 ml) in the presence of *m*-cresol (0.5 ml, 50 eq) in an ice-bath for 90 min, then dry ether was added to give a powder. This treatment was repeated under identical conditions and the deprotected peptide was dissolved in H_2O (4 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 1 g) for 30 min, then filtered and lyophilized. The residue was dissolved in H_2O (4 ml), incubated with DTT (308 mg, 50 eq) at 37 °C for 2 d and subjected to gel-filtration on Sephadex G-25 (3 × 130 cm) with 1 N AcOH. Individual fractions (10 ml each) were examined by ultraviolet (UV) absorption measurement at 280 nm. Fractions corresponding to the main peak (tube Nos. 38—42) were collected and the solvent was removed by lyophilization to give a powder; 48.1 mg (33%).

The product (10.0 mg) in H_2O (1 ml) was next applied to a column of Sephadex G-25 (2.0 × 60 cm) equilibrated with the lower phase of *n*-BuOH–AcOH–H_2O (4 : 1 : 5), and the column was developed with the upper phase of the above solvent system. Each fraction (4 ml) was examined by UV absorption measurement at 280 nm. After elution of a small peak, the main peak fractions (tubes 29—35) were combined, the solvent was removed by evaporation, and the residue was lyophilized to give a fluffy powder; yield 8.20 mg (82%), $[\alpha]_D^{15}$ −127.9° (c=0.4, H_2O), Rf_4 0.30, Rf_5 0.27. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.94, Pro 2.79, Gly 1.00, Met+Met(O) 1.88, Tyr 0.92, Phe 0.94, Arg 0.85 (recovery of Gly, 75%).

Effect of Various Cation Scavengers——Z(OMe)–Met–OH or Boc–Met–OH (0.05 mmol) was treated with TFA (0.5 ml) in the presence of various scavengers (0.25 mmol, Table I) at room temperature (18 °C) for 30 min and CH_2Cl_2 (3 ml) was added. A part of the solution was examined by TLC using the solvent system of benzene–MeOH–AcOH (7 : 2 : 1). After coloration with ninhydrin (90 °C, 20 min), the ratios of two spots in each case were colorimetrically determined with a Shimadzu dual-wavelength TLC scanner: Rf_6 0.21 (Met) and 0.02 (*S-p*-methoxybenzyl–Met sulfonium salt) in the case of Z(OMe)–Met–OH; Rf_6 0.21 (Met) and 0.02 (*S-tert*-butyl–Met sulfonium salt) in the case of Boc–Met–OH as shown in Table I. The above two reference samples were prepared by reaction of Met with the corresponding halides, and their structures were confirmed by NMR, in which the *S*-methyl signal of Met in each compound was shifted downfield ($\Delta\delta$=0.72 in *p*-methoxybenzyl derivative and 0.70 in *tert*-butyl derivative[6]) from the parent signal (δ=2.18).

Boc–Phe–Tyr–Gly–OBzl——The azide [prepared from 5.00 g (11.3 mmol) of Boc–Phe–Tyr–$NHNH_2$] in DMF (5 ml) and Et_3N (1.7 ml) 11.3 mmol were added to an ice-chilled solution of H–Gly–OBzl [prepared from 4.40 g (12.43 mmol) of the tosylate] in DMF (5 ml) and the mixture was stirred at 4 °C for 48 h. After filtration, the filtrate was concentrated and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over Na_2SO_4, and concentrated. The residue was recrystallized from AcOEt and petroleum ether: yield 4.40 g (68%), mp 179—181 °C, Rf_1 0.86, $[\alpha]_D^{30}$ −17.7° (c=1.6, MeOH). *Anal.* Calcd for $C_{32}H_{37}N_3O_7$: C, 66.77; H, 6.48: N, 7.30. Found: C, 67.01; H, 6.49; N, 7.35.

Z–Arg(Mds)–Phe–Tyr–Gly–OBzl——Boc–Phe–Tyr–Gly–OBzl (3.80 g, 6.63 mmol) was treated with TFA–

anisole (15 ml–3 ml) in an ice-bath for 40 min, then dry ether was added. The resulting powder was collected by filtration and dissolved in 3.2 N HCl/DMF (2 ml). Dry ether was added again. The resulting oily precipitate was separated by decantation, dried over KOH pellets for 3 h, and then dissolved in DMF (15 ml) together with Et_3N (0.92 ml, 6.62 mmol), Z–Arg(Mds)–OH (3.35 g, 6.62 mmol), and HOBT (0.90 g, 6.62 mmol). After addition of DCC (1.37 g, 6.65 mmol), the mixture was stirred at room temperature for 24 h, and filtered. The filtrate was concentrated and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography using $CHCl_3$–MeOH (60 : 1, v/v) as an eluant: yield 5.02 g (78%), amorphous powder, Rf_1 0.69, $[\alpha]_D^{32}$ −19.6° (c=0.5, DMF). *Anal.* Calcd for $C_{50}H_{57}N_7O_{11}S$: C, 62.29; H, 5.96; N, 10.17. Found: C, 62.32; H, 6.12; N, 9.98.

Boc–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–OH——In the usual manner, Z–Arg(Mds)–Phe–Tyr–Gly–OBzl (3.50 g, 3.63 mmol) in MeOH–AcOH (50 ml–6 ml) was hydrogenated over Pd black for 8 h. The catalyst was then removed by filtration and the filtrate was concentrated *in vacuo*. The residue was dried over KOH pellets *in vacuo* then dissolved in DMF (12 ml) together with Et_3N (1.0 ml, 7.19 mmol), Boc–Asp(OBzl)–ONp (1.61 g, 3.63 mmol), and HOBT (20 mg, 0.14 mmol). After being stirred for 18 h, the solution was concentrated and the residue was purified by column chromatography on silica gel using $CHCl_3$–MeOH–H_2O (90 : 15 : 5) as an eluant: yield 2.50 g (63%), amorphous powder, Rf_2 0.40, $[\alpha]_D^{32}$ −36.1° (c=1.1, DMF). Amino acid ratios in 6 N HCl hydrolysate: Asp 0.97, Arg 1.04, Phe 1.00, Tyr 0.93, Gly 0.96 (recovery of Phe, 87%). *Anal.* Calcd for $C_{51}H_{64}N_8O_{14}S \cdot 2H_2O$: C, 56.66; H, 6.34; N, 10.36. Found: C, 56.89; H, 6.14; N, 10.22.

Boc–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–Met–Met–NH_2——Boc–Met–Met–NH_2 (1.03 g, 2.71 mmol) was treated with TFA–DMA containing 2% EDT (2 ml–0.5 ml) in an ice-bath for 40 min, then dry ether was added. The resulting H–Met–Met–NH_2 · TFA salt was converted to the corresponding hydrochloride in the usual manner as described above, and the hydrochloride was dissolved in DMF (5 ml) containing Et_3N (0.377 ml, 2.71 mmol). To this ice-chilled solution, Boc–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–OH (2.00 g, 1.80 mmol), HOBT (0.24 g, 1.76 mmol) and DCC (0.37 g, 1.80 mmol) were added. The mixture, after being stirred at room temperature for 24 h, was filtered and the filtrate was concentrated *in vacuo*. The residual solid was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over P_2O_5 and recrystallized from MeOH: yield 1.30 g (53%), mp 197—198 °C (dec.), Rf_2 0.56, $[\alpha]_D^{29}$ −23.5° (c=0.9, DMF). Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Arg 1.07, Phe 0.94, Tyr 0.97, Gly 1.00, Met 2.14, (recovery of Gly, 90%). *Anal.* Calcd for $C_{61}H_{83}N_{11}O_{15}S_3$: C, 56.08; H, 6.40; N, 11.79. Found: C, 55.84; H, 6.37; N, 11.62.

Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–Met–Met–NH_2——Boc–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–Met–Met–NH_2 (1.00 g, 0.73 mmol) was treated with TFA–DMA containing 2% EDT (3 ml–0.5 ml) and the N^{α}-deprotected product was converted to the corresponding hydrochloride as described above. The hydrochloride was then dissolved in DMF (5 ml) containing Et_3N (0.10 ml, 0.73 mmol). To this ice-chilled solution, Boc–Asp(OBzl)–Pro–OH (0.34 g, 0.81 mmol), HOBT (0.11 g, 0.81 mmol) and DCC (0.17 g, 0.83 mmol) were added successively. The reaction mixture, after being stirred at room temperature for 48 h, was filtered and the filtrate was concentrated *in vacuo*. The residual solid was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over P_2O_5 *in vacuo* and precipitated from MeOH with ether: yield 1.00 g (82%), mp 169—172 °C (dec.), Rf_1 0.35, $[\alpha]_D^{29}$ −33.6° (c= 1.1, DMF). Amino acid ratios in 6 N HCl hydrolysate: Asp 1.87, Pro 0.92, Arg 0.97, Phe 1.00, Tyr 0.96, Gly 0.95, Met 1.80 (recovery of Phe, 99%). *Anal.* Calcd for $C_{77}H_{101}N_{13}O_{19}S_3$: C, 57.48; H, 6.33; N, 11.32. Found: C, 57.20; H, 6.29; N, 11.16.

Boc–Asp(OBzl)–Pro–Pro–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–Met–Met–NH_2——Boc–Asp(OBzl)–Pro–Asp(OBzl)–Arg(Mds)–Phe–Tyr–Gly–Met–Met–NH_2 (0.80 g, 0.48 mmol) was treated with TFA–DMA containing 2% EDT (2.4 ml–0.4 ml) and the N^{α}-deprotected product was converted to the corresponding hydrochloride as mentioned above. The hydrochloride was dissolved in DMF (3 ml) containing Et_3N (0.068 ml, 0.49 mmol). To this ice-chilled solution, Boc–Asp(OBzl)–Pro–Pro–OH (0.28 g, 0.54 mmol), HOBT (72 mg 0.53 mmol) and DCC (0.11 g, 0.53 mmol) were added successively. The reaction mixture, after being stirred at room temperature for 48 h, was filtered and the filtrate was concentrated *in vacuo*. The residual solid was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over P_2O_5 *in vacuo* and reprecipitated from MeOH with ether: yield 0.58 g (59%), mp 139—150 °C, Rf_2 0.44, $[\alpha]_D^{19}$ −48.0° (c=0.8, DMF). Amino acid ratios in 6 N HCl hydrolysate: Asp 2.96, Pro 2.78, Arg 1.03, Phe 1.00, Tyr 0.99, Gly 0.98, Met 1.92 (recovery of Phe, 90%). *Anal.* Calcd for $C_{98}H_{126}N_{16}O_{24}S_3 \cdot 2H_2O$: C, 57.58: H, 6.41; N, 10.96. Found: C, 57.52; H, 6.47; N, 10.94.

H–Asp–Pro–Pro–Asp–Pro–Asp–Arg–Phe–Tyr–Gly–Met–Met–NH_2 (Hylambatin)——The above protected dodecapeptide amide (100 ml, 0.048 mmol) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in an ice-bath for 2 h, then dry ether was added. The resulting precipitate was collected by filtration, washed with ether, and dissolved in H_2O (20 ml). The solution was treated with Amberlite IR-45 (acetate form, approximately 2 g) for 30 min, then filtered and lyophilized. The resulting powder was dissolved in 3% AcOH (3 ml) and the solution was applied to a column of Sephadex G-15 (2.5 × 85 cm), which was eluted with 3% AcOH. Individual fractions (10 ml each) were collected and UV absorption at 275 nm was determined. The fractions corresponding to the front peak (tube Nos. 13—22) were combined and the solvent was removed by lyophilization to give a white powder (yield 67.7 mg, 82%). This powder was dissolved in H_2O (20 ml) and the solution was applied to a column of DEAE-cellulose (2.0 × 9.0 cm),

Fig. 4. HPLC of a Mixture of the Synthetic Samples of Hylambatin Prepared by the Two Alternative Routes

which was eluted first with H_2O (140 ml) and then pH 6.9 NH_4OAc buffers; 0.01 M (180 ml) and 0.02 M (200 ml). Next, gradient elution was carried out with pH 6.9, 0.2 M NH_4OAc buffer (500 ml) through a mixing flask containing pH 6.9, 0.02 M NH_4OAc buffer (150 ml). Individual fractions (10 ml each) were collected and the UV absorption at 275 nm was determined. The fractions corresponding to the main peak (tube Nos. 69—74) were combined and the solution was concentrated to approximately 3 ml. This solution, after incubation with 2-mercaptoethanol at 50 °C for 20 h, was applied to a column of Sephadex G-10 (3.5 × 35 cm), which was eluted with 3% AcOH. The desired fractions were collected as described above and the solvent was removed by lyophilization to give a white fluffy powder: yield 24.0 mg (29%), $[\alpha]_D^{29}$ −77.5° (c=1.2, 3% AcOH), Rf_4 0.31, Rf_4 0.50 (cellulose). Amino acid ratios in 6 N HCl hydrolysate: Asp 2.80, Pro 3.13, Gly 1.00, Met 1.94, Tyr 1.00, Phe 1.07, Arg 1.06 (recovery of Gly, 89%).HPLC: retention time 23.4 min. *Anal.* Calcd for $C_{63}H_{90}N_{16}O_{19}S_2 \cdot 2AcOH \cdot 7H_2O$: C, 47.73; H, 6.70; N, 13.29. Found: C, 47.49; H, 6.59; N, 13.53.

Two samples of synthetic hylambatin obtained by the present method and the former method exhibited identical Rf_4 values and identical retention times (23.4 min, flow rate 1 ml/min) on HPLC (Fig. 4).

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References and Notes

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2) Amino acids, peptides and their derivatives mentioned in this report are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Boc=*tert*-butoxycarbonyl, Mds=4-methoxy-2,6-dimethylbenzenesulfonyl, DCC=dicyclohexylcarbodiimide, HOBT=*N*-hydroxybenzotriazole, Np=*p*-nitrophenyl, Su=*N*-hydroxysuccinyl, Mts=mesitylenesulfonyl, TFA=trifluoroacetic acid, DMF=dimethylformamide, EDT=ethanedithiol, DTT=dithiothreitol, DMSO=dimethylsulfoxide, TFMSA=trifluoromethanesulfonic acid, THF=tetrahydrofuran.
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