

Tandem Enzymatic Resolution Yielding L- α -Aminoalkanedioic Acid ω -Esters

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The tandem action of serine protease (α -chymotrypsin or subtilisin BPN') and *Aspergillus* genus aminoacylase on racemic *N*-acetyl- α -aminoalkanedioic acid α,ω -diester produced L- α -aminoalkanedioic acid ω -ester in good yield and high optical purity. L- α -Aminosuberic acid ω -ester thus obtained was conveniently introduced into an oxytocin analog, [Asu^{1,6}]oxytocin, by the solid-phase-synthesis and cyclization–cleavage method with oxime resin.

Key words α -aminoalkanedioic acid; aminosuberic acid; tandem enzymatic resolution; cyclic peptide; oxytocin analog

In the deaminodicarba analogs of some peptide hormones such as [Asu^{1,6}]oxytocin¹⁾ and elcatonin,²⁾ L- α -aminosuberic acid (Asu) replaces a cystine residue. The Asu derivative protected with an ω -carboxyl group is desirable for the synthesis of Asu-containing peptides. Formerly, L-Asu was obtained by enzymatic resolution,³⁾ then the ω -carboxyl group was protected as benzyl ester. To overcome the inefficiency in the ω -selective protection of free aminodicarboxylic acids, enzymatic resolution of the corresponding dibenzyl esters with proteases has been examined.^{4,5)} However, partial hydrolysis of the D-isomer occurred during the use of a single protease.⁴⁾ We tried the tandem use of two enzymes with fully protected racemic amino acid derivatives (Chart 1). The protease hydrolyzes the α -ester in the first step, and the product is the substrate of the aminoacylase. We also report the synthesis of [Asu^{1,6}]oxytocin, to demonstrate our facile syntheses of Asu derivatives.

The fully protected racemic α -aminosuberic acid, Ac-DL-Asu(OMe)–OMe (**1e**), was synthesized through the acetamidomalonate method.⁶⁾ In the enzymatic resolution, **1e** was first subjected to the action of α -chymotrypsin (bovine, Sigma). α -Chymotrypsin (70 mg) and **1e** (73 mmol) were mixed in 350 ml of aqueous NH₃, to which aqueous 1 M NH₃ was added continuously to maintain the pH at 8.0–8.5. The ester hydrolysis of L-**1e** ended within 8 h, when the total of the added NH₃ corresponded to the amount of Ac-L-Asu(OMe)–OMe. Without product isolation, aminoacylase (*Aspergillus* genus, Tokyo Kasei) (2.1 g) (dissolved in H₂O and filtered to remove insoluble materials) and CoCl₂·6H₂O (95 mg) were added to the α -chymotrypsin reaction mixture. This second resolution with the removal of the acetyl group was carried out at

38 °C overnight at pH 7. After concentration of the reaction mixture, the desired L-Asu(OMe) (**2e**) was precipitated by the addition of EtOH. The yield was 75% based on the Ac-L-Asu(OMe)–OMe in the racemic mixture. The unreacted Ac-D-Asu(OMe)–OMe could be extracted with AcOEt from the reaction mixture.

An emulsion of Ac-DL-Asu(OBzl)–OBzl (**1f**) in 20% (v/v) aqueous *N,N*-dimethylformamide (DMF) resisted the action of α -chymotrypsin. Therefore, subtilisin BPN' (Sigma), a bacterial endoprotease with broader specificity, was examined and found to hydrolyze the α -benzyl ester of L-**1f** successfully in 20% DMF. After 10 h with continuous addition of aqueous 1 M NH₃ to keep the pH at 8, the first resolution ended. The second resolution by aminoacylase was performed at 38 °C overnight at pH 7. The desired L-Asu(OBzl) (**2f**) precipitated from the reaction mixture. The yield was 68% based on the Ac-L-Asu(OBzl)–OBzl. Similarly, the tandem enzymatic resolution method was applied for the preparation of ω -esters of L-aminoadipic acid (L-Aad) and L-aminopimelic acid (L-Api). As summarized in Table 1, the ω -methyl and ω -benzyl esters of L-aminoalkanedioic acids were obtained in good yields and high optical purities. The specific rotation of L-Aad(OBzl) was much greater than the reported value.⁵⁾

Some partially protected Asu derivatives were prepared for use in peptide syntheses (Table 2). Asu(OMe) (**2e**) and Asu(OBzl) (**2f**) were fully protected as **4a–d**. The saponification of **4a** gave Boc-Asu–OCHex (**5a**). Hydrogenation of Boc-Asu(OBzl)–OCHex (**4d**) with Pd-charcoal also afforded **5a**. Compound **5b** was previously employed for the synthesis of the cyclic portion of elcatonin.⁷⁾

The linkage of **5a** to Kaiser's oxime resin at the ω -

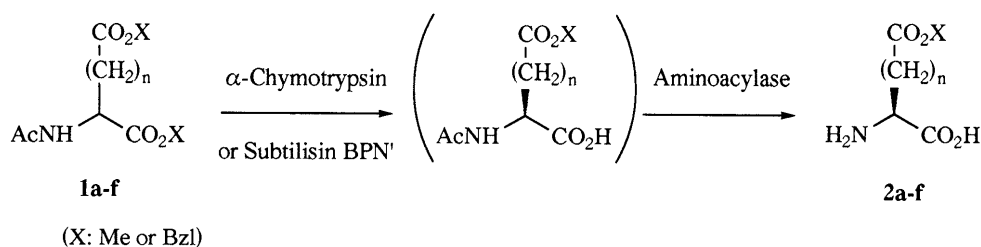


Chart 1

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Table 1. L- α -Aminoalkanedioic Acid ω -Ester Obtained by the Tandem Enzymatic Resolution

L-Amino acid	<i>n</i>	Method ^{a)}	Yield (%) ^{b)}	<i>R_f</i> ^{c)}	mp (°C) (dec.)	$[\alpha]_D^{25}$ (deg.)
Aad (OMe) (2a)	3	A	63	0.44	172—174	+24.9 (<i>c</i> = 1, H ₂ O)
Aad (OBzl) (2b)	3	B	72	0.74	185—187	+22.1 (<i>c</i> = 0.5, HCO ₂ H) ^{d)} +24.4 (<i>c</i> = 1, AcOH)
Api (OMe) (2c)	4	A	67	0.49	201—202	+9.8 (<i>c</i> = 1, H ₂ O)
Api (OBzl) (2d)	4	B	70	0.83	202—204	+19.2 (<i>c</i> = 0.5, HCO ₂ H) +19.1 (<i>c</i> = 1, AcOH)
Asu (OMe) (2e)	5	A	75	0.42	206—207	+7.5 (<i>c</i> = 1, H ₂ O)
Asu (OBzl) (2f)	5	B	68	0.70	208—210	+18.4 (<i>c</i> = 0.5, HCO ₂ H) ^{e)} +17.8 (<i>c</i> = 1, AcOH)

a) A, α -chymotrypsin/aminoacylase; B, subtilisin BPN'/aminoacylase. b) Based on the fully protected L-amino acid. c) *n*-Butanol:AcOH:pyridine:H₂O = 4:1:1:2, v/v. d) +10° in ref 5. e) +18° in ref 5.

Table 2. Asu Derivatives with Different Protections at the α - and ω -Carboxyl Groups^{a)}

Asu derivative	Method	Yield (%)	<i>R_f</i>	$[\alpha]_D^{25}$ (deg.) ^{d)}
Boc-Asu(OMe) (3a)	Boc ₂ O	87	0.54 ^{b)}	+3.8
Boc-Asu(OBzl) (3b)	Boc ₂ O	93	0.54 ^{b)}	+3.5
Boc-Asu(OMe)-OCHex (4a)	cHexOH/DCC/DMAP	83	0.39 ^{c)}	-18.8
Boc-Asu(OMe)-N ₂ H ₂ Z (4b)	Z-NHNH ₂ /DCC	82	0.34 ^{c)}	-26.8
Boc-Asu(OBzl)-OMe (4c)	MeOH/DCC/DMAP	82	0.70 ^{c)}	-9.8
Boc-Asu(OBzl)-OCHex (4d)	cHexOH/DCC/DMAP	74	0.82 ^{c)}	-15.8
Boc-Asu-OCHex (5a)	aq. NaOH/MeOH	85	0.82 ^{b)}	-21.3
Boc-Asu-N ₂ H ₂ Z (5b)	aq. NaOH/MeOH	76	0.78 ^{b)}	-22.3
Boc-Asu-OMe (5c)	H ₂ /Pd-charcoal	94	0.80 ^{b)}	-13.5

a) All Asu derivatives are colorless oily materials. b) CHCl₃-MeOH-AcOH (90:10:2, v/v/v). c) CHCl₃-MeOH (19:1, v/v). d) *c* = 1, MeOH.

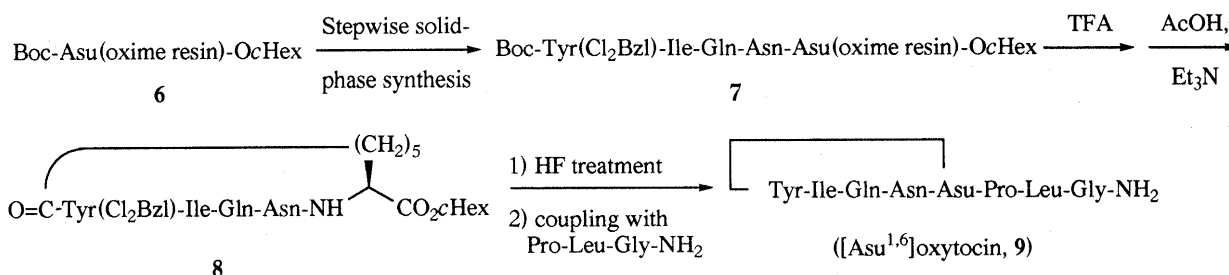


Chart 2

carboxyl position yielded Boc-Asu(oxime resin)-OCHex (**6**) (Chart 2). After the initial stepwise solid-phase synthesis, the protected cyclic portion of [Asu^{1,6}]oxytocin was synthesized by the cyclization-cleavage method.⁸⁾ Condensation of the product with Pro-Leu-Gly-NH₂ afforded the desired [Asu^{1,6}]oxytocin (**9**) in 26% yield after HPLC purification. The HPLC and FAB-MS analyses of the synthetic **9** in comparison with an authentic sample (Peptide Institute, Osaka) clearly indicated the successful synthesis of [Asu^{1,6}]oxytocin.

Experimental

TLC analyses were performed on Wako B-5 plates with the following solvent systems (by volume): *R_f*¹ = *n*-BuOH-AcOH-pyridine-H₂O (4:1:1:2), *R_f*² = CHCl₃-MeOH-AcOH (90:10:2), *R_f*³ = CHCl₃-MeOH (19:1). The specific rotations were measured with a Horiba SEPA-200 polarimeter using a 10 cm cell at 25°C. The melting points were measured on a Yamato MP-21 melting point apparatus without correction. The FAB-MS analyses were performed with a JEOL DX300 mass spectrometer. The HPLC analyses were carried out with a Hitachi L-6200 intelligent pump equipped with an MS-GEL C18 PAC DF-5-120 Å column (10 × 250 mm) and a Hitachi L-4200 UV-Vis detector.

DL- α -Aminosuberic Acid Hydrochloride (DL-Asu·HCl) Diethyl α -

acetoamidomalonate (44 g, 0.20 mol) was added to an EtOH (160 ml) solution of NaOEt (0.22 mmol) and the mixture was refluxed for 1 h. Then, ethyl 6-bromohexanoate (42 g, 0.20 mol) was added and refluxing was continued for a further 9 h. The product was isolated and heated in aqueous 6M HCl (200 ml) for 5 h. The solution was concentrated to dryness to afford a white solid. The yield was 43 g (0.19 mol, 95%). *R_f*² 0.23.

Dimethyl Acetyl-DL- α -aminosuberate (Ac-DL-Asu(OMe)-OMe, 1e) DL-Asu·HCl (25 g, 0.11 mol), *p*-toluenesulfonic acid hydrate (23 g, 0.12 mol), and benzyl alcohol (108 g, 1.0 mol) were mixed in benzene (100 ml) and refluxed for 5 h with a Dean-Stark apparatus. After evaporation, the product was solidified by the addition of ethyl ether-petroleum ether. Then Et₃N (24 g, 0.24 mol) and Ac₂O (18 g, 0.18 mol) was added at 0°C. The mixture was stirred at room temperature for 6 h. After evaporation of the solvent, the product was extracted with AcOEt. The solution was washed, dried, and concentrated to give an oil. The

yield was 26 g (0.10 mmol, 92%). *R_f*³ 0.54.

Dibenzyl Acetyl-DL- α -aminosuberate (Ac-DL-Asu(OBzl)-OBzl, 1f) DL-Asu·HCl (23 g, 0.10 mol), *p*-toluenesulfonic acid hydrate (23 g, 0.12 mol), and benzyl alcohol (108 g, 1.0 mol) were mixed in benzene (100 ml) and refluxed for 5 h with a Dean-Stark apparatus. After evaporation, the product was solidified by the addition of ethyl ether-petroleum ether. Then Et₃N (24 g, 0.24 mol) and Ac₂O (18 g, 0.18 mol) was added at 0°C. The mixture was stirred at room temperature for 6 h. After evaporation of the solvent, the product was extracted with AcOEt. The solution was washed, dried, and concentrated to give an oil. The

yield was 27 g (68 mmol, 68%). R_f^3 0.64.

5-Methyl L- α -Aminosuberate (L-Asu(OMe), 2e) α -Chymotrypsin (bovine, Sigma type II, 40–60 units/mg, 70 mg) was added to a solution of **1e** (19 g, 73 mmol) in water (350 ml), adjusted to pH 8 with aqueous NH_3 , at 25 °C. The pH of the reaction medium was monitored with a pH-meter and maintained at 8.0–8.5 by the continuous addition of aqueous 1 M NH_3 . The pH became constant after 8 h, when the total amount of NH_3 added was 37 mmol. To this solution of the α -chymotrypsin-hydrolyzate (estimated to contain 37 mmol of Ac-L-Asu(OMe)), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (95 mg, 0.40 mmol) and *Aspergillus* genus aminoacylase (Tokyo Kasei, 2.1 g, dissolved in H_2O and filtered before use) were added. The mixture was incubated at 37 °C overnight. TLC analyses indicated the disappearance of Ac-Asu(OMe) and production of Asu(OMe). The reaction mixture was concentrated, and the addition of EtOH to the residue crystallized the desired amino acid. The fully protected D-isomer was left in the mother liquor. The yield was 5.5 g (27 mmol, 75% based on Ac-L-Asu(OMe)-OMe). Recrystallization from H_2O -EtOH gave **2e** as a white solid. R_f^1 0.42, R_f^2 0.06. mp (dec.) 206–207 °C. FAB-MS (glycerol): 204 ($\text{M} + \text{H}^+$). Anal. Calcd for $\text{C}_9\text{H}_{17}\text{NO}_4 \cdot 1/4\text{H}_2\text{O}$: C, 52.04; H, 8.49; N, 6.74. Found: C, 52.17; H, 8.33; N, 6.69. $[\alpha]_D^{25} + 7.5^\circ$ ($c = 1.0$, H_2O).

5-Benzyl L- α -Aminosuberate (L-Asu(OBzl), 2f) Water (400 ml) was mixed to a solution of **1f** (40 g, 0.10 mol) and subtilisin BPN' (100 mg) in DMF (100 ml), to form an emulsion. The pH was adjusted to 8, and maintained at 8.0–8.5 as described above. After 8 h, aminoacylase (3.0 g) and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (130 mg, 0.55 mmol) were added, and the mixture was incubated at 37 °C overnight. The crystalline precipitate was collected, then washed with H_2O and AcOEt. The yield of **2f** was 10 g (36 mmol, 68% based on Ac-L-Asu(OBzl)-OBzl). R_f^1 0.70, R_f^2 0.12. mp (dec.) 208–210 °C. FAB-MS (glycerol): 266 ($\text{M} + \text{H}^+$). $[\alpha]_D^{25} + 18.4^\circ$ ($c = 0.5$, HCOOH), $+ 17.8^\circ$ ($c = 1.0$, AcOH).

Boc-Asu-OcHex (5a) 4-Dimethylaminopyridine (DMAP) (0.12 g, 1.0 mmol), cyclohexanol (3.0 g, 34 mmol), and dicyclohexylcarbodiimide (DCC) (2.4 g, 12 mmol) were added to a CH_2Cl_2 (30 ml) solution of Boc-Asu(OMe) (**3e**) (3.0 g, 10 mmol) at 0 °C.⁹⁾ After 24 h, the precipitate was filtered off and the filtrate was evaporated. Silica gel chromatography with CHCl_3 gave **4a** as an oil. Then, **4a** (1.5 g, 4.0 mmol) was saponified in MeOH-aqueous 1 M NaOH (1:1, v/v, 15 ml) for 3 h at 0 °C. After neutralization, the mixture was evaporated, extracted with AcOEt, and evaporated to give **5a** as an oil. The yield was 1.2 g (3.4 mmol, 85%). The same L-Asu derivative (**5a**) was obtained from **4d** by hydrogenation in MeOH. R_f^2 0.82. FAB-MS (glycerol): 372 ($\text{M} + \text{H}^+$). $[\alpha]_D^{25} - 21.3^\circ$ ($c = 1.0$, MeOH).

Boc-Tyr(Cl_2Bzl)-Ile-Gln-Asn-Asu(Oxime Resin)-OcHex (7)¹⁰⁾ Kaiser's oxime resin (1.3 g) was pre-swollen with CH_2Cl_2 , then a CH_2Cl_2 (30 ml) solution of **5a** (0.50 g, 1.3 mmol) and DCC (0.28 g, 1.3 mmol) was added. The mixture was shaken for 24 h, the resin was removed by filtration, washed with CH_2Cl_2 and CH_2Cl_2 -EtOH (1:1, v/v), and dried to yield Boc-Asu(oxime resin)-OcHex (**6**). After treatment of **6** (1.0 g, 0.39 mmol) with 25% trifluoroacetic acid (TFA) in CH_2Cl_2 (v/v) for

30 min, the resin was washed with CH_2Cl_2 and 2-propanol, then suspended in DMF (15 ml). To this suspension a mixture of Boc-Asn-OH (3 eq), benzotriazol-1-yloxytris(diethylamino)phosphonium hexafluorophosphate (BOP, 3 eq), 1-hydroxybenzotriazole hydrate (HOBt $\cdot \text{H}_2\text{O}$, 3 eq), and Et_3N (6 eq) in DMF (20 ml) was added. The mixture was shaken for 45 min, then the resin was washed with DMF and CH_2Cl_2 , and subjected to the next condensation.

Tyr(Cl_2Bzl)-Ile-Gln-Asn-Asu-OcHex (8) After the removal of the Boc group of **7** by 25% TFA/ CH_2Cl_2 , a DMF (15 ml) solution of AcOH and Et_3N (2 eq each) was added.⁸⁾ After 24 h, the filtrate was concentrated and H_2O was added to the residue to obtain **8** as a white precipitate. The yield was 93 mg (0.10 mmol, 20% based on **7**).

Tyr-Ile-Gln-Asn-Asu-Pro-Leu-Gly-NH₂ ([Asu^{1,6}]oxytocin, 9) The cyclic portion **8** (93 mg, 0.10 mmol) was treated with anhydrous HF-anisole (9:1, v/v) at 0 °C for 1 h. Then, the deprotected cyclic peptide was mixed with Pro-Leu-Gly-NH₂ $\cdot \text{HCl}$ (39 mg, 0.12 mmol), HOBt $\cdot \text{H}_2\text{O}$ (16 mg, 0.10 mmol), Et_3N (10 mg, 0.10 mmol), and DCC (25 mg, 0.12 mmol) in DMF (3.0 ml) at 0 °C. After 24 h, the mixture was filtered and concentrated. The precipitated solid was purified by HPLC (eluting with a linear gradient of $\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{TFA} = 11:89:0.1$ to $33:67:0.1$ (v/v) over 30 min, flow rate, 3.0 ml/min). The peak with the retention time of 14.32 min was collected. The yield was 25 mg (26 μmol , 26%). The HPLC retention time of synthetic **9** was identical with that of an authentic sample. FAB-MS (glycerol): 955 (M^+).

References and Notes

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- 6) Abbreviations used are according to the IUPAC-IUB Commission, *Eur. J. Biochem.*, **138**, 9 (1984). The amino acids are of L form unless otherwise noted. Other abbreviations: Aad, L- α -aminoadipic acid; Api, L- α -aminopimelic acid; Asu, L- α -aminosuberic acid; Boc, *tert*-butoxycarbonyl; cHex, cyclohexyl; Z, benzyloxycarbonyl.
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