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

Norikazu NISHINO,*[,a] Toru ARAI,[a] Yukio UENO,[b] and Masataka OHBA[b]

Department of Applied Chemistry, Faculty of Engineering, Kyushu Institute of Technology,[a] Tobata-ku, Kitakyushu 804, Japan and Central Research Institute, Asahi Glass Co., Ltd.,[b] Kanagawa-ku, Yokohama 221, Japan.
Received June 26, 1995; accepted September 12, 1995

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

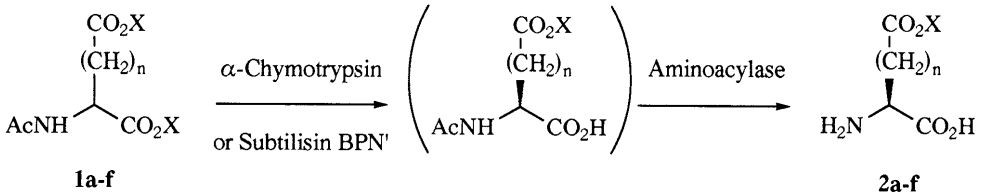
In the deaminodicarba analogs of some peptide hormones such as [Asu[1,6]]oxytocin[1] and elcatonin,[2] L-α-aminosuberic acid (Asu) replaces a cystine residue. The Asu derivative protected with an ω-carboxyl group is desirable for the synthesis of Asu-containing peptides. Formerly, L-Asu was obtained 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 NH_3, to which aqueous 1 M NH_3 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 NH_3 corresponded to the amount of Ac–L-Asu(OMe)–OMe. Without product isolation, aminoacylase (*Aspergillus genus*, Tokyo Kasei) (2.1 g) (dissolved in H_2O and filtered to remove insoluble materials) and $CoCl_2 \cdot 6H_2O$ (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 NH_3 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 empolyed for the synthesis of the cyclic portion of elcatonin.[7]

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



1a-f

(X: Me or Bzl)

2a-f

Chart 1

* To whom correspondence should be addressed.

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

L-Amino acid		n	Method[a]	Yield (%)[b]	Rf[c]	mp (°C) (dec.)	$[\alpha]_D^{25}$ (deg.)
Aad (OMe)	(**2a**)	3	A	63	0.44	172—174	+24.9 (c=1, H_2O)
Aad (OBzl)	(**2b**)	3	B	72	0.74	185—187	+22.1 (c=0.5, HCO_2H)[d]
							+24.4 (c=1, AcOH)
Api (OMe)	(**2c**)	4	A	67	0.49	201—202	+9.8 (c=1, H_2O)
Api (OBzl)	(**2d**)	4	B	70	0.83	202—204	+19.2 (c=0.5, HCO_2H)
							+19.1 (c=1, AcOH)
Asu (OMe)	(**2e**)	5	A	75	0.42	206—207	+7.5 (c=1, H_2O)
Asu (OBzl)	(**2f**)	5	B	68	0.70	208—210	+18.4 (c=0.5, HCO_2H)[e]
							+17.8 (c=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 : H_2O = 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 (%)	Rf	$[\alpha]_D^{25}$ (deg.)[d]
Boc–Asu(OMe)	(**3a**)	Boc_2O	87	0.54[b]	+3.8
Boc–Asu(OBzl)	(**3b**)	Boc_2O	93	0.54[b]	+3.5
Boc–Asu(OMe)–OcHex	(**4a**)	cHexOH/DCC/DMAP	83	0.39[c]	−18.8
Boc–Asu(OMe)–N_2H_2Z	(**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_2H_2Z	(**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) $CHCl_3$–MeOH–AcOH (90 : 10 : 2, v/v/v). c) $CHCl_3$–MeOH (19 : 1, v/v). d) c=1, MeOH.

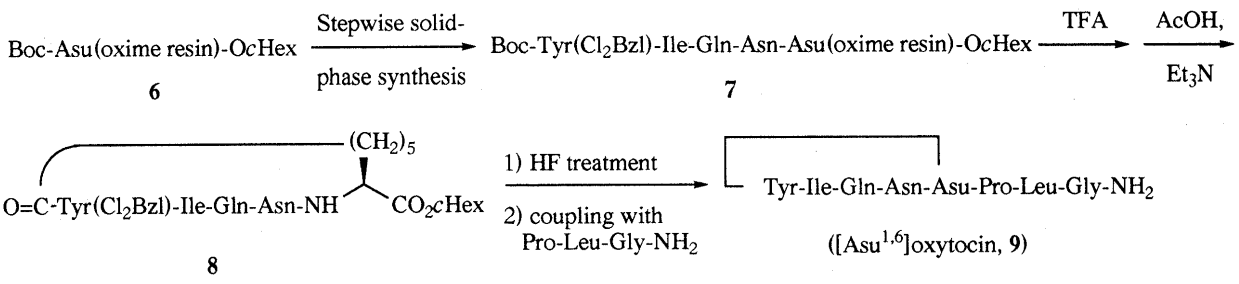


Chart 2

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–NH_2 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): Rf^1 = n-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf^2 = $CHCl_3$–MeOH–AcOH (90 : 10 : 2), Rf^3 = $CHCl_3$–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 6 M 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%). Rf^2 0.23.

Dimethyl Acetyl-DL-α-aminosuberate (Ac–DL-Asu(OMe)–OMe, 1e) DL-Asu·HCl (25 g, 0.11 mol) was converted to the dimethyl ester with the aid of thionyl chloride in methanol. The product was dissolved in $CHCl_3$ (100 ml). To this solution, Et_3N (22 g, 0.22 mol) and Ac_2O (16 g, 0.16 mol) was added at 0 °C, then the mixture was stirred at room temperature for 2 h. After evaporation of the solvent, the residue was dissolved in AcOEt. The solution was washed with water, dried over $MgSO_4$, and concentrated to afford an oil. The yield was 26 g (0.10 mmol, 92%). Rf^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 Et_3N (24 g, 0.24 mol) and Ac_2O (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%). Rf^3 0.64.

ζ-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)), $CoCl_2 \cdot 6H_2O$ (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). Reprecipitation from H_2O–EtOH gave **2e** as a white solid. Rf^1 0.42, Rf^2 0.06. mp (dec.) 206—207 °C. FAB-MS (glycerol): 204 $(M+H^+)$. *Anal.* Calcd for $C_9H_{17}NO_4 \cdot 1/4H_2O$: C, 52.04; H, 8.49; N, 6.74. Found: C, 52.17; H, 8.33; N, 6.69. $[\alpha]_D^{25}$ +7.5° (c=1.0, H_2O).

ζ-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 $CoCl_2 \cdot 6H_2O$ (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). Rf^1 0.70, Rf^2 0.12. mp (dec.) 208—210 °C. FAB-MS (glycerol): 266 $(M+H)^+$. $[\alpha]_D^{25}$ +18.4° (c=0.5, HCOOH), +17.8° (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 $CHCl_3$ give **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. Rf^2 0.82. FAB-MS (glycerol): 372 $(M+H)^+$. $[\alpha]_D^{25}$ −21.3° (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 filteration, 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 ($HOBt \cdot H_2O$, 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–NH_2 ([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–$NH_2 \cdot HCl$ (39 mg, 0.12 mmol), $HOBt \cdot H_2O$ (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 precipirated solid was purified by HPLC (eluting with a linear gradient of $CH_3CN : H_2O : TFA = 11 : 89 : 0.1$ 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

1) Yamanaka T., Hase S., Sakakibara S., Schwartz I. L., Dubais B. M., Walter R., *Mol. Pharmacol.*, **6**, 474 (1970).
2) Japan. Patent JP53-41677 (1978) [*Chem. Abstr.*, **89**, 44248 (1978)].
3) Hase S., Kiyoi R., Sakakibara S., *Bull. Chem. Soc. Jpn.*, **41**, 1266 (1968); Mori K., Sugai T., Maeda Y., Okazaki T., Noguchi T., Naito H., *Tetrahedron*, **41**, 5307 (1985).
4) Chen S. T., Wang K. T., *Synthesis*, **1987**, 581.
5) Pugnière M., Castro B., Domergue N., Previero A., *Tetrahedron Asymmetry*, **3**, 1015 (1992).
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.
7) Nishino N., Xu M., Mihara H., Fujimoto T., Ohba M., Ueno Y., Kumagai H., *J. Chem. Soc., Chem. Commun.*, **1992**, 180.
8) Ösapay G., Profit A., Taylor J. W., *Tetrahedron Lett.*, **31**, 6121 (1990); Xu M., Nishino N., Mihara H., Fujimoto T., Izumiya N., *Chem. Lett.*, **1991**, 191.
9) Tam J. P., Wong T. W., Rieman M. W., Tjiong F. S., Merrifield R. B., *Tetrahedron Lett.*, **1979**, 4033.
10) Nishino N., Xu M., Mihara H., Fujimoto T., *Bull. Chem. Soc. Jpn.*, **65**, 991 (1992).