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175. Yoshifumi Maki, Makoto Sato, and Kazunaga Obata :
Studies of Steroidal α -Amino Acids. I. Synthesis
of Two Epimeric 3-Amino-5 α -cholestane-3-
carboxylic Acids and Their Configuration.

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Since Connors, *et al.*¹⁾ observed the antitumor activity of 1-aminocyclopentanecarboxylic acid, increasing attentions^{2~4)} have been focused on some alicyclic α -amino acids from the view-point of their biological activities.

Recently, Munday, *et al.*^{5,6)} reported their new findings concerning the synthetic method for alkyl substituted 1-aminocyclohexanecarboxylic acids. Their method involves a stereospecific synthesis of the amino acids, which are epimeric to each other, by adopting either the Strecker method or the Bucherer hydantoin method. However, they did not offer any suggestions regarding the mechanism of these stereospecific reactions.

There are only a few literatures^{7,8)} on steroidal α -amino acids prepared from androsterone and testosterone by the Bucherer hydantoin method, but these did not present sufficient informations about synthesis and their stereochemistry.

The purpose of this study is to develop a synthetic method for compounds with an α -amino acids function in several positions of the steroidal skeleton and to establish their stereochemistry, in consideration of Munday's observation in cyclohexane series.

First, we wish to describe the synthesis of two epimeric 3-amino-5 α -cholestane-3-carboxylic acids (IV and V') by two methods, *i.e.*, the one by the modified Strecker method and the other by the Bucherer hydantoin synthesis, as shown in Chart 1. Also the steric configuration of IV and V' was elucidated by chemical and physico-chemical methods.

By the modified Strecker synthesis using sodium cyanide and ammonium chloride in aqueous ether, 5 α -cholestan-3-one (I) was converted into the amino nitrile (II), m.p. 144~146°, in 70% yield. However, a thin-layer chromatographic analysis of the reaction mixture revealed the presence of five substances. Four of them were identified

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1) T. A. Connors, L. A. Elson : *Biochem. Pharmacol.*, **1**, 239 (1958).

2) A. B. Mauger, W. C. J. Ross : *Ibid.*, **11**, 847 (1962).

3) P. TAILLEUR, L. BERLINGUET : *Can. J. Chem.*, **40**, 2214 (1962).

4) S. Aaronson, B. Bensky : *Biochem. Pharmacol.*, **11**, 983 (1962).

5) L. Munday : *J. Chem. Soc.*, **1961**, 4372; *Ibid.*, **1964**, 1413.

6) R. J. W. Cremlyne : *Ibid.*, **1962**, 3977.

7) K. Miescher, A. Wettstein : *Helv. Chim. Acta*, **21**, 1317 (1938).

8) *Idem* : *Brit. Pat.*, 488,829 (1938). (*C. A.*, **33**, 180 (1938)).

through thin-layer chromatography respectively as I, II, and two epimeric cyano-hydrins, which are intermediates of the Strecker reaction. The last one is probably a very small amount of the epimer of II, but could not be separated on a preparative scale.

Chart 1.

As II was decomposed to the original ketone (I) by heating in acid or basic medium, II was led to the corresponding acetate (III), m.p. 231~232.5°, by means of pyridine-acetic anhydride in order to accomplish facile hydrolysis to amino acid (IV). Purity of the acetate (III) was checked by thin-layer and gas chromatography, and its structure was supported by its analytical and spectral data; infrared absorption bands at 3340 (ν_{NH}), 2225 ($\nu_{\text{C}\equiv\text{N}}$) and 1690 ($\nu_{\text{C}=\text{O}}$) cm^{-1} , and peaks at 8.00 τ (CH_3CO) and 3.36 τ (NHCOCH_3) in the nuclear magnetic resonance spectrum (in CDCl_3).

In order to convert III into the amino acid (IV), (III) was heated drastically with conc. hydrochloric acid in a sealed tube at 130~160° for 16 hr. Purification of the resulting IV hydrochloride, m.p. 248~260° (decomp.), was difficult because of its limited solubility in water and organic solvents, but IV sulfate, m.p. 265~270°, was easily recrystallized from methanol. Thus, the desired amino acid (IV), m.p. 278~280°, was obtained by treating IV sulfate with aqueous ammonia.

The presence of α -amino acid function in IV was fully supported by the formation of methyl ester (V), m.p. 115~116°, N-acetate (VI), m.p. 260~261°, and N-benzoate (VII), m.p. 261~261.5°.

Hydrolysis of III to IV was achieved by the following circuitous route: III was easily hydrolysed to the N-acetyl acid amide (VIII), m.p. 248~250°, by treating it with alkaline hydrogen peroxide and, when VIII was heated with a mixture of dioxane and conc. hydrochloric acid for 5 hr., IV hydrochloride was formed, together with the N-acetyl amino acid (VI).

According to the Bucherer hydantoin procedure, I was allowed to react with a mixture of potassium cyanide, ammonium carbonate, and 60% aqueous ethanol in

a sealed tube at 90~100° for 6 hr. to afford a mixture of two isomeric spiro[5 α -cholestane-3,5'-hydantoins] (X), m.p. above 305°, and (X'), m.p. 273~274°, in about 1:9 ratio. Separation of the two epimers was made by utilizing their different solubility in ethyl acetate and their purity was examined by thin-layer chromatography. Infrared spectra of these compounds exhibited characteristic absorption bands corresponding to a hydantoin derivative at 3280, 1775, and 1730 cm⁻¹ in X and at 3220, 1780, and 1735 cm⁻¹ in X'. Furthermore, they exhibited similar ultraviolet absorption spectra with a maximum at 252 m μ (fine structure) in dioxane, while they showed different Rf values in thin-layer chromatography.

Tanabe, *et al.*⁹⁾ previously reported the synthesis of spiro[cholestane-3,5'-hydantoin], m.p. 335°, which seemed to be identical with our compound (X), m.p. above 305°, but they did not describe anything about the isolation of its epimer (X'), m.p. 273~274°.

The alkali fusion of the major product (X') afforded an amino acid (N'), m.p. 264~265°, in a very good yield, which was purified after its conversion to N' sulfate, m.p. 269~271°. Presence of an amino acid function in N' was supported by the formation of its derivatives; methyl ester (V'), m.p. 141~141.5°, N-acetate (W'), m.p. 215~217°, and N-benzoate (W''), m.p. 252~253°. Each of these derivatives is different from the corresponding derivatives of the amino acid (N), prepared by the Strecker method, in melting point, infrared spectra, and Rf values of thin-layer chromatography. Mass spectrum of the methyl ester (V') is nearly identical with that of V, except for the difference in their intensities, as shown in Fig. 3 (a) and (b). These spectra appear reasonable to support the structure of V and V'.

The minor hydantoin derivative (X) was converted into the corresponding amino acid and its derivatives, similarly as in the case of X'. The derivatives were identified as those of the amino acid (N), prepared by the Strecker synthesis, by means of a mixed melting point determination and a comparison of their infrared spectra.

Furthermore, the relation between the minor hydantoin derivative and the Strecker product was established by the following procedure: The amino-nitrile (II) was transformed into the ureide (K), which, without purification, was subjected to ring-closure to form the hydantoin X by heating with conc. hydrochloric acid.

These experimental results support that the amino acids, N and N', are epimeric to each other. Thus, it was revealed that the Bucherer and the Strecker methods introduce an α -amino acid function into the steroidal skeleton with high stereospecificity which works in an opposite orientation. These results are in good agreement with Munday's results in cyclohexane series.

Next, attempts were made to assign configurations of amino acids (N), (N'), and related compounds, and analyse of chemical and physical data described below suggested that they may have configurations as shown in Chart 1.

1) Stoll, *et al.*¹⁰⁾ reported that C-O stretching vibration bands of axial ester group are more complex than those of equatorial one. On the basis of this fact, axial configuration is assigned to the ester group in V and accordingly the carboxyl group in N (See Fig. 1).

2) In the infrared spectra of two epimeric 1-aminocyclohexanecarboxylic acids in solid state, Munday⁵⁾ pointed out that the equatorial ammonium group exhibits well-defined ammonium deformation bands, while bands of axial one are not well resolved. In analogy with this finding, Fig. 2 suggests that N sulfate contains an equatorial ammonium group.

9) K. Tanabe, K. Sakai: Japan. Pat., 4,066 (1963). (C. A., 59, 11,625 (1963)).

10) A. Stoll, T. Petrzilka, J. Rtschmann, A. Hoffmann, H. H. Günthard: Helv. Chim. Acta, 37, 2039 (1954).

Fig. 1. Infrared Spectra of Two Epimeric Esters (V) and (V') in the 1100~1300 cm^{-1} Region (CCl_4)

Fig. 2. Ammonium Deformation Bands of N-sulfate and N'-sulfate (Nujol)

3) Biemann, *et al.*^{11,12)} reported that in the mass spectra of two epimeric alcohols or their acetates, the steric requirements of a hydroxyl or acetoxyl group are responsible for the faster decomposition of a molecular ion of the more crowded species. Accordingly, if the Strecker amino acid (N) has a carboxyl group in the axial position, its methylester (V) may be expected to undergo fragmentation to the $(M-59)^+$ peak more easily than its epimeric methyl ester (V').

As shown in Fig. 3 (a) and (b), both (V) and (V') exhibit very strong $(M-59)^+$ peaks which are characteristic for α -amino acids in their mass spectra, and the intensity of $(M-59)^+$ peak is 28.99% for V, and 27.33% for V'. The difference in the intensities of

Fig. 3. Mass Spectra of Two Epimeric Methyl Esters

(a) V; (b) V' mol. wt. 445

11) K. Biemann, J. Seibl: *J. Am. Chem. Soc.*, **81**, 3149 (1959).

12) K. Biemann: "Mass Spectrometry. Organic Chemical Applications," p. 144 (1962), McGraw-Hill Book Co., New York.

(M-59)⁺ peak seems to support the axial configuration for the ester group of V, but this conclusion is not reliable enough because the difference of their intensities is small.

4) Bird and Cookson¹³⁾ studied the basicity of epimeric equatorial and axial primary amino and dimethylamino groups which are in various positions of the steroidal skeleton and found that the axial amino group is generally less basic than their equatorial epimers because solvation, which would assist salt formation, is more restricted in the axial group. Measurement of pK_a' (in water-methylcellosolve solution) of two epimeric methyl esters (V) and (V') was made by potentiometric titration. V (pK_a' 6.87) is more basic than its epimer (V') (pK_a' 6.74). This suggests that the amino group is equatorial in V and its series.

5) As is well-known, the axial ester group shows resistance to hydrolysis in comparison with the equatorial one. The relative rate of alkaline hydrolysis of the methyl esters (V') and (V) was measured under the identical condition as will be described in the experimental part. It was thereby found that the hydrolysis rate of V' was about 1.7 times faster than that of V. This finding indicates that the ester group in V' is equatorial.

Chart 2.

Munday⁵⁾ suggested the configuration of the α -amino acid groups in the alkyl-substituted 1-aminocyclohexanecarboxylic acids (A and B in Chart 2), prepared respectively by either the Strecker method or the Bucherer-hydantoin method, from dissociation constants and infrared spectra. The conformations of A and B correspond to that of the A ring in our amino acids, IV and IV', respectively, thus suggesting analogy with cyclohexane series in stereospecificity of synthesis. This conclusion seems reasonable, since it is considered that the steric environments of the carbonyl groups in I and in alkyl-substituted cyclohexanone are similar.

Attempts to elucidate the difference of stereochemical course between the Strecker method and the Bucherer method are now in progress, taking into consideration their reaction mechanisms and the synthetic data for α -amino acids in several positions of the steroidal skeleton.

Experimental

3 β -Amino-5 α -cholestane-3 α -carbonitrile (II)—A mixture of 5 α -cholestane-3-one (5 g.), NaCN (1 g.), and NH₄Cl (1 g.) in ether (80 ml.) containing H₂O (50 ml.) was stirred under ice-cooling for 6 hr. The reaction mixture was extracted with ether, and the ether extract was washed with H₂O, dried over Na₂SO₄, and evaporated *in vacuo*. The residue was dissolved in MeOH (250 ml.) with slight warming and the MeOH solution was saturated with dry ammonia gas at 10°. The solution was set aside in a refrigerator for 2 days in order to complete the reaction. The crude crystals deposited on standing, which were collected and recrystallized from MeOH to 3.7 g. of colorless needles (II), m.p. 144~146°. *Anal.* Calcd. for C₂₈H₄₈N₂: C, 81.49; H, 11.72; N, 6.79. Found: C, 81.34; H, 11.82; N, 6.52. IR cm⁻¹: ν_{NH} 3375, 3300; ν_{CN} 2210; δ_{NH} 1620 (CCl₄).

13) C. W. Bird, R. C. Cookson: J. Chem. Soc., 1960, 2343.

3 β -Acetamido-5 α -cholestane-3 α -carbonitrile (III)—II (1 g.) in a mixture of Ac₂O (4 ml.) and pyridine (7 ml.) was allowed to stand at room temperature overnight and the solvent was evaporated *in vacuo*. The residue was washed with H₂O, dried, and recrystallized from Ac₂O to 0.95 g. of colorless needles, m.p. 231~232.5°. *Anal.* Calcd. for C₃₀H₅₀ON₂ (III): C, 79.24; H, 11.08; N, 6.16. Found: C, 79.06; H, 11.05; N, 6.13. IR cm⁻¹: ν_{NH} 3340; ν_{CN} 2225; ν_{CO} 1690 (KBr). NMR (τ): 8.00 (CH₃CO), 3.36 (NHCO-CH₃) (CDCl₃).

3 β -Acetamido-5 α -cholestane-3 α -carboxamide (VIII)—A mixture of III (1.0 g.), 10% NaOH (6 ml.), 15% H₂O (10 ml.), and Ac₂O (200 ml.) was stirred at room temperature for 10 hr. Resulting crystals were collected and recrystallized from Ac₂O to colorless needles, m.p. 248~250°. Yield, 280 mg. *Anal.* Calcd. for C₃₀H₅₂O₂N₂ (VIII): C, 76.22; H, 11.09; N, 5.93. Found: C, 75.46; H, 11.44; N, 5.97. IR cm⁻¹: ν_{NH} 3550~3310; ν_{CO} 1685, 1635 (Nujol).

Hydrolysis of III to 3 β -Amino-5 α -cholestane-3 α -carboxylic Acid (IV)—A mixture of III (2 g.) and conc. HCl (50 ml.) was heated in a sealed tube at 130~160° for 16 hr. IV hydrochloride, m.p. 248~260°, separated from the solution. It was insoluble in H₂O and in usual organic solvents. This hydrochloride was neutralized with conc. NH₄OH to afford a colorless amorphous substance which was treated with conc. H₂SO₄ in MeOH. The precipitate thus obtained was recrystallized from MeOH to IV sulfate as colorless crystals, m.p. 265~270°. *Anal.* Calcd. for C₂₈H₅₁O₆NS: C, 63.52; H, 9.64; N, 2.64. Found: C, 63.73; H, 9.73; N, 2.34. IR cm⁻¹: $\nu_{\text{NH}_3^+, \text{OH}}$ 3400~2500; ν_{CO} 1760.

IV sulfate was treated with conc. NH₄OH and the resulting substance was washed with H₂O and MeOH, and dried over P₂O₅ to give IV as colorless amorphous substance, m.p. 278~280°. *Anal.* Calcd. for C₂₈H₄₉O₂N: C, 77.96; H, 11.44; N, 3.24. Found: C, 77.16; H, 11.38; N, 3.60. IR cm⁻¹: $\nu_{\text{NH}_3^+}$ 2300~3400; $\delta_{\text{NH}_3^+}$ 1630, 1600, 1520; ν_{COO^-} 1565 (Nujol).

Hydrolysis of 3 β -Acetamido-5 α -cholestane-3 α -carboxamide (VIII)—A mixture of VIII (0.3 g.), 35% HCl (5 ml.), H₂O (1 ml.), and dioxane (20 ml.) was heated on a steam-bath for 5 hr. The precipitate was collected by filtration and washed with H₂O and ether to colorless crystals, m.p. 250~260°(decomp.), which were identical with a specimen of IV-HCl obtained as above in IR spectra. When the filtrate was concentrated to a small volume under a reduced pressure, a second product VI precipitated. VI was identical with a specimen obtained by acetylation of IV by admixture and comparison of the IR spectra.

Two Epimeric Spiro[5 α -cholestane-3,5'-hydantoin] (X and X')—A mixture of I (5 g.), KCN (2.5 g.), and (NH₄)₂CO₃ (9.0 g.) in 60% EtOH (50 ml.) was heated in a sealed tube at 90~100° for 6 hr. After allowing to stand at room temperature overnight, the precipitated product was collected, washed with H₂O, dried, and extracted repeatedly with EtOAc. The EtOAc extract was concentrated to a small volume to give colorless feathery crystals, m.p. 273~274°. Yield, 4.5 g. *Anal.* Calcd. for C₂₉H₄₈O₂N₂: C, 76.26; H, 10.59; N, 6.13. Found: C, 76.26; H, 10.70; N, 6.31. IR cm⁻¹: ν_{NH} 3280 (broad); ν_{CO} 1775, 1730 (Nujol). UV $\lambda_{\text{max}}^{\text{dioxane}}$ m μ (ϵ): 252 (6,200) (fine structure).

The material insoluble in EtOAc was collected and recrystallized from tetrahydrofuran to colorless crystals (X), m.p. above 305°. Yield, 0.45 g. *Anal.* Calcd. for C₂₉H₄₈O₂N₂: C, 76.26; H, 10.59; N, 6.13. Found: C, 76.41; H, 10.51; N, 5.88. IR cm⁻¹: ν_{NH} 3220 (broad), ν_{CO} 1780, 1735 (Nujol). UV spectrum of X was identical with that of X'.

Conversion of II to Spiro[5 α -cholestane-3,5'-hydantoin] (X)—A solution of II (0.5 g.) and KNCO (1.0 g.) in 70% AcOH (10 ml.) was heated at 70° for 1 hr. After dilution with H₂O, the resulting precipitate (X) was collected and refluxed with 20% HCl (20 ml.) for 3 hr. to give a crystalline solid. It was washed with H₂O and recrystallized from tetrahydrofuran to X as colorless crystals, m.p. 300°, which was identical by IR comparison with the higher melting hydantoin (X) obtained as above.

Hydrolysis of Two Epimeric Spiro[5 α -cholestane-3,5'-hydantoin] (X and X')—X (1 g.) was submitted to alkaline fusion with a mixture of NaOH (10 g.) and H₂O (5 ml.) in a fusion-pot. Heating was continued for 0.5 hr. by adding occasionally a small amount of H₂O. After the resulting solid mass was treated with H₂O (30 ml.), the white precipitate formed was collected, washed with H₂O, and dried. Treatment of the precipitate with 70% H₂SO₄ (10 ml.) afforded IV sulfate, which was recrystallized from MeOH to colorless crystals. Identity of IV sulfate was ascertained by IR comparison with a specimen obtained by the hydrolysis of III. Its epimeric amino acid, IV' sulfate, was prepared from X' by the same method as described above, as colorless powder, m.p. 269~271°. *Anal.* Calcd. for C₂₈H₅₁O₆NS: C, 63.52; H, 9.64; N, 2.64. Found: C, 63.22; H, 9.50; N, 2.59. IR cm⁻¹: $\nu_{\text{NH}_3^+, \text{OH}}$ 3300~2500; ν_{CO} 1760, 1725 (Nujol). IV' sulfate was treated with conc. NH₄OH to give IV' as amorphous substance, m.p. 262~264°. *Anal.* Calcd. for C₂₈H₄₉O₂N: C, 77.96; H, 11.44; N, 3.24. Found: C, 77.30; H, 11.30; N, 3.50. IR cm⁻¹: $\nu_{\text{NH}_3^+}$ 3400~2400; $\delta_{\text{NH}_3^+}$ 1635, 1590, 1515; ν_{COO^-} 1570 (Nujol).

Methyl 3 β -Amino-5 α -cholestane-3 α -carboxylate (V) and Its Epimer (V')—A solution of IV sulfate (0.5 g.) in absolute MeOH (50 ml.) containing conc. H₂SO₄ (7 ml.) was refluxed for 10 hr. The residue obtained by evaporation of MeOH was extracted with ether after neutralization with Na₂CO₃ solution. The ether extract was washed with H₂O, dried over anhyd. Na₂SO₄, and evaporated to dryness. Repeated recrystallization of the residue from MeOH gave V as colorless plates, m.p. 115~116°. *Anal.* Calcd.

for $C_{29}H_{51}O_2N$: C, 78.14; H, 11.53; N, 3.14. Found: C, 77.71; H, 11.56; N, 3.09. IR cm^{-1} : ν_{NH} 3340 (broad); ν_{CO} 1730 (CCl_4).

Its epimeric ester (V') was prepared by the same method as above. Colorless prisms, m.p. 141~141.5°. *Anal.* Calcd. for $C_{29}H_{51}O_2N$: C, 78.14; H, 11.53; N, 3.14. Found: C, 78.01; H, 11.49; N, 3.10. IR cm^{-1} : ν_{NH} 3340 (broad); ν_{CO} 1730 (CCl_4).

3 β -Acetamido-5 α -cholestane-3 α -carboxylic Acid (VI) and Its Epimer (VI')—A mixture of V (0.2 g.), AcONa (0.5 g.), and AcCl (15 ml.) was refluxed for 3 hr. The crystalline mass obtained by removal of excess AcCl was taken up in ether and the ether solution was washed with H_2O , dried over anhyd. Na_2SO_4 , and evaporated to dryness. The residue was recrystallized from EtOAc to VI as colorless crystals, m.p. 259~261°. *Anal.* Calcd. for $C_{30}H_{51}O_3N$: C, 76.06; H, 10.35; N, 2.96. Found: C, 75.80; H, 10.85; N, 2.66. IR cm^{-1} : ν_{NH} 3350; ν_{CO} 1718, 1640 (Nujol).

By treating V' as described above for the preparation of the acetate (VI), N-acetate (VI') was obtained as colorless crystals, m.p. 215~217°. *Anal.* Calcd. for $C_{30}H_{51}O_3N$: C, 76.06; H, 10.85; N, 2.96. Found: C, 75.76; H, 11.12; N, 2.90. IR cm^{-1} : ν_{NH} 3350; ν_{CO} 1725, 1655 (Nujol).

3 β -Benzamido-5 α -cholestane-3 α -carboxylic Acid (VII) and Its Epimer (VII')—A mixture of BzCl (15 ml.) and V (0.2 g.) was heated in a water bath for 5 hr. After removal of excess reagent the crystalline solid was taken up in ether and the solution was washed with 5% KOH and H_2O , and dried over anhyd. Na_2SO_4 . In order to remove a persistent impurity, the residue dissolved in $CHCl_3$ was chromatographed on silica gel. The material thus obtained was recrystallized from EtOAc to VII as colorless crystals, m.p. 261°. *Anal.* Calcd. for $C_{35}H_{53}O_3N$: C, 78.46; H, 9.97; N, 2.61. Found: C, 78.42; H, 9.86; N, 2.33. IR cm^{-1} : ν_{NH} 3340; ν_{CO} 1740, 1635 (Nujol).

Its epimeric N-benzoate (VII') was prepared as above for preparation of VII. Colorless crystals (from MeOH), m.p. 252~253°. *Anal.* Calcd. for $C_{35}H_{53}O_3N$: C, 78.46; H, 9.97; N, 2.61. Found: C, 78.40; H, 9.88; N, 2.30. IR cm^{-1} : ν_{NH} 3330; ν_{CO} 1695, 1640 (Nujol).

Determination of pKa' of Two Epimeric Methyl Esters (V and V')—Determination of pKa' was carried out in H_2O -methylcellosolve (1:9) solution at 10°. Twenty ml. of 0.05M solution of the methyl esters (V) and (V') was half-neutralized with 0.05M hydrochloric acid solution. The pH was determined with a glasscalomel electrode in conjunction with a Yanagimoto Model 42-A pH-meter. The pKa' values were 6.87 for V and 6.74 for V'.

Measurement of the Relative Rate of Hydrolysis of the Methyl Esters (V and V')—To a solution of 11.2 mg. of V (or V') in 5.0 ml. of MeOH 0.1N KOH (0.30 ml.) was added the mixture was refluxed. Hydrolysis of the two isomers was effected under the same conditions and the time required for complete hydrolysis was measured by thin-layer chromatography. The spot of methyl ester (V) disappeared completely after 7 hr., while that of V' did after 4 hr.

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Summary

Two epimeric 3-amino-5 α -cholestane-3-carboxylic acids were synthesized by both the Strecker method and the Bucherer-hydantoin synthesis as shown in Chart 1. The configurations of these amino acids were established by comparing the hydrolysis rate of their methyl esters. Additionally, their configurations were also supported by some other data such as the pKa' values, mass spectra, and infrared spectra of their derivatives.

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