

VANOXONIN, A NEW INHIBITOR OF THYMIDYLATE SYNTHETASE

II. STRUCTURE DETERMINATION AND TOTAL SYNTHESIS

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Acid hydrolysis of vanoxonin yielded one mol each of 2,3-dihydroxybenzoic acid, L-threonine, L-*N*^ω-hydroxyornithine. Presence of acetyl group in vanoxonin was suggested by the ¹H NMR. Periodate oxidation of vanoxonin liberated one mol of acetic acid suggesting that the acetyl group bound to the ω-nitrogen of *N*^ω-hydroxyornithine. The sequence of three components was determined to be L-*N*-(2,3-dihydroxybenzoyl)threonyl-L-*N*^ω-acetyl-*N*^ω-hydroxyornithine by mass spectrometric analysis. This structure was confirmed by the total synthesis of vanoxonin.

In a previous paper¹⁾, we reported the isolation and characterization of vanoxonin, which was produced by a strain of *Saccharopolyspora hirsuta*. We also reported its vanadium complex which inhibited thymidylate synthetase. In this paper, the structure determination and total synthesis of vanoxonin are reported.

Structural Studies of Vanoxonin

Vanoxonin is obtained as colorless amorphous powder as reported previously¹⁾. The molecular formula was determined to be C₁₈H₂₅N₃O₈ (MW 427) by field desorption mass spectrometry, elementary analysis, and ¹³C NMR spectrometry. It showed ultraviolet absorption maxima at 247 nm (ε 8,750) and 311 (2,560) in H₂O; at 345 (3,290) in 0.01 N NaOH. The IR spectrum showed an absorption at 1720 cm⁻¹ attributed to a carboxylic acid, because this absorption band disappeared in the sodium salt and the potentiometric titration gave the *pKa'* value at 2.8 in addition to 7.0 and 9.0. The IR spectrum also showed absorption bands at 1640 and 1540 cm⁻¹ attributable to amide bonds. Vanoxonin gave positive Rydon-Smith but negative ninhydrin reaction suggesting a peptide structure with a masked *N*-terminus.

Hydrolysis of vanoxonin with 2 N HCl at 100°C for 8 hours gave one ethyl acetate-extractable substance and two ninhydrin positive products (Scheme 1). The solvent soluble substance was purified by Sephadex LH-20 column chromatography and identified with 2,3-dihydroxybenzoic acid. Two ninhydrin positive

Scheme 1. Acid hydrolysis of vanoxonin.

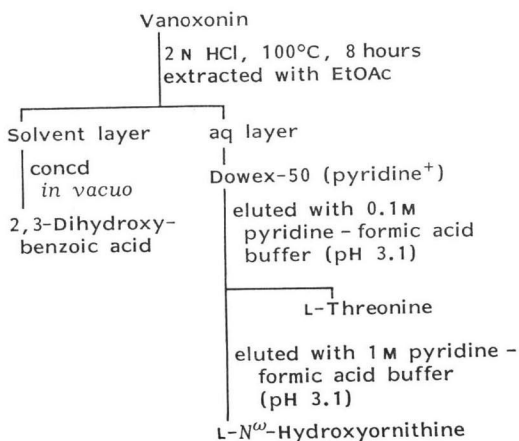
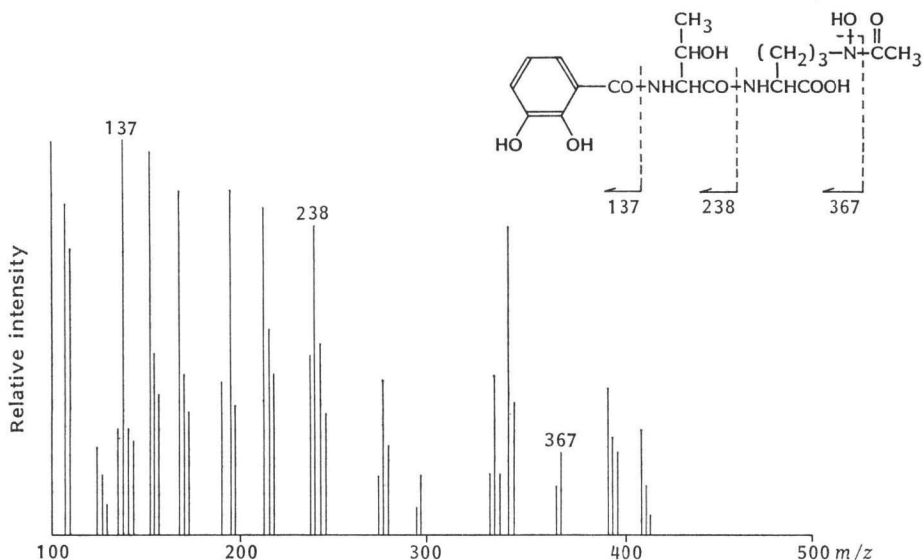


Fig. 1. EI mass spectrum of vanoxonin.



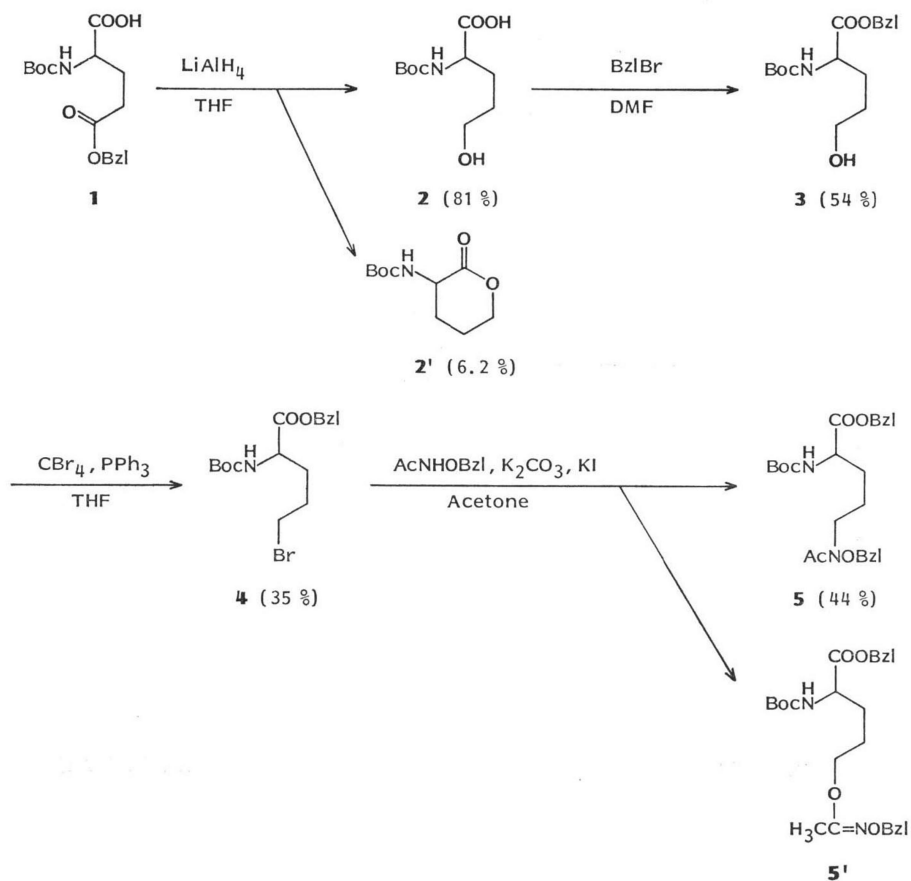
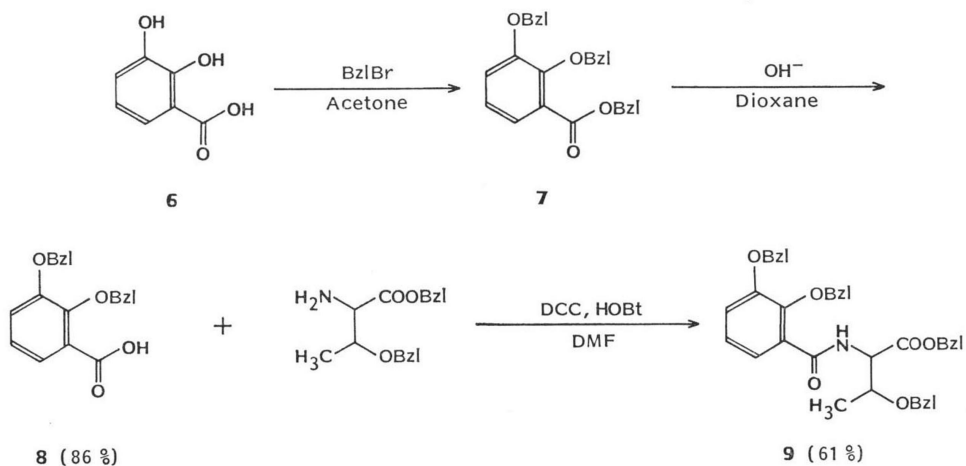
products were separated by a column chromatography of Dowex 50WX4 (pyridine form). One of them was eluted with 0.1 M pyridine-formate buffer (pH 3.1) and identified as L-threonine, and the other, which was eluted with 1 M pyridine-formate buffer (pH 3.1), was identical with L-*N*^ω-hydroxyornithine.

The ¹H NMR spectrum of vanoxonin in D₂O indicated the presence of an acetyl group (δ 2.55, (s, 3H)) in addition to the above-described three constituents. Periodate oxidation of vanoxonin liberated one mol of acetic acid which was identified by gas chromatography. It has been reported by NEILANDS²⁾ that periodate attacked hydroxamic acids causing the liberation of the acyl group as the free acid. Therefore, it was suggested that the acetyl group bound to the hydroxyamino group of *N*^ω-hydroxyornithine to form an acetohydroxamic acid. As a control experiment, acetohydroxamic acid (CH₃CONHOH) and acetamide were treated with periodate in the same way. One mol of acetic acid was liberated from acetohydroxamic acid but not from acetamide.

The sequence of the constituents was determined by mass spectrometry as shown in Fig. 1. The presence of dihydroxybenzoyl moiety (m/z 137), dihydroxybenzoyl-threonyl moiety (m/z 238), and dihydroxybenzoyl-threonyl-ornithine moiety (m/z 367) was revealed by the fragmentation ions. These results suggested that the structure of vanoxonin should be L-*N*-(2,3-dihydroxybenzoyl)threonyl-L-(*N*^ω-acetyl-*N*^ω-hydroxy)ornithine.

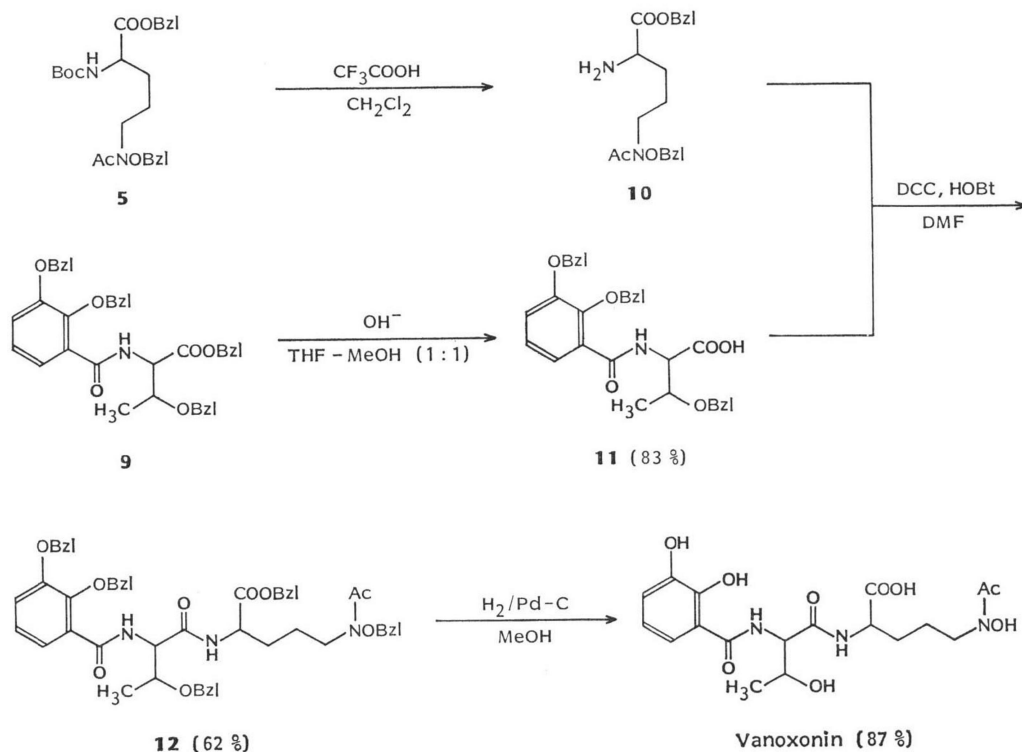
Synthesis of Vanoxonin

In order to confirm the above proposed structure, we carried out a synthesis of vanoxonin. L-(*N*^α-Boc-*N*^ω-Acetyl-*N*^ω-benzyloxy)ornithine benzyl ester (**5**) was synthesized from γ -benzyl ester of *N*-Boc-L-glutamic acid through 4 steps according to a modified procedure for the preparation of *N*^ω-acetyl-*N*^ω-hydroxylysine by MILLER *et al.*^{3,4)} (Scheme 2). L-(*N*-Boc- ω -Hydroxy)norvaline (**2**) was prepared from L-*N*-Boc-glutamic acid γ -benzyl ester by hydrogenolysis of the benzyl ester with lithium aluminum hydride. A small amount (6.2%) of the δ -lactone (**2'**) was also formed, which was easily separated by solvent extraction. The remaining carboxyl group of **2** was treated with benzyl bromide to give L-

Scheme 2. Synthesis of L-(*N*^α-Boc-*N*^ω-acetyl-*N*^ω-benzyloxy)ornithine benzyl ester.Scheme 3. Synthesis of L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonine benzyl ester.

(*N*-Boc- ω -hydroxy)norvaline benzyl ester (**3**). The hydroxyl group of **3** was converted to the bromide group by the treatment with triphenyl phosphine and carbon tetrabromide in dry THF to give **4**. The bromide (**4**) was reacted with *O*-benzyl acetohydroxamate in dry acetone with an excess of K_2CO_3 and a

Scheme 4. Synthesis of vanoxonin from L-(*N*^α-Boc-*N*^ω-acetyl-*N*^ω-benzyloxy)ornithine benzyl ester and L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonine benzyl ester.



catalytic amount of KI to form L-(*N*^α-Boc-*N*^ω-acetyl-*N*^ω-benzyloxy)ornithine benzyl ester (**5**). The *O*-alkyl derivative (**5'**) was also formed in a small amount (9.2%), but this isomer was easily separated by silica gel chromatography.

The protected derivative of L-*N*-(2,3-dihydroxybenzoyl)threonyl moiety of vanoxonin was synthesized as shown in Scheme 3. 2,3-Dibenzyloxybenzoic acid (**8**) was prepared from 2,3-dihydroxybenzoic acid (**6**) by reaction with benzylbromide in dry acetone followed by hydrolysis of the resulting benzyl ester (**7**) with sodium hydroxide. The compound **8** was condensed with L-(*O*-benzyl)threonine benzyl ester in dry DMF with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) to form L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonine benzyl ester (**9**).

The final steps of the synthesis of vanoxonin from compounds **5** and **9** are shown in Scheme 4. Selective removal of the α-amino protective group of compound **5** was accomplished by mild treatment with CF₃CO₂H to give **10**, and alkaline hydrolysis of the benzyl ester (**9**) gave the free acid (**11**). Condensation of **10** and **11** in dry DMF with DCC and HOBT to form tetra-*O*-benzylvanoxonin benzyl ester (**12**). Finally, hydrogenolysis of **12** with palladium-carbon gave vanoxonin (L-*N*-(2,3-dihydroxybenzoyl)-threonyl-L-(*N*^ω-acetyl-*N*^ω-hydroxy)ornithine). The physico-chemical properties of synthetic vanoxonin were the same as those of the natural product. Thus, the structure of vanoxonin was conclusively determined.

Experimental

General

Melting points were determined on a Yanaco hot-stage microscope MP-3 and were uncorrected. UV spectra were determined on a Hitachi EPS-3T recording spectrophotometer, and IR spectra on a Hitachi 260-10 spectrophotometer. NMR spectra were recorded on a Varian XL-100 spectrometer. Chemical shifts were expressed in values (ppm) with tetramethylsilane as an internal standard. Proton-noise decoupled FT-¹³C NMR spectra were taken at 25.2 MHz on a Varian XL-100 spectrometer using tetramethylsilane as reference. The mass spectra were taken by a Hitachi mass spectrometer RMU-6M. Optical rotations were determined by a Carl Zeiss LEPA2. *pKa* values were measured by a Metrohm Herisau Potentiograph E436. Precoated silica gel F-254 layers (E. Merck, Darmstadt) were used for TLC. High voltage paper electrophoresis was carried out in formic acid - AcOH - H₂O (25: 75: 900) under 3,500 V for 15 minutes and R_m values (alanine=1) were calculated. GLC was performed with Shimadzu gas chromatograph GC-4CM (one meter steel column with 10% diethylene glycol succinate polymer on Chromosorb W 80~100 mesh; FID; temperatures: 210/160/210°C inject-column-detection).

Periodate Oxidation of Vanoxonin, Acetohydroxamic Acid and Acetamide

To a 5 ml of aq solution (pH 7.0) of vanoxonin (20 mg) 1.0 ml of 0.1 M periodic acid was added and the mixture stirred for about 1 minute. After 2 drops of ethylene glycol were added, the solution was extracted with 10 ml of ether 10 times. The ether extracts were concentrated to a small volume without heating and analyzed by gas chromatograph: 2.5 mg (87%) of AcOH (retention time: 2.1 minutes) was detected. Only AcOH and ethylene glycol (retention time: 3.1 minutes) were found in the extracts.

Acetohydroxamic acid and acetamide (5 mg each) were treated with periodate in the same way, respectively: 3.6 mg (91%) of AcOH formed from acetohydroxamic acid but none from acetamide.

2,3-Dihydroxy Benzoic Acid

Vanoxonin (80 mg) was hydrolyzed with 2 N HCl (30 ml) for 8 hours at 100°C. After concentration under reduced pressure, 10 ml of H₂O was added, and the mixture extracted with EtOAc (10 ml × 2). The extracts were concentrated under reduced pressure and the residue dissolved in 1 ml of MeOH. Further purification by Sephadex LH-20 chromatography (1.1 × 53 cm) and crystallization from H₂O gave 16 mg (55%) of white crystals. R_f 0.56 (CHCl₃ - MeOH, 20: 1), mp 199~202°C (ref⁶), mp 203~205°C), MS *m/z* 154 (M, C₇H₆O₄), 136 (M - H₂O). IR (KBr) cm⁻¹ 3400, 1730, 1540, 1220, 1140, 900. ¹H NMR (100 MHz, MeOH-*d*₄) δ 6.72 (1H, t, *J*=8.0 Hz), 7.01 (1H, d, *J*=8.0 Hz), 7.35 (1H, d, *J*=8.0 Hz). ¹³C NMR (25.2 MHz, MeOH-*d*₄) δ 114.2, 120.0, 121.6, 121.9, 146.9, 151.7, 173.9. Anal Calcd for C₇H₆O₄: C 55.55, H 3.90. Found: C 55.10, H 3.72.

L-Threonine and L-*N*^ω-Hydroxyornithine

The aq layer of the acid hydrolysate after the EtOAc extraction was applied to Dowex 50WX4 (pyridine form) column (1.2 × 8 cm). L-Threonine was eluted with 0.1 M pyridine - formate buffer (pH 3.1). It was crystallized from MeOH - H₂O (4: 1) to give white crystals (15 mg, 67%). R_m 0.81, mp 252~254°C (ref⁶), mp 255~257°C), [α]_D²⁵ -27.3° (*c* 1.0, H₂O) (ref⁶), [α]_D²⁵ -28.3° (*c* 1.1, H₂O)), IR (KBr) cm⁻¹ 3400, 3200, 1640, 1480, 1420, 1360, 1250, 1120, 1050, 940, 710. ¹H NMR (90 MHz, D₂O), δ 1.35 (3H, d, *J*=7.0 Hz, CH₃), 3.62 (1H, d, *J*=7.0 Hz, NCH), 4.29 (1H, m, *J*=7.0 Hz OCH). Anal Calcd for C₄H₉NO₃: C 40.31, H 7.61, N 11.80. Found: C 40.19, H 7.32, N 11.83.

L-*N*^ω-Hydroxyornithine was eluted with 1 M pyridine-formate buffer (pH 3.1). The eluate was concentrated under reduced pressure and dissolved in 1 ml of H₂O. 2-Nitro-1,3-indanedione (11 mg) was added and the pH of the solution was raised to 4.0 with dil KOH, when pale yellow needles precipitated. Recrystallization from H₂O gave 30.7 mg (48%) of mono-2-nitro-1,3-indanedione salt of L-*N*^ω-hydroxyornithine: R_m 1.30, mp 231~236°C (dec) (ref⁷), mp 232~235°C), [α]_D²⁵ +11.5° (*c* 1.0, 2 N HCl) (ref⁷), [α]_D²⁵ +11.9° (2 N HCl)). Anal Calcd for C₁₄H₁₇N₃O₇: C 49.56, H 5.05, N 12.09. Found: C 49.32, H 5.21, N 11.95. IR and ¹H NMR spectra were taken after desalting with Dowex 50WX4 (NH₄⁺) column (1.2 × 4 cm). IR (KBr) cm⁻¹ 3400, 2950, 1740, 1620, 1500, 1220, 1120, 1030. ¹H NMR (90 MHz, D₂O) δ 2.31 (4H, m, CCH₂CH₂C), 3.68 (2H, t, *J*=7.0 Hz, CCH₂N), 4.24 (1H, t, *J*=6.0 Hz, CH).

O-Benzyl Acetohydroxamate

O-Benzyl acetohydroxamate was prepared by the method of NICOLAUS *et al.* (bp 130°C/0.3 Torr). (ref⁹), bp 125°C/0.2 Torr).

L-(N-Boc- ω -Hydroxy)norvaline Benzyl Ester (3)

To an ice bath-cooled solution prepared from 3.0 g of L-N-Boc-glutamic acid γ -benzyl ester (1) and 60 ml of dry THF was added 0.473 g of lithium aluminum hydride under stirring. After stirring for 3 hours at room temp, the solution was again cooled in an ice bath and 100 ml of EtOAc was added dropwise followed by the addition of 100 ml of distilled H₂O. The pH of the H₂O layer was adjusted to 2.0 with 1 N HCl. After discarding the H₂O layer, 80 ml of cold 0.1 N NaOH was added dropwise to the solvent layer with stirring to adjust the pH of this H₂O layer to 7.3. The solvent layer was washed with H₂O, dried (Na₂SO₄) and evaporated. The residue was applied to silica gel column (3.5 \times 10 cm) and eluted with benzene - EtOAc (5: 1). Evaporation of the main fractions gave 0.31 g (6.2%) of amorphous powder (compound 2', L- α -*t*-butoxycarbonylamino- δ -valerolactone). The H₂O layer was washed with 50 ml of benzene and lyophilized to give 1.68 g (81%) of colorless powder (2). To the solution prepared from the powder and 30 ml of dry DMF was added 0.92 ml of benzylbromide, and stirred for 14 hours at room temp. The reaction mixture was poured into 150 ml of EtOAc and washed consecutively with saturated NaHCO₃ and NaCl, and dried (Na₂SO₄) and evaporated. The residue was applied to silica gel column (3.5 \times 18 cm) and eluted with benzene - EtOAc (3: 1). Evaporation of the main fractions gave 1.25 g (44%) of colorless, oily compound (3). Rf 0.43 (benzene - EtOAc, 1: 1), $[\alpha]_D^{25}$ -3.2° (*c* 1.0, CHCl₃), MS (SIMS) *m/z* 324 (M+1), IR (KBr) cm⁻¹ 3400, 2960, 2920, 1740, 1705, 1500, 1457, 1390, 1363, 1160, 1045, 750, 693. UV ($\lambda_{\max}^{\text{MeOH}}$ nm (ϵ)) 213 (5,400), 257 (635). ¹H NMR (100 MHz, CDCl₃) δ 1.42 (9H, s, C(CH₃)₃), 1.40~1.95 (4H, m, 3-CH₂, 4-CH₂), 2.33 (1H, s, OH), 3.60 (2H, t, *J*=6.0 Hz, 5-CH₂), 4.20~4.50 (1H, m, 2-CH), 5.17 (2H, s, ArCH₂), 5.30 (1H, d, *J*=7.5 Hz, NH), 7.36 (5H, s, ArH). Anal Calcd for C₁₇H₂₅NO₅: C 63.14, H 7.79, N 4.33. Found: C 63.05, H 7.51, N 4.47.

Compound 2': Rf 0.54 (benzene - EtOAc, 1: 1), mp 106~108°C, $[\alpha]_D^{25}$ +81.9° (*c* 1.0, CHCl₃), MS *m/z* 215 (M), 114 (M-COOC(CH₃)₃), IR (KBr) cm⁻¹ 3370, 1770, 1743, 1690, 1535, 1162. ¹H NMR (90 MHz, CDCl₃) δ 1.42 (9H, s, C(CH₃)₃), 1.6~2.7 (4H, m, 3-CH₂, 4-CH₂), 4.27~4.52 (3H, m, 2-CH, 5-CH₂), 5.43 (1H, br, NH). Anal Calcd for C₁₀H₁₇NO₄: C 55.80, H 7.96, N 6.51. Found: C 56.12, H 7.96, N 6.25.

L-(N-Boc- ω -Bromo)norvaline Benzyl Ester (4)

To an ice bath-cooled solution prepared from 1.20 g of compound 3, 10 ml of dry THF, and 1.83 g of carbon tetrabromide was added dropwise 5 ml of dry THF containing 1.45 g of triphenyl phosphine under stirring. After stirring for 14 hours at room temp, the resulting precipitate was removed by filtration. To the filtrate, 75 ml of benzene was added, the solution washed with saturated NaHCO₃ and NaCl, dried (Na₂SO₄) and evaporated. The residue was applied to silica gel column (3.5 \times 27 cm) and eluted with benzene - EtOAc (50: 1). Evaporation of the main-fractions gave 0.495 g (34.8%) of colorless, oily compound (4). Rf 0.74 (benzene - EtOAc, 5: 1), $[\alpha]_D^{25}$ -0.33° (*c* 1.0, CHCl₃), MS (SIMS) *m/z* 386, 388 (M+1). IR (KBr) cm⁻¹ 3400, 2960, 1740, 1710, 1500, 1450, 1365, 1160, 750, 695. UV $\lambda_{\max}^{\text{MeOH}}$ nm (ϵ) 218 (8,360), 253 (2,361), 325 (194). ¹H NMR (100 MHz, CDCl₃) δ 1.43 (9H, s, C(CH₃)₃), 1.70~2.00 (4H, m, 3-CH₂, 4-CH₂), 3.38 (2H, t, *J*=6.0 Hz, 5-CH₂), 4.20~4.45 (1H, m, 2-CH), 4.90~5.20 (1H, br, NH), 5.19 (2H, s, ArCH₂), 7.39 (5H, s, ArH). Anal Calcd for C₁₇H₂₄NO₄Br: C 52.86, H 6.26, N 3.63, Br 20.69. Found: C 52.89, H 6.16, N 3.49, Br 20.42.

L-(N $^{\alpha}$ -Boc-N $^{\omega}$ -Acetyl-N $^{\omega}$ -benzyloxy)ornithine Benzyl Ester (5)

To a solution prepared from 0.49 g of compound 4, 0.305 g of O-benzyl acetohydroxamate, and 10 ml of dry Me₂CO, 0.70 g of anhydrous potassium carbonate and 0.03 g of potassium iodide were added with stirring. After stirring for 13 hours at 60°C, the undissolved material was removed by filtration and 100 ml of EtOAc was added to the filtrate. The solution was washed with saturated NaCl and dried (Na₂SO₄) and evaporated. The residue was applied to silica gel column (2 \times 10 cm) and eluted with benzene - EtOAc (10: 1). The evaporation of the main fractions gave 0.265 g (44%) of colorless oily compound (5) and 0.055 g (9.2%) of the isomer (5').

Compound **5**: Rf 0.35 (benzene - EtOAc, 2: 1), $[\alpha]_D^{25} +1.5^\circ$ (*c* 1.0, CHCl₃), MS (SIMS) *m/z* 471 (M+1), 371 (M-COOC(CH₃)₃). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm⁻¹ 1735, 1710, 1650, 1500, 1370, 1160, 695. UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ) 252 (351), 257 (426), 263 (370). ¹H NMR (100 MHz, CDCl₃) δ 1.41 (9H, s, C(CH₃)₃), 1.55~1.85 (4H, m, 3-CH₂, 4-CH₂), 2.04 (3H, s, NCOCH₃), 3.82 (2H, t, *J*=6.0 Hz, 5-CH₂), 4.2~4.45 (1H, m, 2-CH), 4.78 (2H, s, NOCH₂Ar), 4.95~5.15 (1H, br, NH), 5.14 (2H, s, COOCH₂Ar), 7.34 (5H, s, ArH), 7.38 (5H, s, ArH). Anal Calcd for C₂₈H₃₄N₂O₆: C 66.36, H 7.28, N 5.95. Found: C 65.97, H 7.26, N 5.72.

Compound **5'**: Rf 0.62 (benzene - EtOAc, 2: 1), ¹H NMR (100 MHz, CDCl₃) δ 1.42 (9H, s, C(CH₃)₃), 1.55~1.95 (4H, m, 3-CH₂, 4-CH₂), 1.87 (3H, s, N=CCH₃), 4.02 (2H, t, *J*=6.0 Hz, 5-CH₂), 4.15~4.45 (1H, m, 2-CH), 4.94 (2H, s, NOCH₂Ar), 5.00~5.52 (1H, br, NH), 5.16 (2H, s, COOCH₂Ar), 7.35 (10H, s, ArH). Anal Calcd for C₂₀H₃₄N₂O₆: C 66.36, H 7.28, N 5.95. Found: C 66.12, H 7.03, N 5.72.

L-(N-2,3-Dibenzoyloxybenzoyl-O-benzyl)threonine Benzyl Ester (9)

To a solution prepared from 0.502 g of 2,3-dihydroxybenzoic acid, 1.8 g of potassium carbonate and 15 ml of dry Me₂CO was added dropwise 1.2 ml of benzylbromide and refluxed for 14 hours. The undissolved material was removed by filtration and the filtrate was evaporated and dissolved in 5 ml of dioxane. To the solution, 4 ml of 2 N NaOH was added. After refluxing for an hour, 4 ml of 2 N HCl was added and evaporated. To the residue, 50 ml of CHCl₃ was added and washed with H₂O and evaporated. Crystallization from EtOH gave 0.94 g (86%) of compound **8**. Rf 0.34 (toluene - EtOAc, 1: 1), mp 124.5~125°C (ref⁹), mp 124°C), MS *m/z* 334 (M, C₂₁H₁₈O₄), 243 (M-C₇H₇), 91 (C₇H₇), IR (KBr) cm⁻¹ 3430, 1690, 1580, 1470, 1450, 1410, 1375, 1300, 1255, 1030. To a solution prepared from 0.664 g of compound **8**, 0.688 g of L-(O-benzyl)threonine benzyl ester $\frac{1}{2}$ C₂H₂O₄, and 10 ml of dry DMF were added consecutively 0.28 ml of triethylamine, 0.32 g of 1-hydroxybenzotriazole, and 0.49 g of dicyclohexylcarbodiimide under stirring. After stirring for 8 hours at room temp, the undissolved material was removed by filtration and the filtrate was evaporated and dissolved in 50 ml of benzene and, washed with saturated NaHCO₃ and H₂O, and dried (Na₂SO₄) and applied to silica gel column (3.5 × 18 cm). Elution with benzene - EtOAc (19: 1) and evaporation of the main fractions gave 0.746 g (61%) of oily compound (**9**). Rf 0.20 (benzene - EtOAc, 19: 1), $[\alpha]_D^{25} +138^\circ$ (*c* 1.0, CHCl₃), MS *m/z* 615 (M, C₃₉H₃₇NO₆), 524 (M-C₇H₇), 91 (C₇H₇), IR (KBr) cm⁻¹ 3360, 1740, 1660, 1578, 1515, 1457, 1380, 1320, 1265, 1210, 1090, 963, 920, 755, 690. UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ) 251 (57,100), 292 (33,200). ¹H NMR (100 MHz, CDCl₃) δ 1.15 (3H, d, *J*=6.5 Hz, CH₃), 4.17 (1H, d, *J*=11.6 Hz, ArCH·H), 4.18 (1H, dd, *J*=6.5 Hz, *J*=2.9 Hz, CHCH₃), 4.36 (1H, d, *J*=11.6 Hz, ArCH·H), 4.96 (1H, dd, *J*=9.3 Hz, *J*=2.9 Hz, NCH), 5.06 (1H, d, *J*=4.7 Hz, ArCH·H), 5.07 (2H, s, ArCH₂), 5.10 (2H, s, ArCH₂), 5.22 (1H, d, *J*=4.7 Hz, ArCH·H), 7.0~7.37 (22H, m, ArH), 7.77 (1H, t, *J*=2.4 Hz, ArH), 8.90 (1H, d, *J*=9.3 Hz, NH). Anal Calcd for C₃₉H₃₇NO₆: C 76.08, H 6.06, N 2.27. Found: C 75.83, H 6.01, N 2.12.

Tetra-O-benzylvanoxonin Benzyl Ester (12)

To a solution prepared from 0.615 g of compound **9**, 5 ml of distilled THF and 5 ml of MeOH was added 1 ml of 2 N NaOH. After stirring for 12 hours at room temp, 2 ml of 1 N HCl was added and evaporated. To the residue, 40 ml of CHCl₃ was added and washed with H₂O and evaporated to give 0.436 g (83%) of L-(N-2,3-dibenzoyloxybenzoyl-O-benzyl)threonine (compound **11**, Rf 0.2 (CHCl₃ - MeOH, 10: 1), MS *m/z* 525 (M, C₃₉H₃₁NO₆)).

To an ice bath-cooled solution prepared from 65 mg of compound **5** and 5 ml of dry dichloromethane was added 0.5 ml of trifluoroacetic acid under stirring. Stirring was continued for 2 hours at room temp and the dichloromethane was removed *in vacuo*. The residue was extracted with 50 ml of EtOAc, washed with cold, saturated NaHCO₃ and NaCl, dried (Na₂SO₄) and evaporated to give 49 mg of ninhydrin positive compound (**10**). To an ice bath-cooled solution prepared from this compound (**10**), 72 mg of compound **11** and 4 ml of dry THF were added 19 mg of 1-hydroxybenzotriazole and 30 mg of dicyclohexylcarbodiimide. After stirring for 15 hours at room temp, the resulting precipitate was removed by filtration. 50 ml of EtOAc was added to the filtrate, washed with saturated NaCl and dried (Na₂SO₄) and evaporated. The residue was extracted with benzene and applied to silica gel column (2 × 10 cm) and eluted with benzene - EtOAc (2: 1). Evaporation of the main fractions gave 74 mg (62%) of a colorless oily compound (**12**). Rf 0.53 (benzene - EtOAc, 1: 1), $[\alpha]_D^{25} +12.8^\circ$ (*c* 2.0,

CHCl₃), MS (SIMS) m/z 878 (M+1), IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm⁻¹ 1740 (ester C=O), 1650 (amide C=O). ¹H NMR (100 MHz, CDCl₃) δ 1.05 (3H, d, $J=6.3$ Hz, CHCH₃), 1.35~1.80 (4H, m, CCH₂CH₂C), 1.98 (3H, s, COCH₃), 3.53 (2H, t, $J=6.0$ Hz, NCH₂), 4.13 (1H, dd, $J=3.4$ Hz, $J=6.3$ Hz, CHCH₃), 4.0~4.35 (1H, m, NCHCH₂), 4.56 (2H, s, ArCH₂), 4.70 (2H, s, ArCH₂), 4.40~4.75 (1H, m, NH), 4.82 (1H, dd, $J=3.4$ Hz, $J=6.9$ Hz, NCHCH), 5.07 (1H, d, $J=10.5$ Hz, ArCH·H), 5.15 (4H, s, ArCH₂ × 2), 5.23 (1H, d, $J=10.5$ Hz, ArCH·H), 7.05~7.45 (27H, m, ArH), 7.69 (1H, t, $J=4.5$ Hz, ArH), 8.73 (1H, d, $J=6.9$ Hz, NH). Anal Calcd for C₃₃H₃₅N₃O₉: C 72.49, H 6.31, N 4.81. Found: C 72.20, H 6.09, N 4.52.

L-N-(2,3-Dihydroxybenzoyl)threonyl-L-(N^ω-acetyl-N^ω-hydroxy)ornithine (Vanoxonin)

To a solution prepared from 50 mg of compound **12** and 3 ml of MeOH, 20 mg of 10% palladium carbon was added. The atmosphere was replaced with hydrogen at atmospheric pressure and stirring was continued for 48 hours at room temp. The undissolved material was removed by filtration and the filtrate was evaporated to give reddish brown residue. Further purification by Sephadex LH-20 column chromatography (4 × 150 cm) gave 21 mg (87%) of vanoxonin. Rf 0.34 (BuOH - MeOH - H₂O, 4: 1: 2), $[\alpha]_{\text{D}}^{25} + 6.2^\circ$ (c 1.0, MeOH), mp 108~109°C, UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ) 247 (8,750), 311 (2,560), UV $\lambda_{\text{max}}^{0.1N \text{ NaOH}}$ nm (ϵ) 345 (3,300). IR (KBr) cm⁻¹ 3350, 1720, 1640, 1540, 1340. FD-MS m/z 427 (M). ¹H NMR (100 MHz, D₂O) δ 1.76 (3H, d, $J=6.0$ Hz, CHCH₃), 2.10~2.50 (4H, m, CCH₂CH₂C), 2.56 (3H, s, COCH₃), 4.05 (2H, m, NCH₂), 4.75 (1H, m, CH(OH)CH₃), 4.88 (1H, m, CHCH₂), 5.0 (1H, d, $J=5.5$ Hz, CHCH(OH)), 7.24 (1H, t, $J=7.0$ Hz, ArH), 7.4 (1H, d, $J=7.0$ Hz, ArH), 7.74 (1H, d, $J=7.0$ Hz, ArH). Anal Calcd for C₁₈H₂₅N₃O₉: C 50.58, H 6.00, N 9.83. Found: C 50.24, H 6.22, N 9.58.

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