VANOXONIN, A NEW INHIBITOR OF THYMIDYLATE SYNTHETASE

III. INHIBITION OF THYMIDYLATE SYNTHETASE BY VANOXONIN-VANADIUM COMPLEX

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Quinquevalent vanadium complex with two mol of vanoxonin ligated by the two catechols was shown to be the active structure for inhibition of thymidylate synthetase. The catechol group of vanoxonin as the essential moiety for the inhibition of enzyme was further confirmed by studies of structure-activity relationships using the enzyme obtained from Ehrlich ascites carcinoma cells of mice. Vanoxonin-vanadium complex showed competitive inhibition with respect to deoxyuridylic acid but uncompetitive to 5,10-methylenetetrahydrofolate.

As reported in previous papers[1,2], a vanoxonin-vanadium complex had inhibitory activity against thymidylate synthetase and the structure of vanoxonin was determined to be L-*N*-(2,3-dihydroxybenzoyl)-threonyl-L-(N^{ω}-acetyl-N^{ω}-hydroxy)ornithine. In this paper, we report the structure and properties of vanoxonin-vanadium complexes, the active species as the inhibitor of thymidylate synthetase, the structure-activity relationships of vanoxonin-related compounds, and kinetic studies of inhibition of thymidylate synthetase by a vanoxonin-vanadium complex.

Results and Discussion

Structure and Properties of Vanoxonin-vanadium Complexes and the Active Species for the Inhibition of Thymidylate Synthetase

Vanoxonin formed a green complex with absorption maxima at 437 and 660 nm with quadrivalent vanadium ($VOSO_4$) under anaerobic condition. The spectrum was changed by exposure to air and a purple complex with absorption maxima at 534 and 845 nm was finally formed (Fig. 1). Addition of cysteine in equimolar amount to the vanadium to a solution of the purple complex regenerated the original green complex. The purple complex was also prepared by reaction of vanoxonin with quinquevalent vanadium ($NaVO_3$). Thus, the green and purple complexes were indicated to contain quadri- and quinquevalent vanadium, respectively.

BRITTON[3] has reported the redox reaction between quadri- and quinquevalent vanadiums in detail. He showed that in acidic solution quadrivalent vanadium (VO^{2+}) is stable but it is easily oxidized by air above pH 3. This air oxidation has also been observed in oxovanadium complexes with uridine[4] and catechol[5]. Vanoxonin-vanadium complex inhibited thymidylate synthetase under aerobic condition, but under anaerobic condition no inhibition was shown by a complex prepared from vanoxonin and quadrivalent vanadium (Table 1). These results indicated that vanadium must be quinquevalent for the manifestation of the inhibitory activity.

The structure of vanoxonin is characterized by two types of ligands: catechol and hydroxamic acid.

Fig. 1. Absorption spectra of vanoxonin-vanadium complex.

Vanadyl sulfate (0.6 μmol in 1 ml of H_2O) and vanoxonin (1.2 μmol in 2 ml of H_2O) were mixed under nitrogen. After the visible absorbance spectrum was taken (a), the solution was exposed to air until the absorbance at 845 nm was constant and the spectra was taken (b).

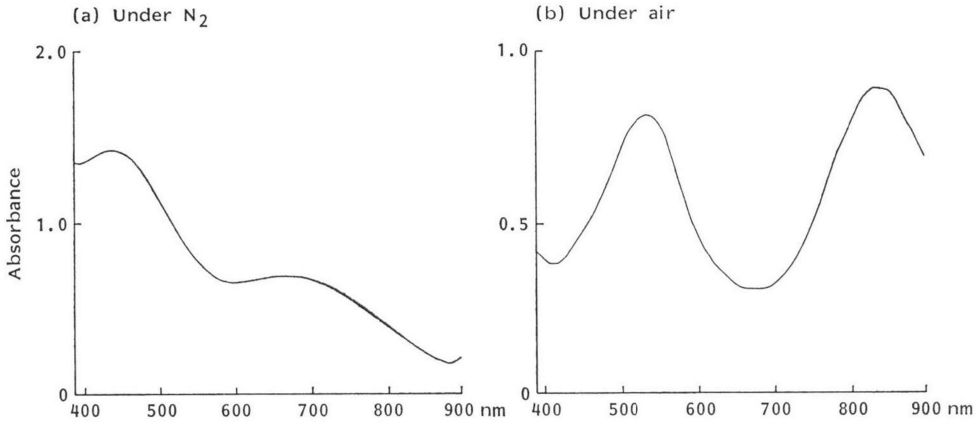


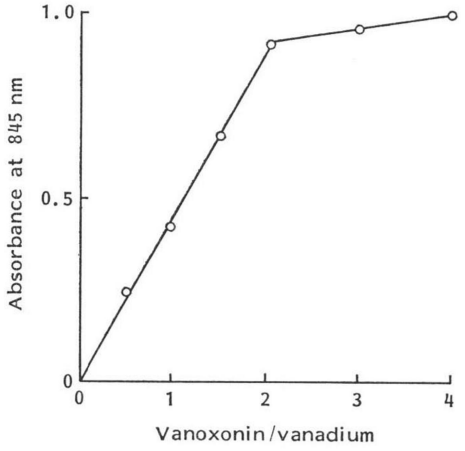
Table 1. Inhibitory activity of vanoxonin-vanadium complexes against thymidylate synthetase.

Vanadium	Inhibition (%)			
	Under air		Under N_2	
	Vanoxonin (μM)		Vanoxonin (μM)	
	3.0	0.3	3.0	0.3
$V^{4+}(VOSO_4)$	89	34	0	0
$V^{5+}(NaVO_3)$	89	43	50	12

Inhibition (%) against the enzyme were shown in the table. The complex, of which the ratio of the ligand to the metal was two, was prepared under air or under the stream of nitrogen by mixing the aq solutions of vanoxonin with the aq solution of vanadyl sulfate or sodium metavanadate. Thymidylate synthetase was assayed as described in experimental section.

Fig. 2. Spectrophotometric titration of vanadium (V) with vanoxonin.

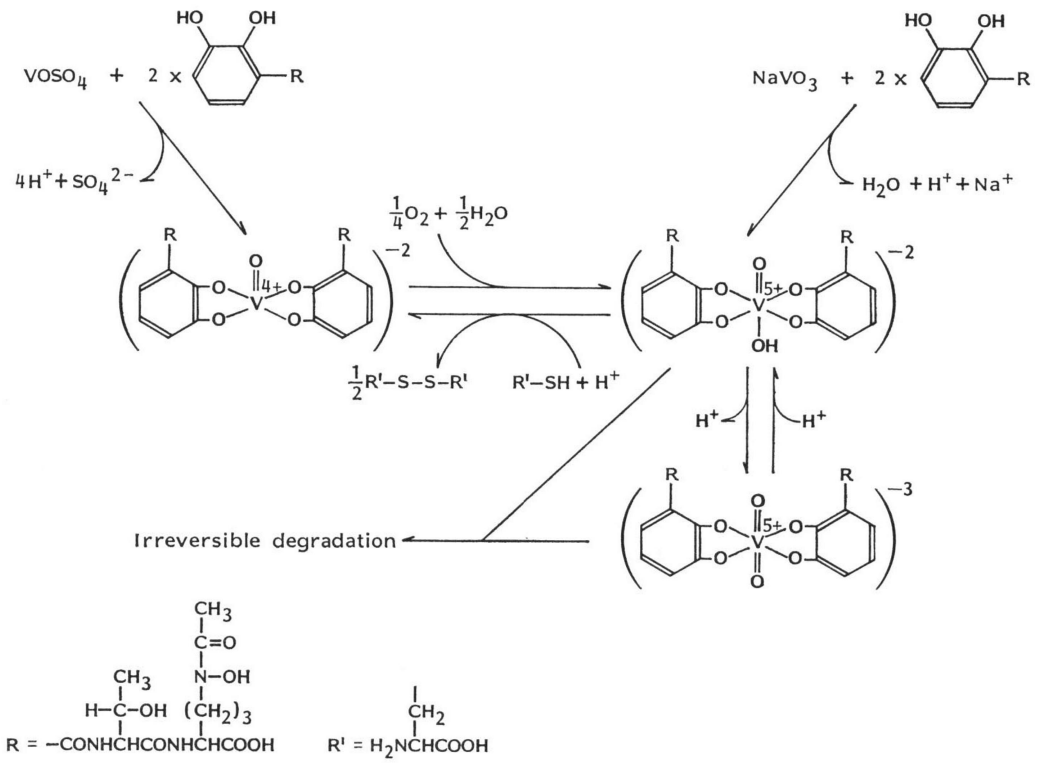
The concentration of sodium metavanadate was 0.2 mM. The absorbance at 845 nm was measured 2 minutes after mixing vanoxonin and vanadium aq solutions.



A number of vanadium complexes with compounds having catechol or hydroxamic acids have been reported[4~8]. Therefore, there was a question as to the ligands involved in the complexation of vanoxonin with vanadium. Potentiometric titration of the vanoxonin-vanadium (V^{5+}) complex showed that the *pKa'* value around 7 attributed to a hydroxyl group on the catechol of vanoxonin could not be observed. Moreover, L-(*N*-2,3-dihydroxybenzoyl-*O*-benzyl)threonine benzyl ester also formed a purple complex with absorption maxima at 534 and 840 nm with vanadium (V^{5+}). These results suggested that vanoxonin-vanadium (V^{5+}) complex was ligated by the hydroxyl groups of the catechols. The molar ratio of vanadium (V^{5+}) to vanoxonin in the complex was found to be 1 : 2 by the intensity of absorbance at 845 nm (Fig. 2).

The solution of the purple vanadium (V^{5+})-vanoxonin complex was weakly acidic (pH 3) and color

Fig. 3. Possible reaction scheme for the formation of vanoxonin-vanadium complex.



of the complex varied depending on the pH; a brown complex with an absorption maximum at 435 nm formed at neutral pH. The color change was not completely reversible, probably due to partial irreversible oxidation of the ligands. The mid-point of the absorbance change was pH 3.9, which well agreed with the *pKa* value of hydroxo ligand on dioxovanadium ($HOVO^{2+} \rightleftharpoons VO_2^+ + H^+$)[9]. Therefore, the color change of vanoxonin-vanadium (V^{5+}) complex depending on pH appears to be deprotonation of the hydroxo ligand.

Based on these results, the dioxovanadium complex with two mol of vanoxonin was assumed to be the active species for the thymidylate synthetase inhibitor and a possible reaction scheme for the complexation of vanadium with vanoxonin can be proposed as shown in Fig. 3. When a mixture of one mol of vanadyl sulfate and two mol of vanoxonin was adjusted to pH 7.0 under air, five equivalents of hydroxide were consumed in addition to two mol of hydroxide necessary for neutralizing the carboxylic acid of vanoxonin. This agreed with the amount expected from the reaction scheme. The vanadium (V^{5+})-vanoxonin complex was unstable in aqueous solution and lost the inhibitory activity against the enzyme in a few hours at room temperature.

Structure-activity Relationships of Vanoxonin-related Compounds for the Inhibition of Thymidylate Synthetase

Vanoxonin-related compounds were prepared and tested for the inhibitory activity against thymidylate synthetase in order to know the structure-activity relationships. The synthesis of L-*N*-(2,3-dihydroxybenzoyl)threonyl-L-N^{ω}-acetylornithine is illustrated in Fig. 4. The IC_{50} values are shown in Tables 2, 3 and 4. (The IC_{50} was defined as the inhibitor concentration necessary to reduce the reaction rate by

Fig. 4. The synthesis of L-*N*-(2,3-dihydroxybenzoyl)threonyl-L-N^{ω}-acetylornithine.

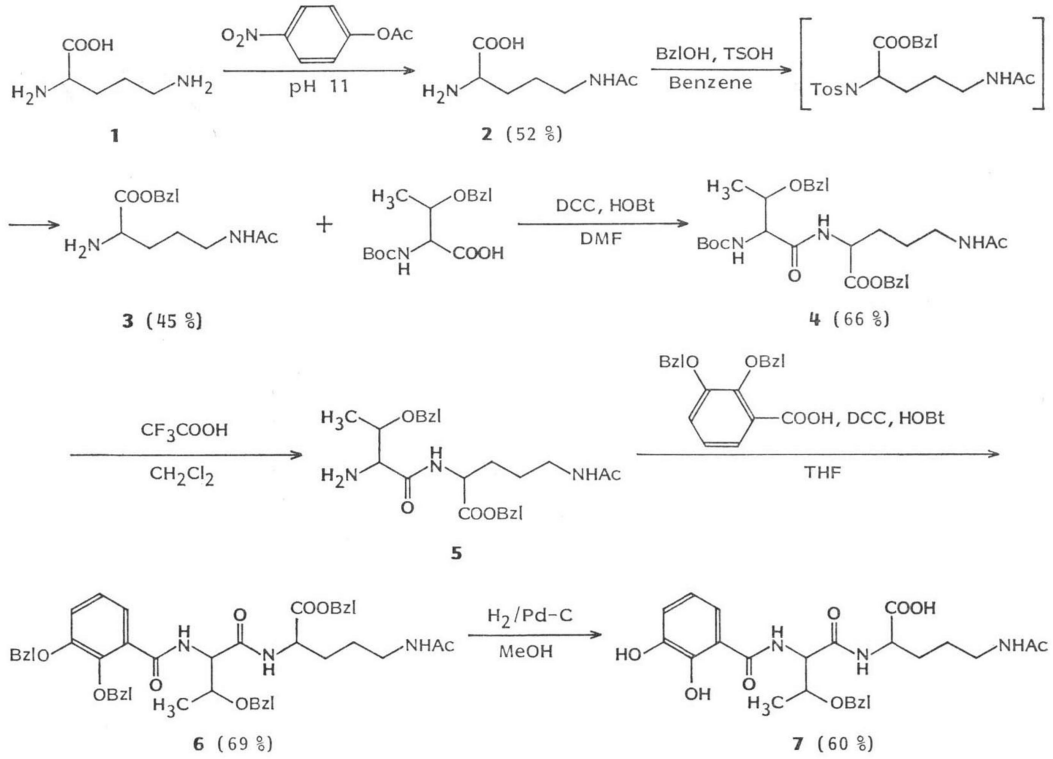
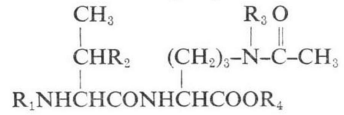


Table 2. Structure-activity relationships (1). Effects of functional group.



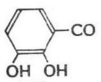
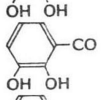
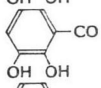
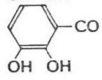
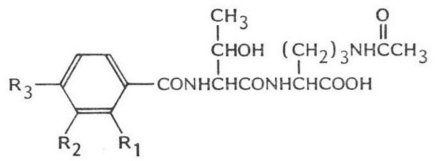
R_1	R_2	R_3	R_4	IC_{50} (μM)
	OH	OH	H (Vanoxonin)	1.9
	OH	H	H	3.1
	OH	H	CH_3	3.1
	CH_3	H	H	3.2
H	OH	OH	H	>200

Table 3. Structure-activity relationships (2). Effects of OH functions on the benzoyl group.

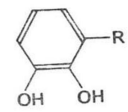


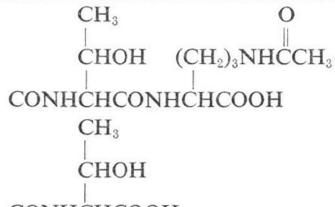
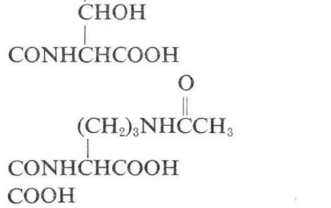
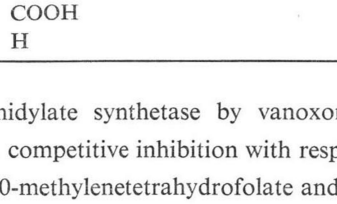
R_1	R_2	R_3	IC_{50} (μM)
H	OH	OH	3.1
OH	OH	H	3.3
OH	H	OH	>200
OH	OCH_3	H	71

50%). From Table 2, it is apparent that hydroxyl groups on threonine and N^{ω}-hydroxyornithine, and free carboxylic acid of vanoxonin have relatively little effect on the activity, but the catechol is essential for the inhibitory activity. Then, the effects of hydroxyl groups on the benzoyl moiety were investigated (Table 3). Compound with 3,4-dihydroxybenzoyl group showed almost the same activity as compound with 2,3-dihydroxybenzoyl group, but compounds with 2,4-dihydroxy- and 2-hydroxy-3-methoxybenzoyl group exhibited

much lower activity. These results indicate that vicinal hydroxyl groups on the benzene ring are essential for the activity. But, as shown in Table 4, catechol itself or 2,3-dihydroxybenzoic acid showed little activity which indicated that the peptide moiety of vanoxonin is also important for the inhibitory activity.

Table 4. Structure-activity relationships (3). Catechol derivatives.



R	IC_{50} (μM)
	3.1
	12.5
	7.5
COOH	650
H	>1,000

Kinetic Studies of Inhibition of Thymidylate Synthetase by Vanoxonin-vanadium Complex

Kinetic analysis of inhibition of thymidylate synthetase by vanoxonin-vanadium complex was performed with 1,680-fold purified enzyme isolated from Ehrlich ascites carcinoma cells of mice by the method of SHIMIZU *et al.*[10] with slight modification consisting of Blue-Sepharose and methotrexate-bound affinity column chromatography. Lineweaver-Burk plot of inhibition of thymidylate synthetase by vanoxonin-vanadium complex was shown in Fig. 5. The inhibitor showed competitive inhibition with respect to deoxyuridylate and uncompetitive inhibition with respect to 5,10-methylenetetrahydrofolate and the apparent *Ki* was shown to be 4.4 μM.

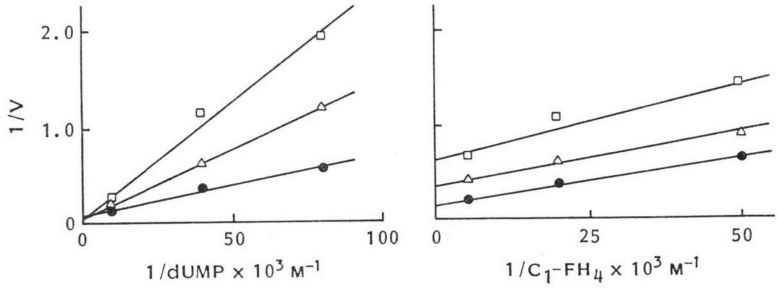
It is well known that vanadate and oxovanadium complexes with nucleosides have inhibitory activities against (Na^+, K^+)-ATPase[11] and ribonuclease[4], respectively. Vanadium in these inhibitors is assumed to be phosphate analogues. The mechanism of inhibition of thymidylate synthetase by vanoxonin-vanadium complex may have some relation to these inhibitors because the inhibition is reversed by higher concentration of deoxyuridylate.

Experimental

General

UV spectra were determined on a Hitachi EPS-3T. NMR spectra were recorded on a Varian XL-100 or Varian EM-390 spectrometer. Chemical shifts were expressed in values (ppm) with tetramethyl-

Fig. 5. Lineweaver-Burk plot of inhibition of thymidylate synthetase by vanoxonin-vanadium complex.
5,10-Methylenetetrahydrofolate 50 μM, dUMP 25 μM. Inhibitor ● none, △ 5 μM, □ 10 μM.
V was expressed as nmol/minute/ml of thymidylate synthetase.



silane as an internal standard. The mass spectra were recorded on a Hitachi mass spectrometer RMU-6M. Metrohm Herisau Potentiograph E436 was used for the measurement of *pKa* values and titration experiments. Precoated Silica gel F-254 layers (E. Merck, Darmstadt) were used for TLC. Thymidylate synthetase was assayed as reported previously[1] except that purified enzyme was used for kinetic studies.

L-(*N*-2,3-Dihydroxybenzoyl-*O*-benzyl)threonine Benzyl Ester

To the solution of *O*-benzyl-L-threonine benzyl ester $\frac{1}{2}C_2H_2O_4$ (344.4 mg, 1.0 mmol) in 5 ml of dry DMF, 0.14 ml (1.0 mmol) of triethylamine was added. After nitrogen gas was passed through the solution, 2,3-dihydroxybenzoic acid (154.1 mg, 1.0 mmol), 1-hydroxybenzotriazole (HOBt) (162 mg, 1.2 mmol), and dicyclohexylcarbodiimide (DCC) (248 mg, 1.2 mmol) were added and stirred for 10 hours at room temp. After concentration under reduced pressure and dissolution in toluene (5 ml), it was applied to silica gel column (4×13 cm) and eluted with toluene - EtOAc (8: 1). The eluate was evaporated to dryness to give white powder (0.71 mmol, 71%). Rf 0.6 (toluene - EtOAc, 2: 1), mp 39~40°C, $[\alpha]_D^{21}$ +17° (*c* 1.0, MeOH), MS *m/z* 435 (M, $C_{25}H_{25}NO_6$), 344 (M−C_7H_7). ^{1}H NMR (100 MHz CD_3OD) δ 1.28 (3H, d, *J*=7 Hz, CH_3), 4.12 (1H, m, C*H*CH_3), 4.48 (1H, s, ArC*H*·H), 4.54 (1H, m, NC*H*CH), 4.65 (1H, s, ArCH·*H*), 5.2 (2H, s, $ArCH_2$), 6.81 (1H, t, *J*=7 Hz, ArH), 6.94 (1H, dd, *J*=2 Hz, *J*=7 Hz, ArH), 7.3 (10H, br, ArH), 7.34 (1H, dd, *J*=2 Hz, *J*=7 Hz, ArH). *Anal* Calcd for $C_{25}H_{25}NO_6$: C 68.95, H 5.79, N 3.22. Found: C 68.57, H 5.32, N 3.25.

L-(*N*-Boc-*O*-Benzyl)threonyl-L-N^ω-acetylornithine Benzyl Ester

N^ω-Acetylornithine was synthesized from ornithine by the method of LECLERC *et al.*[12] as follows. L-Ornithine hydrochloride (6.75 g, 40 mmol) was dissolved in H_2O (400 ml). After pH of the solution was raised to 11 with 2 N NaOH, 4-nitrophenyl acetate (14.5 g, 80 mmol) was added, and stirred for an hour at pH 11 (pH-stat). The solution was removed from the pH-stat and then stirred for an additional hour in the presence of 200 ml of Dowex 50 (H^+ form). The resin was filtered off, washed several times with H_2O, and stirred in 3 N NH_4OH (500 ml) for 15 minutes. The resin was removed by filtration, the filtrate evaporated to dryness, and crystallization twice from H_2O - EtOH gave white crystalline powder (3.46 g, 20.8 mmol, 52%). N^ω-Acetylornithine hydrochloride was prepared from the crystals by adding an equivalent amount of 1 N HCl and evaporating to dryness; Rf 0.12 (BuOH - AcOH - H_2O, 4: 1: 2), mp 277~281°C (dec), $[\alpha]_D^{27}$ +1° (*c* 1.0, H_2O). ^{1}H NMR (90 MHz, D_2O) δ 1.9~2.4 (4H, m, 3-CH_2, 4-CH_2), 2.48 (3H, s, CH_3CO), 3.7 (2H, t, *J*=6 Hz, 5-CH_2), 4.24 (1H, t, *J*=6 Hz, 2-CH). *Anal* Calcd for $C_7H_{14}N_2O_3$: C 48.26, H 8.10, N 16.08. Found: C 47.98, H 8.28, N 16.16. L-N^ω-Acetylornithine hydrochloride; ^{1}H NMR (90 MHz, D_2O) δ 1.92~2.62 (4H, m, 3-CH_2, 4-CH_2), 2.47 (3H, s, CH_3CO), 3.72 (2H, t, *J*=6 Hz, 5-CH_2), 4.62 (1H, t, *J*=6 Hz, 2-CH).

As shown in the ^{1}H NMR spectra, the α-CH signal showed strong shift in the hydrochloride suggesting the acetylation of the ω-amino group. The benzyl ester of L-N^ω-acetylornithine was synthesized by the method of IZUMIYA[13] as follows. The crystals of L-N^ω-acetylornithine (3.08 g, 18.25 mmol) and *p*-toluene sulfonic acid (5.2 g, 27.4 mmol) were dissolved in benzyl alcohol (50 ml) at 110~120°C. After benzene (300 ml) was added, the solution was refluxed with Dean-Stark water separator for 5 hours. After concentration under reduced pressure the residue was dissolved in $CHCl_3$ (200 ml) and extracted with H_2O (200 ml). The H_2O layer was washed with $CHCl_3$, saturated $NaHCO_3$ (200 ml) and extracted with $CHCl_3$ (200 ml), dried (Na_2SO_4) and evaporated to an oil (L-N^ω-acetylornithine benzyl ester, 2.13 g, 8.24 mmol, 45%). This (1.03 g, 4.0 mmol) was dissolved in distilled THF (40 ml) and L-(*N*-Boc-*O*-benzyl)threonine (1.0 g, 4.0 mmol), DCC (0.99 g, 4.8 mmol) and HOBt (0.63 g, 4.8 mmol) were added to the solution and the mixture was stirred at room temp for 14 hours. The resulting precipitate was removed by filtration, EtOAc (200 ml) was added to the filtrate, the solution washed with saturated NaCl, dried (Na_2SO_4) and evaporated. The residue was extracted with $CHCl_3$ (100 ml), applied to silica gel column (5×40 cm), eluted with $CHCl_3$ and evaporated to give white powder (1.46 g, 2.64 mmol, 66%). Rf 0.33 ($CHCl_3$ - MeOH, 10: 1), mp 135~138°C, $[\alpha]_D^{25}$ −11.4° (*c* 1.0, $CHCl_3$). ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.2 (3H, d, *J*=7.5 Hz, CHC*H*$_3$), 1.52~1.83 (4H, m, CH(C*H*$_2$)$_2$), 1.9 (3H, s, CH_3CO), 3.12 (2H, q, *J*=6 Hz, NCH_2), 4.1~4.4 (2H, m, CH×2), 4.58 (1H, s, ArC*H*·H), 4.62 (1H, s, ArCH·*H*), 4.65 (1H, m, CH), 5.18 (2H, s, $ArCH_2$), 5.5 (2H, m, NH×2), 7.35 (10H, s, ArH). *Anal* Calcd for C_{30}-

$H_{41}N_3O_7$: C 64.85, H 7.44, N 7.56. Found: C 64.59, H 7.23, N 7.73.

L-*N*-Boc-Valyl-L-N^{ω}-acetylornithine Benzyl Ester

The same treatment of L-*N*-Boc-valine (1.0 g, 4.0 mmol) instead of L-(*N*-Boc-*O*-benzyl)threonine as in the synthesis of L-(*N*-Boc-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine benzyl ester gave white powder of L-*N*-Boc-valyl-L-N^{ω}-acetylornithine benzyl ester (1.2 g, 2.56 mmol, 64%). Rf 0.44 ($CHCl_3$ - MeOH, 10: 1), mp 134~136°C, $[\alpha]_D^{23}$ +7.0° (*c* 1.0, $CHCl_3$). ^1H NMR (90 MHz, $CDCl_3$) δ 0.85 (3H, d, J=4 Hz, CH_3CCH_3), 0.95 (3H, d, J=4 Hz, CH_3CCH_3), 1.43 (9H, s, $C(CH_3)_3$), 1.45~1.9 (4H, m, $CH(CH_2)_2$), 1.92 (3H, s, CH_3CO), 3.2 (2H, q, J=6 Hz, NCH_2), 3.9 (1H, m, $CHCH_3$), 4.6 (1H, m, $CHCH_2$), 5.05 (1H, m, $NCHCH$), 5.15 (2H, s, $ArCH_2$), 5.9 (2H, m, NH×2), 6.7 (1H, m, NH), 7.35 (5H, s, ArH). *Anal* Calcd for $C_{24}H_{37}N_3O_6$: C 62.18, H 8.05, N 9.06. Found: C 62.39, H 8.20, N 9.05.

2,4-Dibenzyloxybenzoic Acid

2,4-Dihydroxybenzoic acid (1.54 g, 10 mmol), anhydrous K_2CO_3 (22.1 g, 40 mmol) and benzylbromide (1.71 g, 10 mmol) were added in dry Me_2CO (20 ml), refluxed for 10 hours and evaporated. The residue was extracted with $CHCl_3$ (200 ml), dried (Na_2SO_4), applied to silica gel column (5×20 cm), eluted with toluene - EtOAc (19: 1) and evaporated to give an oil. The oily material was dissolved in dioxane (18 ml), 1 N NaOH (18 ml) was added to the solution and refluxed for 1 hour. 1 N HCl (18 ml) was added to the solution and extracted with $CHCl_3$ (200 ml), washed with saturated NaCl, dried (Na_2SO_4) and evaporated. Crystallization from EtOH gave white crystals in the form of needles (2.4 g, 73 mmol, 73%). Rf 0.48 (toluene - EtOAc, 1: 1), mp 93.5°C. ^1H NMR (90 MHz, $CDCl_3$) δ 5.1 (2H, s, $ArCH_2$), 5.2 (2H, s, $ArCH_2$), 6.61 (1H, s, 3-CH), 6.72 (1H, dd, J=3 Hz, J=9 Hz, 5-CH), 7.43 (10H, s, ArH), 8.13 (1H, d, J=9 Hz, 6-CH). *Anal* Calcd for $C_{21}H_{18}O_4$: C 75.43, H 5.42. Found: C 75.21, H 5.59.

3,4-Dibenzyloxybenzoic Acid

The same treatment of 3,4-dihydroxybenzoic acid (1.54 g, 10 mmol) instead of 2,4-dihydroxybenzoic acid as in the synthesis of 2,4-dibenzyloxybenzoic acid gave white crystalline powder (3.11 g, 9.3 mmol, 93%). Rf 0.17 (toluene - EtOAc, 1: 1), mp 174~176°C. ^1H NMR (90 MHz, $CDCl_3$) δ 5.2 (2H, s, $ArCH_2$), 5.23 (2H, s, $ArCH_2$), 6.95 (1H, d, J=9 Hz, 6-CH), 7.35~7.5 (10H, m, ArH), 7.6~7.8 (2H, m, 2-CH, 5-CH). *Anal* Calcd for $C_{21}H_{18}O_4$: C 75.43, H 5.42. Found: C 75.59, H 5.60.

2-Benzyloxy-3-methoxybenzoic Acid

O-Vanillin (3.04 g, 20 mmol), anhydrous K_2CO_3 (11.04 g, 20 mmol) and benzylbromide (3.42 g, 20 mmol) were added in dry Me_2CO (40 ml), refluxed for 8 hours and evaporated. The residue was dissolved in benzene (50 ml), applied to silica gel column (4×10 cm), eluted with benzene - EtOAc (4: 1) and evaporated to give 2-benzyloxy-3-methoxybenzaldehyde (3.29 g, 13.6 mmol, 68%), an oil. Rf 0.75 (toluene - EtOAc, 1: 1). ^1H NMR (90 MHz, $CDCl_3$) δ 3.93 (3H, s, OCH_3), 5.2 (2H, s, $ArCH_2$), 7.1~7.5 (8H, m, ArH), 10.3 (1H, s, CHO). The aldehyde was oxidized to the acid by the method of RASTETTER *et al.*[14] as follows. The aldehyde (0.97 g, 4 mmol) was dissolved in Me_2CO (5 ml) and the solution was diluted with H_2O (3 ml). To the cloudy mixture were added sulfamic acid (0.46 g, 4.7 mmol) and sodium chlorite (0.4 g, 4.4 mmol) in portions over 30 minutes. After stirring for 1 hour, the solution was evaporated, the residue was extracted with $CHCl_3$ (200 ml), dried (Na_2SO_4) and re-evaporated. The residue was dissolved in toluene (50 ml), applied to silica gel column (4×15 cm), eluted with toluene - EtOAc (1: 1) and evaporated. Crystallization from EtOH gave white crystals in the form of needles (0.49 g, 1.9 mmol, 47%). Rf 0.2 (toluene - EtOAc, 1: 1), mp 81°C. ^1H NMR (90 MHz, $CDCl_3$) δ 3.92 (3H, s, OCH_3), 5.2 (2H, s, $ArCH_2$), 7.1~7.6 (8H, m, ArH). *Anal* Calcd for $C_{15}H_{14}O_4$: C 69.76, H 5.46. Found: C 69.71, H 5.56.

L-(*N*-2,4-Dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine Benzyl Ester

L-(*N*-Boc-*O*-Benzyl)threonyl-L-N^{ω}-acetylornithine (0.56 g, 1 mmol) was dissolved in dry dichloromethane (10 ml), cooled in an ice-bath and added trifluoroacetic acid (2.0 ml). The solution was stirred for 2 hours at room temp and evaporated. The residue was extracted with EtOAc (200 ml), washed with saturated $NaHCO_3$ and NaCl, dried (Na_2SO_4) and evaporated to give a ninhydrin positive compound.

This was dissolved in distilled THF (20 ml), 2,4-dibenzyloxybenzoic acid (0.33 g, 1.0 mmol), DCC (0.25 g, 1.2 mmol) and HOBt (0.16 g, 1.2 mmol) were added and the mixture stirred for 12 hours at room temp. The resulting precipitate was removed by filtration, EtOAc (200 ml) was added to the filtrate, washed with saturated $NaHCO_3$ and NaCl, dried (Na_2SO_4) and evaporated. The residue was extracted with $CHCl_3$ (100 ml), the extract applied to silica gel column (5×20 cm), eluted with $CHCl_3$ - MeOH (50: 1) and the eluate evaporated. The residue was dissolved in MeOH and applied to Sephadex LH-20 column (2×50 cm), eluted with MeOH and evaporated. Crystallization from EtOH gave white crystals (0.61 g, 0.8 mmol, 80%). Rf 0.17 (EtOAc), mp 80°C, $[\alpha]_D^{26}$ +30° (*c* 1.0, $CHCl_3$). ^1H NMR (90 MHz, $CDCl_3$) δ 1.05 (3H, d, J=7 Hz, CHCH_3), 1.3~1.8 (4H, m, CH(CH_2)$_2$), 1.8 (3H, s, CH_3CO), 3.1 (2H, q, J=6 Hz, NCH_2), 4.17 (1H, m, CHCH$_3$), 4.55 (2H, s, $ArCH_2$), 4.61 (1H, m, CHCH$_2$), 4.81 (1H, m, NCH-CH), 5.1 (2H, s, $ArCH_2$), 5.15 (4H, s, $ArCH_2$×2), 5.68 (1H, m, NH), 6.62 (1H, s, ArH), 6.68 (1H, dd, J=3 Hz, J=7.5 Hz, ArH), 7.2~7.5 (1H, m, NH), 7.25~7.4 (20H, m, ArH), 8.15 (1H, d, J=7.5 Hz, ArH), 8.62 (1H, d, J=7 Hz, NH). *Anal* Calcd for $C_{46}H_{49}N_3O_8$: C 71.57, H 6.40, N 5.44. Found: C 71.16, H 6.58, N 5.23.

L-(*N*-2,3-Dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine Benzyl Ester

The same treatment of 2,3-dibenzyloxybenzoic acid[2] (0.33 g, 1.0 mmol) instead of 2,4-dibenzyloxybenzoic acid as in the synthesis of L-(*N*-2,4-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine benzyl ester gave a white crystalline powder (0.53 g, 0.69 mmol, 69%). Rf 0.47 ($CHCl_3$ - MeOH, 10: 1), mp 128~130°C, $[\alpha]_D^{26}$ +21° (*c* 1.0, $CHCl_3$). ^1H NMR (90 MHz, $CDCl_3$) δ 1.13 (3H, d, J=6 Hz, CHCH_3), 1.2~1.8 (4H, m, CH(CH_2)$_2$), 1.85 (3H, s, CH_3CO), 3.12 (2H, q, J=6 Hz, NCH_2), 4.15 (1H, m, CHCH$_3$), 4.55 (1H, m, CHCH$_2$), 4.57 (4H, s, $ArCH_2$×2), 4.75 (1H, m, NCHCH), 5.18 (4H, s, $ArCH_2$× 2), 5.77 (1H, m, NH), 7.18~7.8 (23H, m, ArH, 1H, m, NH), 8.9 (1H, d, J=7 Hz, NH). *Anal* Calcd for $C_{46}H_{49}N_3O_8$: C 71.57, H 6.40, N 5.44. Found: C 71.22, H 6.12, N 5.66.

L-(*N*-3,4-Dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine Benzyl Ester

The same treatment of 3,4-dibenzyloxybenzoic acid (0.33 g, 1.0 mmol) instead of 2,4-dibenzyloxybenzoic acid as in the synthesis of L-(*N*-2,4-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine benzyl ester gave white crystals in the form of columns (0.56 g, 0.72 mmol, 72%). Rf 0.22 (EtOAc), mp 142~144°C, $[\alpha]_D^{26}$ +29.4° (*c* 1.0, $CHCl_3$). ^1H NMR (90 MHz, $CDCl_3$) δ 1.21 (3H, d, J=6 Hz, CHCH_3), 1.3~1.8 (4H, m, CH(CH_2)$_2$), 1.88 (3H, s, CH_3CO), 3.13 (2H, q, J=6 Hz, NCH_2), 4.25 (1H, m, CHCH$_3$), 4.6 (1H, m, CHCH$_2$), 4.7 (2H, s, $ArCH_2$), 4.8 (1H, m, NCHCH), 5.18 (2H, s, $ArCH_2$), 5.21 (2H, s, $ArCH_2$), 5.24 (2H, s, $ArCH_2$), 5.5 (1H, m, NH), 6.9~7.6 (23H, m, ArH, 2H, m, NH×2). *Anal* Calcd for $C_{46}H_{49}N_3O_8$: C 71.57, H 6.40, N 5.44. Found: C 71.99, H 6.77, N 5.05.

L-(*N*-(2-Benzyloxy-3-methoxy)benzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine Benzyl Ester

The same treatment of 2-benzyloxy-3-methoxybenzoic acid (0.26 g, 1.0 mmol) instead of 2,4-dibenzyloxybenzoic acid as in the synthesis of L-(*N*-2,4-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine benzyl ester gave an oily compound (0.44 g, 0.63 mmol, 63%). Rf 0.14 (EtOAc), $[\alpha]_D^{26}$ +15.9° (*c* 1.0, $CHCl_3$). ^1H NMR (90 MHz, $CDCl_3$) δ 1.13 (3H, d, J=6 Hz, CHCH_3), 1.22~1.8 (4H, m, CH(CH_2)$_2$), 1.88 (3H, s, CH_3CO), 3.13 (2H, q, J=6 Hz, NCH_2), 3.9 (3H, s, OCH_3), 4.2 (1H, m, CH-CH_3), 4.57 (2H, s, $ArCH_2$), 4.6 (1H, m, CHCH$_2$), 4.8 (1H, m, NCHCH), 5.18 (4H, s, $ArCH_2$×2), 5.72 (1H, m, NH), 7.15~7.72 (18H, m, ArH, 1H, m, NH), 8.82 (1H, d, J=7 Hz, NH). *Anal* Calcd for $C_{40}H_{45}N_3O_8$: C 69.04, H 6.52, N 6.04. Found: C 68.73, H 6.23, N 5.89.

L-*N*-(2,3-Dibenzyloxybenzoyl)valyl-L-N^ω-acetylornithine Benzyl Ester

The same treatment of L-*N*-Boc-valyl-L-N^ω-acetylornithine benzyl ester (0.46 g, 1.0 mmol) instead of L-(*N*-Boc-*O*-benzyl)threonyl-L-N^ω-acetylornithine benzyl ester as in the synthesis of L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^ω-acetylornithine benzyl ester gave a white crystalline powder (0.51 g, 0.75 mmol, 75%). Rf 0.47 ($CHCl_3$ - MeOH, 10: 1), mp 93~94°C, $[\alpha]_D^{27}$ +8.9° (*c* 1.0, $CHCl_3$). ^1H NMR (90 MHz, $CDCl_3$) δ 0.8 (3H, d, J=4 Hz, CH_3CCH$_3$), 0.9 (3H, d, J=4 Hz, CH$_3$CCH_3), 1.4~2.2 (4H, m, CH(CH_2)$_2$), 1.8 (3H, s, CH_3CO), 3.13 (2H, q, J=6 Hz, NCH_2), 3.7 (1H, m, CH), 4.4~4.7 (2H, m, CH×2), 5.2 (6H, s, $ArCH_2$×3), 6.4 (1H, m, NH), 7.15~7.6 (18H, m, ArH, 1H, m, NH), 8.72 (1H, d, J=7 Hz, NH). *Anal* Calcd for $C_{40}H_{45}N_3O_7$: C 70.67, H 6.67, N 6.18. Found: C 70.29, H

6.21, N 6.45.

L-(*N*-2,3-Dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine Methyl Ester

L-N^{ω}-Acetylornithine (0.35 g, 2 mmol) was dissolved in 10% HCl - MeOH (20 ml), stirred for 10 hours at room temp and evaporated. The residue was dissolved in H_2O and applied to Diaion HP-20 column (2×10 cm), eluted with 50% aq Me_2CO, and evaporated to give the methyl ester of L-N^{ω}-acetylornithine (0.24 g, 1.3 mmol, 65%). Rf 0.29 (BuOH - AcOH - H_2O, 4: 1: 1). ^{1}H NMR (90 MHz, D_2O) δ 1.5~2.3 (4H, m, 3-CH_2, 4-CH_2), 2.15 (3H, s, CH_3CO), 3.47 (2H, t, J=7 Hz, 5-CH_2), 4.02 (3H, s, OCH_3), 4.4 (1H, t, J=6 Hz, 2-CH). The methyl ester and L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)-threonine[2] (0.68 g, 1.3 mmol) were dissolved in 5 ml dry DMF. To the solution were added DCC (0.3 g, 1.6 mmol) and HOBt (0.19 g, 1.6 mmol) and stirred for 12 hours at room temp. The resulting precipitate was removed by filtration, EtOAc (100 ml) was added to the filtrate, washed with saturated $NaHCO_3$ and NaCl, dried (Na_2SO_4) and evaporated. The residue was dissolved in $CHCl_3$ (50 ml) and applied to silica gel column (4×10 cm), eluted with $CHCl_3$ - MeOH (50: 1) and evaporated to give an oily compound (0.44 g, 0.6 mmol, 49%). Rf 0.62 ($CHCl_3$ - MeOH, 10: 1), $[\alpha]_D^{27}$ +12.9° (*c* 1.0, $CHCl_3$). ^{1}H NMR (90 MHz, CD_3OD) δ 1.2 (3H, d, J=6 Hz, $CHCH_3$), 1.35~1.9 (4H, m, $CH(CH_2)_2$), 1.88 (3H, s, CH_3CO), 3.15 (2H, t, J=7 Hz, NCH_2), 3.73 (3H, s, OCH_3), 4.1 (1H, m, $CHCH_3$), 4.35 (2H, s, $ArCH_2$), 4.4 (1H, m, $CHCH_2$), 4.65 (1H, m, NCHCH), 5.12 (2H, s, $ArCH_2$), 5.18 (2H, s, $ArCH_2$), 5.45 (1H, m, NH), 7.2~7.6 (18H, m, ArH), 8.28 (1H, m, NH), 8.9 (1H, m, NH). *Anal* Calcd for $C_{40}H_{45}N_3O_4$: C 69.05, H 6.52, N 6.04. Found: C 69.43, H 6.56, N 5.99.

L-N^{α}-2,3-Dibenzyloxybenzoyl-L-N^{ω}-acetylornithine Benzyl Ester

2,3-Dibenzyloxybenzoic acid (0.33 g, 1 mmol) and L-N^{ω}-acetylornithine benzyl ester (0.26 g, 1 mmol) were dissolved in distilled THF (10 ml). To the solution were added DCC (0.25 g, 1.2 mmol) and HOBt (0.16 g, 1.2 mmol), and the mixture was stirred for 7 hours at room temp. The resulting precipitate was removed by filtration, EtOAc (100 ml) was added to the filtrate, washed with saturated $NaHCO_3$ and NaCl, dried (Na_2SO_4) and evaporated. The residue was extracted with EtOAc (100 ml), applied to silica gel column (4×10 cm), eluted with EtOAc and evaporated to give white powder (0.53 g, 0.91 mmol, 91%). Rf 0.21 (EtOAc), mp 195~200°C, $[\alpha]_D^{26}$ −4.3° (*c* 1.0 $CHCl_3$). ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.1~1.67 (4H, m, $CH(CH_2)_2$), 1.92 (3H, s, CH_3CO), 3.15 (2H, t, J=6 Hz, NCH_2), 4.7 (1H, m, CH), 5.2 (6H, s, $ArCH_2$×3), 5.6 (1H, m, NH), 7.18~7.82 (18H, m, ArH), 8.55 (1H, d, J=6 Hz, NH). *Anal* Calcd for $C_{35}H_{36}N_2O_6$: C 72.39, H 6.25, N 4.82. Found: C 72.05, H 6.37, N 5.13.

L-*N*-(2,4-Dihydroxybenzoyl)threonyl-L-N^{ω}-acetylornithine

To a solution prepared from L-(*N*-2,4-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine benzyl ester (0.54 g, 0.7 mmol) and MeOH (10 ml), was added 10% palladium carbon (50 mg). After the atomosphere was replaced with hydrogen at atomospheric pressure, stirring was continued for 16 hours at room temp. The undissolved material was removed by filtration and the MeOH was removed *in vacuo* to give yellow residue. Further purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from EtOH gave crystals (0.12 g, 0.3 mmol, 43%). Rf 0.50 (BuOH - MeOH - H_2O, 4: 1: 1), mp 102~103°C, $[\alpha]_D^{26}$ +17.6° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 1.25 (3H, d, J=6 Hz, $CHCH_3$), 1.35~1.8 (4H, m, $CH(CH_2)_2$), 1.93 (3H, s, CH_3CO), 3.15 (2H, t, J=6 Hz, NCH_2), 4.2 (1H, m, $CHCH_3$), 4.4 (1H, m, $CHCH_2$), 4.57 (1H, d, J=5 Hz, NCHCH), 6.3 (1H, s, ArH), 6.35 (1H, dd, J=3 Hz, J=7 Hz, ArH), 7.7 (1H, d, J=7 Hz, ArH). *Anal* Calcd for $C_{18}H_{25}N_3O_8$: C 52.55, H 6.13, N 10.21. Found: C 52.32, H 5.76, N 9.87.

L-*N*-(3,4-Dihydroxybenzoyl)threonyl-L-N^{ω}-acetylornithine

Hydrogenolysis of L-(*N*-3,4-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine benzyl ester (0.46 g, 0.6 mmol) with 10% palladium carbon in the same way as the hydrogenolysis of L-(*N*-2,4-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine benzyl ester, purification by Sephadex LH-20 column chromatography (4×150 cm), and crystallization from MeOH gave crystals (0.14 g, 0.33 mmol, 55%). Rf 0.41 (BuOH - MeOH - H_2O, 4: 1: 1), mp 112~115°C, $[\alpha]_D^{20}$ +23.2° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 1.25 (3H, d, J=6 Hz, $CHCH_3$), 1.3~1.8 (4H, m, $CH(CH_2)_2$), 1.88 (3H, s, CH_3CO), 3.15 (2H, t, J=6 Hz, NCH_2), 4.2 (1H, m, $CHCH_3$), 4.4 (1H, m, $CHCH_2$), 4.55 (1H, d, J=5 Hz,

NC*H*CH), 6.82 (1H, d, J=9 Hz, ArH), 7.28 (1H, dd, J=3 Hz, J=9 Hz, ArH), 7.3 (1H, s, ArH). *Anal* Calcd for $C_{18}H_{25}N_3O_8$: C 52.55, H 6.13, N 10.21. Found: C 52.39, H 6.08, N 10.43.

L-*N*-(2,3-Dihydroxybenzoyl)threonyl-L-N^{ω}-acetylornithine

Hydrogenolysis of L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine benzyl ester (0.46 g, 0.6 mmol) as described above, purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from MeOH gave a crystalline powder (0.28 g, 0.36 mmol, 60%). Rf 0.4 (BuOH - MeOH - H_2O, 4: 1: 1), mp 147~150°C, $[\alpha]_D^{24}$ −9.3° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 1.25 (3H, d, J=6 Hz, CHCH_3), 1.3~1.8 (4H, m, CH(CH_2)$_2$), 1.85 (3H, s, CH_3CO), 3.12 (2H, t, J=6 Hz, NCH_2), 4.2 (1H, m, C*H*CH$_3$), 4.4 (1H, m, C*H*CH$_2$), 4.6 (1H, d, J=5 Hz, NC*H*CH), 6.91 (1H, t, J=9 Hz, ArH), 7.18 (1H, dd, J=3 Hz, J=9 Hz, ArH), 7.43 (1H, dd, J=3 Hz, J=9 Hz, ArH). *Anal* Calcd for $C_{18}H_{25}N_3O_8$: C 52.55, H 6.13, N 10.21. Found: C 52.07, H 6.50, N 9.77.

L-*N*-(2-Hydroxy-3-methoxy)benzoylthreonyl-L-N^{ω}-acetylornithine

Hydrogenolysis of L-(*N*-(2-benzyloxy-3-methoxy)benzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine benzyl ester (0.4 g, 0.57 mmol) as described above, purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from MeOH gave a crystalline powder (0.2 g, 0.47 mmol, 82%). Rf 0.5 (BuOH - MeOH - H_2O, 4: 1: 1), mp 77~78°C, $[\alpha]_D^{20}$ +22.3° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 1.25 (3H, d, J=6 Hz, CHCH_3), 1.38~1.8 (4H, m, CH(CH_2)$_2$), 1.88 (3H, s, CH_3CO), 3.17 (2H, t, J=6 Hz, NCH_2), 3.86 (3H, s, OCH_3), 4.28 (1H, m, C*H*CH$_3$), 4.4 (1H, m, C*H*CH$_2$), 4.6 (1H, d, J=4.5 Hz, NC*H*CH), 6.85 (1H, t, J=9 Hz, ArH), 7.12 (1H, dd, J=2 Hz, J=9 Hz, ArH), 7.5 (1H, dd, J=2 Hz, J=9 Hz, ArH). *Anal* Calcd for $C_{19}H_{27}N_3O_8$: C 53.64, H 6.40, N 9.88. Found: C 53.76, H 6.18, N 9.70.

L-*N*-(2,3-Dihydroxybenzoyl)valyl-L-N^{ω}-acetylornithine

Hydrogenolysis of L-*N*-(2,3-dibenzyloxybenzoyl)valyl-L-N^{ω}-acetylornithine benzyl ester (0.4 g, 0.6 mmol) as described above, purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from MeOH gave a crystalline powder (0.1 g, 0.25 mmol, 41%). Rf 0.42 (BuOH - MeOH - H_2O, 4: 1: 1), mp 115~116°C, $[\alpha]_D^{22}$ +2.7° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 0.92 (3H, d, J=3 Hz, CH_3CCH$_3$), 1.05 (3H, d, J=3 Hz, CH$_3$CCH_3), 1.3~1.8 (4H, m, CH(CH_2)$_2$), 1.8 (3H, s, CH_3CO), 3.12 (2H, t, J=6 Hz, NCH_2), 3.9 (1H, m, C*H*CH$_3$), 4.28~4.5 (2H, m, CH×2), 6.65 (1H, t, J=9 Hz, ArH), 6.87 (1H, dd, J=3 Hz, J=9 Hz, ArH), 7.28 (1H, dd, J=3 Hz, J=9 Hz, ArH). *Anal* Calcd for $C_{19}H_{27}N_3O_7$: C 55.74, H 6.65, N 10.26. Found: C 55.39, H 6.21, N 10.43.

L-*N*-(2,3-Dihydroxybenzoyl)threonyl-L-N^{ω}-acetylornithine Methyl Ester

Hydrogenolysis of L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonyl-L-N^{ω}-acetylornithine methyl ester (0.35 g, 0.5 mmol) as described above, purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from MeOH gave a crystalline powder (0.14 g, 0.34 mmol, 68%). Rf 0.64 (BuOH - MeOH - H_2O, 4: 1: 1), mp 74~75°C, $[\alpha]_D^{20}$ −0.72° (*c* 1.0, MeOH), ^{1}H NMR (90 MHz, CD_3OD) δ 1.25 (3H, d, J=6 Hz, CHCH_3), 1.35~1.85 (4H, m, CH(CH_2)$_2$), 1.90 (3H, s, CH_3CO), 3.12 (2H, t, J=6 Hz, NCH_2), 3.74 (3H, s, OCH_3), 4.22 (1H, m, C*H*CH$_3$), 4.4 (1H, m, C*H*CH$_2$), 4.6 (1H, d, J=5 Hz, NC*H*CH), 6.67 (1H, t, J=9 Hz, ArH), 6.95 (1H, dd, J=3 Hz, J=9 Hz, ArH), 7.35 (1H, dd, J=3 Hz, J=9 Hz, ArH). *Anal* Calcd for $C_{19}H_{27}N_3O_8$: C 53.64, H 6.40, N 9.88. Found: C 53.19, H 6.06, N 9.53.

L-(N^{α}-2,3-Dihydroxybenzoyl-N^{ω}-acetyl)ornithine

Hydrogenolysis of L-(N^{α}-2,3-dibenzyloxybenzoyl-N^{ω}-acetyl)ornithine benzyl ester (0.46 g, 0.8 mmol) as described above, purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from MeOH gave crystals in the form of plates (0.17 g, 0.55 mmol, 69%). Rf 0.44 (BuOH - MeOH - H_2O, 4: 1: 1), mp 83~85°C, $[\alpha]_D^{20}$ +8.3° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 1.2~1.8 (4H, m, CH(CH_2)$_2$), 1.9 (3H, s, CH_3CO), 3.2 (2H, t, J=7 Hz, NCH_2), 4.65 (1H, t, J=7 Hz, CH), 6.73 (1H, t, J=9 Hz, ArH), 6.95 (1H, dd, J=3 Hz, J=9 Hz, ArH), 7.35 (1H, dd, J=3 Hz, J=9 Hz, ArH). *Anal* Calcd for $C_{14}H_{18}N_2O_6$: C 53.79, H 6.05, N 9.61. Found: C 54.19, H 5.85, N 9.22.

L-Threonyl-L-(N^{ω}-acetyl-N^{ω}-hydroxy)ornithine

L-(*N*-Boc-*O*-Benzyl)threonine (0.25 g, 1.0 mmol) and L-(N^{ω}-acetyl-N^{ω}-benzyloxy)ornithine benzyl ester[2] (0.37 g, 1.0 mmol) were dissolved in distilled THF (10 ml). To the solution were added DCC (0.25 g, 1.2 mmol) and HOBt (0.16 g, 1.2 mmol), and the mixture was stirred for 7 hours at room temp. The resulting precipitate was removed by filtration, EtOAc (150 ml) was added to the filtrate, washed with saturated $NaHCO_3$, and NaCl, dried (Na_2SO_4) and evaporated. The residue was extracted with toluene (50 ml), applied to silica gel column (2×10 cm), eluted with toluene - EtOAc (5: 1) and evaporated. The residue was dissolved in dichloromethane (10 ml), cooled in an ice-bath and trifluoroacetic acid (0.5 ml) was added. Stirring was continued for 2 hours at room temp and the solvent was removed *in vacuo*. The residue was dissolved in MeOH (5 ml) and 10% palladium carbon (50 mg) was added to the solution. After the atomosphere was replaced with hydrogen at atomospheric pressure, stirring was continued for 12 hours at room temp. The undissolved material was removed by filtration and the filtrate was applied to Sephadex LH-20 column (3×40 cm), eluted with MeOH and evaporated to give white powder (0.17 g, 0.60 mmol, 60%). Rf 0.28 (BuOH - MeOH - H_2O, 4: 1: 2), mp 177~180°C, $[\alpha]_D^{25}$ −1.2° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, D_2O) δ 1.36 (3H, d, J=6.7 Hz, $CHCH_3$), 1.7~2.0 (4H, m, $CH(CH_2)_2$), 2.15 (3H, s, CH_3CO), 3.68 (2H, t, J=5.3 Hz, NCH_2), 3.88 (1H, d, J=5.3 Hz, $CHNH_2$), 4.17 (1H, m, $CHCH_3$), 4.38~4.45 (1H, m, $CHCH_2$). *Anal* Calcd for $C_{11}H_{21}N_3O_6$: C 45.35, H 7.27, N 14.42. Found: C 45.11, H 7.54, N 14.03.

L-(*N*-2,3-Dihydroxybenzoyl)threonine

Hydrogenolysis of L-(*N*-2,3-dibenzyloxybenzoyl-*O*-benzyl)threonine benzyl ester[2] (0.62 g, 1.0 mmol) as described above, purification by Sephadex LH-20 column chromatography (4×150 cm) and crystallization from MeOH gave white crystals in the form of needles (0.186 g, 0.73 mmol, 73%). Rf 0.54 (BuOH - AcOH - H_2O, 4: 1: 1), mp 179~180°C, $[\alpha]_D^{25}$ +26° (*c* 1.0, MeOH). ^{1}H NMR (90 MHz, CD_3OD) δ 1.25 (3H, d, J=6.9 Hz, CH_3), 4.45 (1H, m, $CHCH_3$), 4.75 (1H, d, J=5 Hz, NCH), 6.76 (1H, t, J=7.5 Hz, ArH), 6.98 (1H, dd, J=7.5 Hz, J=2 Hz, ArH), 7.38 (1H, dd, J=7.5 Hz, J=2 Hz, ArH). *Anal* Calcd for $C_{11}H_{13}NO_6$: C 51.77, H 5.13, N 5.49. Found: C 51.50, H 5.09, N 5.62.

Methotrexate Bound AH-Sepharose 4B

Methotrexate bound AH-Sepharose 4B was prepared by the method of KAUFMAN *et al.*[15] as follows. AH-Sepharose 4B (25 g) was reacted with methotrexate (0.23 g, 0.5 mmol) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.46 g, 2 mmol) at room temp for 2 hours while stirring and maintaining the pH at 6.4. Then, AcOH (1 ml) was added and stirred for an additional hour. The bright yellow-orange gel was washed with large volumes of H_2O on a glass filter until the resulting filtrates were colorless. The final product was stored as an aq suspension in the cold and protected from light.

Purification of Thymidylate Synthetase from Ehrlich Ascites Carcinoma Cells of Mice

The enzyme purification was carried out by the method of SHIMIZU *et al.*[10] modified as follows. Ehrlich ascites carcinoma cells from 30 mice were harvested 7 days after intraperitoneal transplantation (1×10^6) and disrupted for two minutes with Ultra-Turrax (Janke & Kunnkel KG, IKA-Werk, TP18/2N) in 20 ml of 0.01 M potassium phosphate - 0.005 M dithiothreitol - 25 μM deoxyuridylate (pH 6.5) (Buffer A) and centrifuged at 105,000×*g* for 1 hour. The supernatant was applied to Blue-Sepharose CL-6B column (10 ml) and eluted with a linear gradient from Buffer A to Buffer A containing 1 M NaCl. Ammonium sulfate (37 g/100 ml) were added to the active fraction, stirred for 30 minutes and centrifuged at 12,000×*g* for 20 minutes to precipitate the enzyme. The precipitate was dissolved in 10 ml of 0.3 M Tris/HCl - 0.005 M dithiothreitol - 0.1 mM deoxyuridylate (pH 7.4) (Buffer B) and applied to methotrexate bound affinity column. After washing with Buffer B, the enzyme was eluted with 0.3 M Tris/HCl - 0.005 M dithiothreitol (pH 7.4) and concentrated by placing the eluate in a dialysis bag covered with solid sucrose and dialyzed against Buffer A. The enzyme at this step was purified 1,680-fold over the initial extract and was used in the kinetic studies of inhibition of the vanoxonin-vanadium complexes.

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