STUDIES ON NEW DEHYDROPEPTIDASE INHIBITORS

II. STRUCTURAL ELUCIDATION AND SYNTHESIS OF WS1358A1 AND B1

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The structures of WS1358A1 and B1, new dehydropeptidase inhibitors isolated from *Streptomyces parvulus* subsp. *tochigiensis* No. 1358, have been established to be 2-hydroxy-2-hydroxyaminocarbonyl-3-methylglutaric acid (**1**) and 2-hydroxy-2-hydroxyaminocarbonylglutaric acid (**2**), respectively, on the basis of spectroscopic evidence and synthesis of the racemates.

In spite of their outstanding antimicrobial activities, carbapenem antibiotics are known to be mostly unstable under physiological conditions and, in particular, rapidly metabolized by renal dehydropeptidase (DHP) in kidney[1]. One approach to overcome this instability of the antibiotics for therapeutic use is co-administration with a specific inhibitor of DHP; a combination of imipenem and cilastatin has been demonstrated to be of practical value[2].

In the course of our screening program for DHP inhibitors of microbial origin, we isolated two new products, WS1358A1 and B1, from a *Streptomyces* as described in the preceding paper[3]. Herein we report the structural elucidation and synthesis of these natural products.

Results and Discussion

WS1358A1 (**1**) was isolated as a colorless powder from the fermentation broth of *Streptomyces* sp. No. 1358: Na salt, $C_7H_9NO_7Na_2$ (elemental analysis); free acid, $C_7H_{11}NO_7$ (FAB-MS); $[\alpha]_D^{23}$ $-14.0°$ (*c* 0.9, H_2O, Na salt). From the same culture broth, WS1358B1 (**2**) was isolated as a colorless powder: Na salt, $C_6H_7NO_7Na_2$ (elemental analysis); free acid, $C_6H_9NO_7$ (FAB-MS); $[\alpha]_D^{23}$ $+2.5°$ (*c* 1.0, H_2O, Na salt).

The ^{13}C NMR spectrum of **2** (D_2O, pD 7.0) revealed all the carbon signals, which were assignable to three carbonyls (δ 185.3 (s, C-5), 178.2 (s, C-1), 173.6 (s, C-6)), one tertiary alcohol (δ 82.7 (s, C-2)), and two methylenes (δ 35.6, (t, C-3), 34.8 (t, C-4)). The proton signals corresponding to the two methylenes were observed at δ 2.25~2.11 (4H) as multiplet in the ^1H NMR spectrum of **2** (D_2O, pD 7.0). The linkage of all the six carbons in **2** was determined by using a 2D INADEQUATE technique[4], revealing the carbon framework of **2** as depicted in Fig. 1.

Fig. 1. The partial structure of **2** and its ^{13}C NMR data (chemical shifts in ppm).

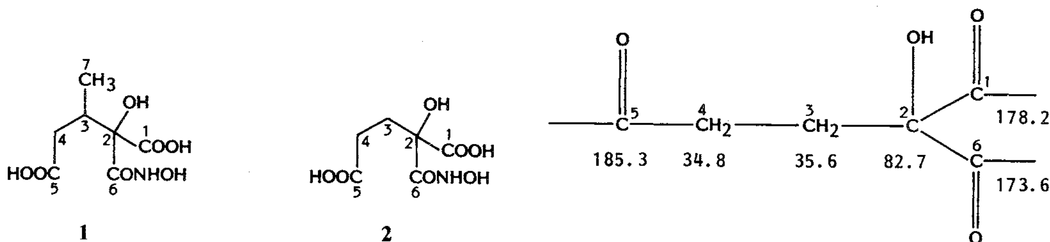

Fig. 2. Synthesis of WS1358A1 and B1 racemates.

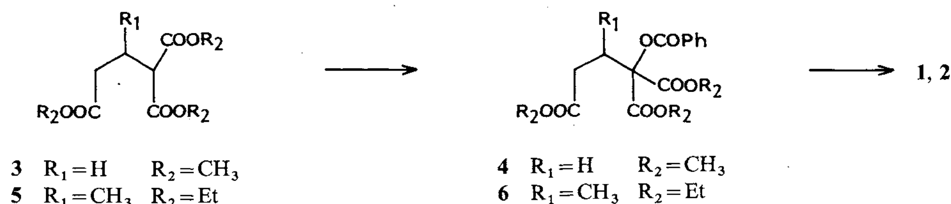

Fig. 3. The partial structure of **1** and its NMR data (chemical shifts in ppm and ^{1}H-^{13}C relationships).

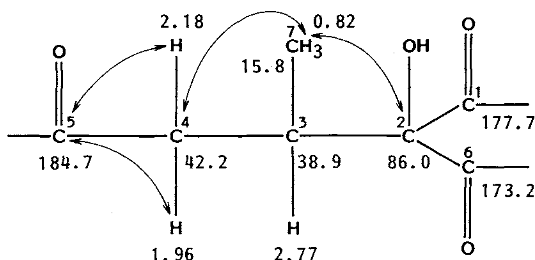

Table 1. IC_{50} of synthetic and natural WS1358 compounds against porcine DHP.

Compound	IC_{50} (ng/ml)	
	Synthetic (DL)	Natural
A1 (**1**)	1.5	0.83
B1 (**2**)	340	150

The remaining problem is to elucidate the position of the function containing nitrogen. As described in the preceding paper[3], **2** showed a positive color reaction (purple) against ferric chloride, suggesting the presence of a hydroxamic acid function in **2**. In the ^{13}C NMR spectrum, the carbonyl of a hydroxamic acid usually resonates at higher-field region than that of the corresponding carboxylic acid†. Therefore, the C-6 signal at δ 173.6 (the highest-field signal in the carbonyl region) was assigned to the hydroxamic carbonyl carbon. This was also corroborated by the mechanistic consideration in an alternative synthesis of this series of compounds as will be discussed in the following paper[5]. The structure of WS1358B1 was thus deduced to be **2** without stereochemistry.

The presumed structure of **2** was confirmed by a synthesis as follows (Fig. 2). Compound **3**, prepared by Michael condensation of methyl acrylate and diethyl malonate, was subjected to oxidation with benzoyl peroxide to give compound **4**. Treatment of **4** with hydroxylamine (2 equiv), followed by a sequence of purification processes described in the preceding paper provided B1 (**2**, racemate) as the Na salt.

The structure of WS1358A1 (**1**) was deduced in a similar manner by analysis of its ^{13}C NMR spectrum (D_2O, pD 7.0), which showed seven carbon signals attributable to three carbonyls (δ 184.7 (s, C-5), 177.7 (s, C-1), 173.2 (s, C-6)), one tertiary alcohol (δ 86.0 (s, C-2)), one methylene (δ 42.2 (t, C-4)), one methine (δ 38.9 (d, C-3)), and one methyl (δ 15.8 (q, C-7)). The methyl proton signal was observed at δ 0.82 (3H, d, $J=6.5$ Hz) and the methylene and methine signals appeared at δ 2.18 (1H, dd, $J=13.5$ and 3 Hz), 1.96 (1H, dd, $J=13.5$ and 11 Hz) and at δ 2.77 (1H, m) in the ^{1}H NMR spectrum (D_2O, pD 7.0) of **1**.

The carbon skeleton of A1 (**1**) was deduced to be the one depicted in Fig. 3 by the following NMR studies. The down-field shifts observed for C-2, C-3 and C-4 (3.3, 3.3 and 7.4 ppm, respectively) of **1** as compared with the corresponding carbon signals of **2** were attributable to a substitution effect of the methyl group[6], indicating that the carbon bearing the methyl group is adjacent to C-2 and C-4. This was

† This was studied using model compounds: In the ^{13}C NMR spectra (CD_3OD), the carbonyl of 3-phenylpropionic acid resonated at δ 176.7, while conversion to the corresponding hydroxamic acid shifted the resonance of the carbonyl upfield to δ 171.9.

corroborated by a correlation *via* long range coupling (COLOC) experiment[7], which showed that the methyl protons are coupled to C-2 and C-4, and further both methylene protons of the C-4 are coupled to the C-5 carbonyl. These data supported the carbon framework depicted in Fig. 3. As in the case of WS1358B1, the highest-field signal (C-6, δ 173.2) of the three carbonyls was assignable to the hydroxamic acid and, consequently, the structure of A1 was proposed to be **1** without stereochemistry.

The presumed structure of **1** was ascertained by a sequence of synthetic reactions similar to that used for B1 (Fig. 2). Michael condensation of ethyl crotonate and diethyl malonate gave compound **5**, which was similarly subjected to oxidation with benzoyl peroxide to yield benzonate **6**. Treatment of **6** with hydroxylamine and subsequent purification afforded WS1358A1 (**1**, racemate) as a major product together with a minor diastereoisomer in a ratio of about 3:2.

The structures of WS1358A1 and B1 were thus established as being **1** and **2**, respectively. As shown in Table 1, the biological activities of these synthetic compounds were found to be about half of those of the corresponding natural products, respectively.

Experimental

General

IR spectra were recorded on a Jasco A-102 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM400 spectrometer. MS were determined with a VG ZAB-SE spectrometer. Optical rotations were measured on a Jasco DIP-400 polarimeter. Analytical HPLC was performed on a Hitachi 655 liquid chromatograph using a YMC ODS-5 column (4.6 × 250 mm) and preparative HPLC was carried out using a YMC ODS-5 column (20 × 250 mm).

WS1358A1 (1)

For isolation of WS1358A1, see ref 3: Na salt, $[\alpha]_D^{23}$ −14.0° (*c* 0.9, H_2O); IR (KBr) cm^{-1} 1660, 1580; ^{1}H NMR (D_2O) δ 2.77 (1H, m), 2.18 (1H, dd, J=13.5 and 3 Hz), 1.96 (1H, dd, J=13.5 and 11 Hz), 0.82 (3H, d, J=6.5 Hz); ^{13}C NMR (D_2O) δ 184.7 (s), 177.7 (s), 173.2 (s), 86.0 (s), 42.2 (t), 38.9 (d), 15.8 (q); *Anal* Calcd for $C_7H_9NO_7Na_2 \cdot H_2O$: C 29.69, H 3.92, N 4.95, Na 16.24; Found: C 30.05, H 3.84, N 4.84, Na 15.89; free acid, FAB-MS *m/z* 222 ($M^+ + 1$); ^{1}H NMR (D_2O-H_2O) δ 2.89 (1H, m), 2.39 (1H, dd, J=15 and 3.5 Hz), 2.23 (1H, dd, J=15 and 10 Hz), 0.95 (3H, d, J=6.5 Hz); ^{13}C NMR (D_2O-H_2O) δ 182.2 (s), 177.4 (s), 173.0 (s), 85.8 (s), 40.5 (t), 38.5 (d), 15.9 (q).

WS1358B1 (2)

For isolation of WS1358B1, see ref 3: Na salt, $[\alpha]_D^{23}$ +2.5° (*c* 1.0, H_2O): IR (KBr) cm^{-1} 1660, 1620, 1580; ^{1}H NMR (D_2O) δ 2.25~2.11 (4H, m); ^{13}C NMR (D_2O) δ 185.3 (s), 178.2 (s), 173.6 (s), 82.7 (s), 35.6 (t), 34.8 (t); *Anal* Calcd for $C_6H_7NO_7Na_2 \cdot H_2O$: C 26.78, H 3.37, N 5.20, Na 17.08; Found: C 26.44, H 3.28, N 5.10, Na 16.88; free acid, FAB-MS *m/z* 208 ($M^+ + 1$); ^{13}C NMR (D_2O) δ 180.3 (s), 175.9 (s), 171.4 (s), 81.4 (s), 34.0 (t), 31.2 (t).

Dimethyl 2-(Methoxycarbonyl)glutarate (3)

A solution of dimethyl malonate (13.2 g, 0.1 mol) in benzene (30 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 400 mg, 0.01 mol) in benzene (50 ml) at 0°C under an atmosphere of nitrogen. To this mixture a solution of methyl acrylate (8.6 g, 0.1 mol) in benzene (30 ml) was added and the mixture was stirred at 80°C for 1 hour. The reaction mixture was cooled to room temperature poured into ice water, and extracted with ether. The extract was washed with water, dried over magnesium sulfate and concentrated to give **3** as an oil (19.4 g): EI-MS *m/z* 218 (M^+); ^{1}H NMR ($CDCl_3$) δ 3.73 (6H, s), 3.69 (3H, s), 3.51 (1H, t, J=7 Hz), 2.5~2.2 (4H, m).

Dimethyl 2-Benzoyloxy-2-(methoxycarbonyl)glutarate (**4**)

A solution of **3** (2.18 g, 10 mmol) in benzene (30 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil, 400 mg, 10 mmol) in benzene (50 ml) under an atmosphere of nitrogen. The mixture was reflexed for 1 hour, during which the mixture became clear, and then cooled to room temperature. To this mixture a solution of benzoyl peroxide (1.82 g, 7.5 mmol) in benzene (30 ml) was added dropwise under ice-bath cooling and the mixture was stirred at room temperature for 15 hours. The reaction mixture was poured into ice water and extracted with ether. The extract was washed with water, dried over magnesium sulfate and evaporated to give an oil (3.85 g), which was purified by chromatography on silica gel, eluting with 20% ethyl acetate in hexane. The fractions containing the desired product were combined and concentrated to give **4** as an oil (1.83 g): FAB-MS *m/z* 339 ($M^+ + 1$); ^1H NMR ($CDCl_3$) δ 8.09 (2H, br d, J=8 Hz), 7.61 (1H, br t, J=8 Hz), 7.47 (2H, br t, J=8 Hz), 3.84 (6H, s), 3.64 (3H, s), 2.74 (2H, t, J=7 Hz), 2.49 (2H, t, J=7 Hz).

WS1358B1 (**2**, Racemate)

Hydroxylamine hydrochloride (139 mg, 2 mmol) was dissolved in 50% aqueous methanol (0.8 ml) and 10 N NaOH (0.4 ml, 4 mmol) was added. To this mixture a solution of **4** (338 mg, 1 mmol) in methanol (2 ml) was added and the mixture was stirred at room temperature for 3 hours. The reaction mixture, after being acidified with 1 N hydrochloric acid to pH 2, was washed with ethyl acetate. The aqueous solution was neutralized with 1 N NaOH and concentrated to remove the organic solvents. The resulting aqueous layer was passed through a column of Dowex 50WX2 (H^+, 30 ml) and eluted with water. The eluate was lyophilized to give a powder (435 mg), which was purified by preparative HPLC on YMC ODS-5 eluting with 0.1% TFA in water. The fraction containing the product corresponding to WS1358B3 (equivalent to WS1358A3[3]) was collected and lyophilized to give a powder (60 mg), which was treated with 0.1 N NaOH (60 ml) for 2 hours and neutralized to pH 7 with Dowex 50WX2 (H^+). The solution was lyophilized to give WS1358B1 (**2**, racemate, 52 mg). This synthetic product was physically identical with the natural B1 in all respects except for optical rotation.

Diethyl 2-(Ethoxycarbonyl)-3-methylglutarate (**5**)

Compound **5** was prepared from diethyl malonate (16 g, 0.1 mmol) and ethyl crotonate (11.4 g, 0.1 mmol) in a manner similar to that for **3**: Yield 25.1 g (oil); EI-MS *m/z* 274 (M^+); ^1H NMR ($CDCl_3$) δ 4.27~4.09 (6H, m), 3.40 (1H, d, J=7 Hz), 2.75 (1H, m), 2.55 (1H, dd, J=16 and 5 Hz), 2.30 (1H, dd, J=16 and 8.5 Hz), 1.32~1.22 (9H, m), 1.08 (3H, d, J=7 Hz).

Diethyl 2-Benzoyloxy-2-(ethoxycarbonyl)-3-methylglutarate (**6**)

Compound **6** was prepared from **5** (274 mg, 1 mmol) in a manner similar to that for **4**: Yield 250 mg; EI-MS *m/z* 394 (M^+); ^1H NMR ($CDCl_3$) δ 8.08 (2H, m), 7.67~7.42 (3H, m), 4.37~4.23 (4H, m), 4.17 (2H, q, J=7 Hz), 3.05 (1H, m), 2.81 (1H, dd, J=16 and 3.5 Hz), 2.32 (1H, dd, J=16 and 10 Hz), 1.34~1.23 (9H, m), 1.19 (3H, d, J=7 Hz).

WS1358A1 (**1**, Racemate)

Compound **1** was prepared from **6** (394 mg, 1 mmol) in a manner similar to that for **2** (racemate). The reaction mixture, after being acidified with 1 N HCl, was washed with ether. The aqueous layer was neutralized with 1 N HCl and washed with ether. The aqueous layer was neutralized with 1 N NaOH and evaporated to remove the organic solvents. The resulting aqueous solution was lyophilized and the residue was purified by chromatography on Diaion SP-207 (20 ml), eluting with water to give a product corresponding to WS1358A3. This product was treated with 0.5 N NaOH for 2 hours and neutralized to pH 7 with Dowex 50WX2 (H^+). The solution was lyophilized to give WS1358A1 (**1**, racemate, Na salt, 15 mg). This synthetic product was physically identical with the natural one in all respects except for optical rotation.

On purification by the Diaion SP-207 chromatography, another product corresponding A3′ (a diastereoisomer of A3) was also obtained, which was similarly treated with 0.5 N NaOH and neutralized to pH 6.5. The solution was lyophilized to give a diastereoisomer (racemate, Na salt, 10 mg) of A1: ^1H NMR (D_2O) δ 2.76 (1H, m), 2.08 (1H, dd, J=14 and 3 Hz), 1.99 (1H, dd, J=14 and 11 Hz), 0.87 (3H,

d, J=6.5 Hz); ^{13}C NMR (D_2O) δ 184.5 (s), 177.6 (s), 173.2 (s), 86.0 (s), 41.9 (t), 38.9 (d), 16.0 (q).

Biological Evaluation

Inhibitory activity (IC_{50}) of the synthetic compounds against porcine DHP was measured by the method described in the preceding paper[3].

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