VOL. 56 NO. 8, AUG. 2003 THE JOURNAL OF ANTIBIOTICS pp. 694 — 699

The Novel Gluconeogenesis Inhibitors FR225659 and FR225656 from *Helicomyces* sp. No. 19353

III. Structure Determination

TATSUYA ZENKOH, YOSHIHIRO OHTSU, SEIJI YOSHIMURA*, NOBUHARU SHIGEMATSU, SHIGEHIRO TAKASE and MOTOHIRO HINO

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3, Tokodai, Tsukuba 300-2698, Japan

(Received for publication March 3, 2003)

During the course of screening for novel gluconeogenesis inhibitors,. FR225659 and its related compounds were isolated from a fermentation broth of *Helicomyces* sp. No. 19353. Spectroscopic analysis concluded that FR225659 is an *N*-acyl tripeptide consisting of a novel acyl, a 3-chloro-4-hydroxyarginine, a 3-hydroxy-3-methylproline and a dehydrovaline. Degradation study allowed assignment of the absolute configuration of the 3-hydroxy-3-methylproline to be (2*S*,3*R*). FR225656 was shown to possess a dehydroisoleucine instead of the dehydrovaline of FR225659.

At present, over 150 millions people are suffering from diabetes in the world. While the cause of diabetes is still unclear, it is certain that high blood glucose level damages various human tissues, leading to complications such as neuropathy, retinopathy and kidney diseases. Thus, control of blood glucose level is prerequisite for diabetes patients. In assumption that gluconeogenesis inhibitors should be useful for down-regulation of blood glucose, we screened those inhibitors from microbial products and found FR225659, FR225656, and their three minor congeners in a cultured broth of *Helicomyces* sp. No. 19353[1,2]. Herein, structure determination of the two major metabolites, FR225659 (**1**) and FR225656 (**2**), will be discussed (Fig. 1).

Results and Discussion

Since FR225659 (**1**) exerted the most promising *in vivo* data among its congeners, our initial structure elucidation was focused on **1**. In the ESI-MS spectrum, a protonated ion of **1** was observed at *m/z* 736 and 738 at a ratio of 3 : 1, indicating the existence of Cl. Analysis of HRESI-MS and ^{13}C NMR data concluded its molecular formula to be $C_{36}H_{42}ClN_7O_8$ (calcd. for $C_{36}H_{43}ClN_7O_8$ 736.2862, found 736.2861). IR bands at 3390 (br), 1630 and 770 cm^{-1} were indicative of carboxyl, amide and alkyl chloride functions, respectively. The ^1H NMR spectrum in DMSO-d_6 showed eight aromatic protons, two protons from an olefinic methylene, twelve protons from four singlet methyl, two amide protons, and a hydroxyl proton. The existence of a fused heterocyclic ring was indicated from a characteristic singlet proton observed at 8.86 ppm. A pair of terminal olefinic protons resonating at 5.16 and 4.96 ppm showed weak cross-peaks with a methyl group (δ_H 1.83) in COSY, suggesting an isopropenyl group. The ^{13}C NMR and HSQC spectra in DMSO-d_6 showed four methyl, four methylene, twelve methine and sixteen quaternary carbons, explaining 36 carbons and 32 protons. The unexplained ten protons were supposed to be exchangeable ones. Four carbonyl carbons (δ_C 170.0, 168.2, 166.1 and 165.4), a guanidine (δ_C 157.4), a phenol (δ_C 158.3) and eighteen more sp^2 carbons were observed in the downfield area. A carbon resonating at 69.2 ppm bearing a singlet proton at 3.96 ppm was

* Corresponding author: seiji_yoshimura@po.fujisawa.co.jp

Fig. 1. Structures of FR225659 (**1**), FR225656 (**2**) and a degradative derivative (**3**).

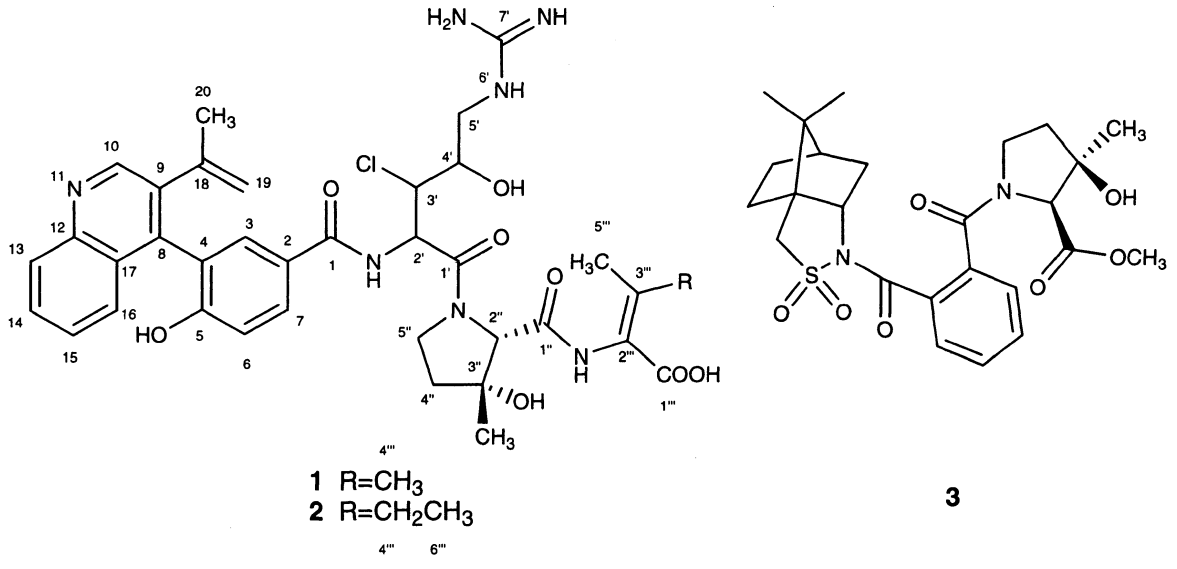
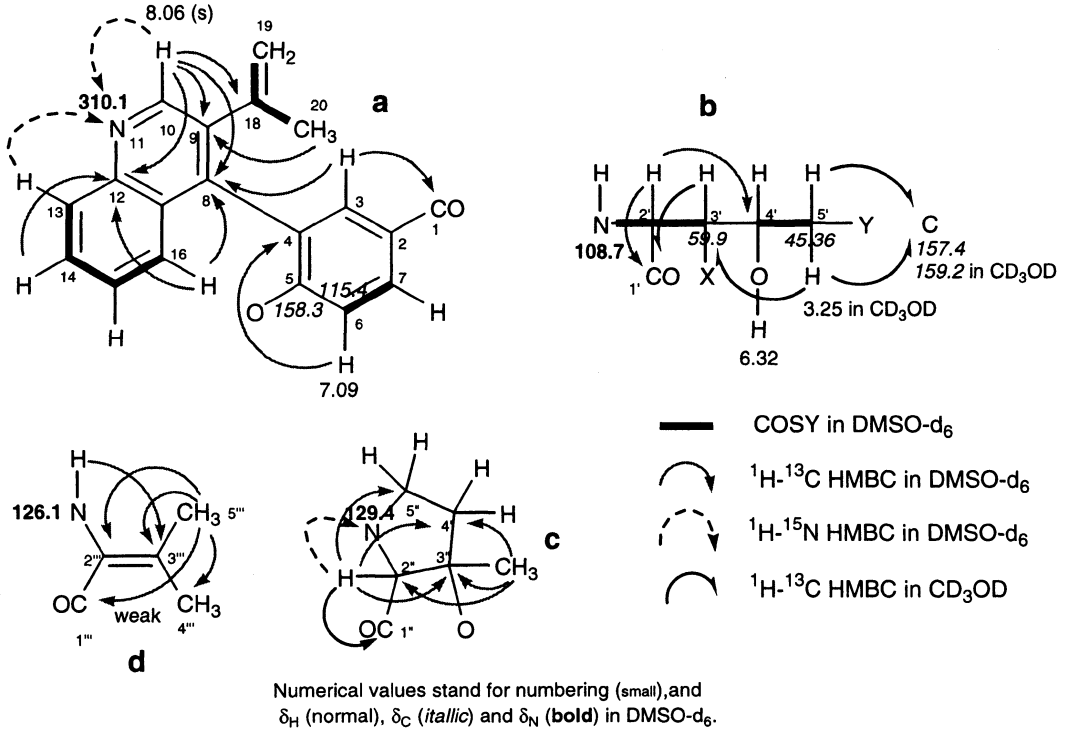


Fig. 2. Partial structures (**a**, **b**, **c** and **d**) of FR225659 (**1**).



considered to be a downfield shifted α-carbon of an α-amino acid. NMR analysis shown below clarifies that **1** consists of an unique acyl group (**a**), an arginine analogue (**b**), a proline analogue (**c**) and a dehydrovaline (**d**) (see Fig. 2). These partial structures account for all the nineteen unsaturation degrees requested from the molecular formula

of **1**, which suggests **1** is a linear acylated tripeptide.

Analysis of COSY data allowed junctions shown as bold lines in Fig. 2. Further elucidation was carried out mainly using ^{1}H-^{13}C HMBC and ^{1}H-^{15}N HMBC in DMSO-d_6. The acyl structure (**a**) including quinoline and phenol was deduced as follows. Two quaternary carbons, C-12 and C-17, were assigned to be orthogonal of a di-substituted benzene by strong HMBC cross peaks from $^{3}J_{CH}$ (14-H/C-12 and 15-H/C-17). A strong HMBC cross peak of 10-H/C-12 and ^{1}H-^{15}N HMBC correlations of 10-H/N-11 and 13-H/N-11 clarified that N-11 is adjacent to C-12 and C-10. Bonds of C-9/C-10 and C-9/C-18 were deduced from HMBC correlations of 10-H/C-9, 10-H/C-18 and 20-H_3/C-9. HMBC correlations of 10-H/C-8 and 16-H/C-8 allowed linkages of C-9/C-8/C-17, giving the quinoline structure. The tri-substituted phenol was assigned with ^{1}H NMR coupling patterns, characteristic ^{13}C NMR chemical shifts (δ_C 158.3 for C-5 and 115.4 for C-6) and HMBC correlation of 6-H/C-4. HMBC correlation of 3-H/C-1 clarifies that C-1 is connected to the phenol ring at position 2. The biaryl linkage between C-4 and C-8 was shown by HMBC correlation of H-3/C-8.

The arginine analogue (**b**), the most characteristic amino acid residue in **1**, was elucidated as below. As $^{3}J_{HH}$ of 3′-H/4′-H was nearly zero, linkage of C-3′/C-4′ was deduced from HMBC correlations of 2′-H/C-4′ and 5′-H/C-3′. An exchangeable proton at 6.32 ppm is weakly coupled with 4′-H, suggesting that the functionality of 4′-O should be hydroxyl. Though the methylene protons at 5′ did not give any correlations to the carbon resonating at 157.4 ppm in DMSO-d_6 presumably because of line broadening, they showed HMBC correlations to a carbon at 159.2 ppm upon using CD_3OD as an NMR solvent. These HMBC data and $^{1}H/^{13}C$ NMR chemical shift values at 5′ indicate that the guanidine group should attach C-5′. Functionality at C-3′ was presumed to be chloride in view of ^{13}C NMR (δ_C 59.9). HMBC correlation of 2′-H/C-1′ allowed assignment of C-1′, yielding 3-chloro-4-hydroxyarginine structure.

The proline analogue residue (**c**) was inferred as follows. HMBC correlations from 3″-CH_3 to C-2″, C-3″ and C-4″ allowed C–C bonds around the quaternary carbon at 3″. Chemical shift of C-3″ (75.7 ppm) indicates that this carbon bears O-function. HMBC correlation from 2″-H to C-5″ and ^{1}H-^{15}N HMBC correlations from 2″-H and 4″-H_2 to 5″-N (δ_N 129.4) links C-2″ to C-5″ *via* 5″-N. C-1″ should attach C-2″ as C-1″ correlates with 2″-H in the HMBC spectrum.

The dehydrovaline (**d**) was deduced mainly using HMBC data. Strong HMBC cross peaks from singlet methyl at position 5‴ to C-3‴, C-2‴ and C-4‴ showed linkages of C-5‴/C-3‴, C-3‴/C-2‴ and C-3‴/C-4‴. A weak HMBC correlation of 5‴-H_3/C-1‴ suggested C-1‴/C-2‴ bond. Similar HMBC correlations were also observed from singlet methyl protons of position 4‴ (not shown in Fig. 2). An amide proton resonating at 8.16 ppm was correlated with C-3‴ in the HMBC spectrum, which unveiled the dehydrovaline residue structure.

The sequence of these substructures (**a-b-c-d**) was determined by sequential HMBC data and supported by NOE data (Fig. 3). HMBC correlations of 2′-NH/C-1 and 2′-H/C-1 (weak) showed the C-1/2′-N bond to allow the acyl-3-chloro-4-hydroxyarginine sequence, which was supported by NOEs of 3-H/2′-NH and 7-H/2′-NH. Similarly, HMBC of 2″-H/C-1′ and 2‴-NH/C-1″, and ^{1}H-^{15}N HMBC of 2″-H/2‴-N (δ_N 126.1) clarified the sequence

Fig. 3. Sequential HMBC and NOE data.

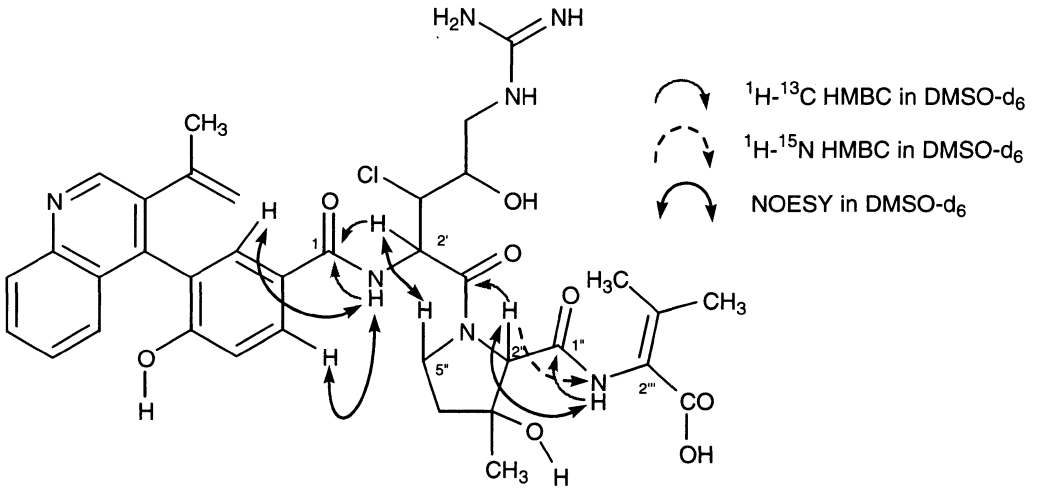


Table 1. NMR assignment of FR225659 (**1**) and FR225656 (**2**).

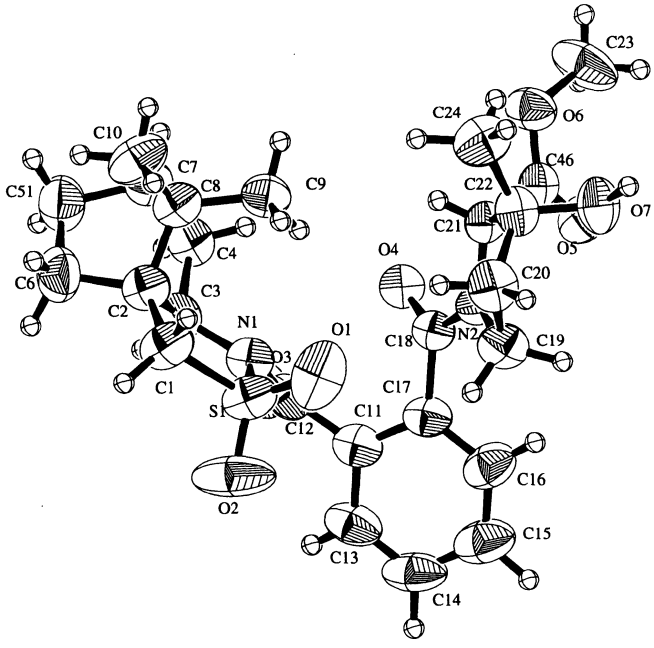
	FR225659 (**1**)					FR225656 (**2**)	
position	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)	δ_N (DMSO-d_6)	δ_H (CD_3OD)	δ_C (CD_3OD)	δ_H (DMSO-d_6)	δ_C (DMSO-d_6)
1		165.4			169.1		165.4
2		123.9			125.4		123.9
3	7.45 (d, 2)	129.8		7.59	131.8	7.45	129.8
4		123.5			125.1		123.5
5		158.3			160.0		158.3
6	7.09 (d, 8)	115.4		7.01	116.6	7.09	115.4
7	7.74 (dd, 8&2)	128.8		7.86	130.6	7.74	128.7
8		140.9			143.7		140.9
9		135.4			137.7		135.4
10	8.86 (s)	150.2		8.80	151.2	8.86	150.2
11			310.1				
12		146.6			147.7		146.6
13	8.06 (d, 8)	129.0		8.05	129.2	8.06	129.0
14	7.73 (dd, 8&8)	128.9		7.71	130.5	7.73	128.9
15	7.51 (dd, 8&8)	126.9		7.47	128.3	7.51	126.9
16	7.35 (d, 8)	126.0		7.44	127.5	7.35	126.0
17		126.6			128.8		126.6
18		142.0			143.6		142.0
19	5.16 (br s)	117.7		5.15	118.7	5.16	117.7
	4.96 (br s)			4.99		4.96	
20	1.83 (3H, br s)	23.4		1.86	23.9	1.83	23.4
1'		168.2			170.0		168.2
2'	5.07 (dd, 7&4)	56.1		5.27	57.8	5.07	56.2
3'	4.76 (br d, 4)	59.9		4.50	60.2	4.76	59.8
4'	4.59 (m)	67.6		4.17	69.9	4.61	67.6
4'-OH	6.32 (br d, 5)					6.33	
5'	3.30 (m)	45.36		3.41	46.8	3.31	45.4
	3.00 (m)			3.25		3.00	
7'		157.4			159.2		157.4
2'-NH	9.04 (br d, 7)		108.7			9.05	
1"	3.96 (s)	166.1			169.8	3.97	165.9
2"		69.2		4.29	70.2		69.1
3"		75.7			78.5		75.7
3"-CH_3	1.29 (s)	26.9		1.51	26.5	1.28	27.0
4"	2.04 (m)	38.3		2.15	40.0	2.05	38.2
	1.82 (m)			2.00		1.80	
5"	4.11 (m)	45.41		4.00	47.1	4.11	45.4
	3.98 (m)			3.90		3.98	
2"-N			129.4				
1"'		170.0			172.4		169.8
2"'		126.9			128.4		126.4
3"'		132.6			136.2		137.6
4"'	2.06 (s)	20.5		2.02	21.0	2.67	26.2
						2.46	
4"'-CH_3						0.95	13.2
5"'	1.64 (s)	22.9		1.75	21.5	1.63	19.9
2"'-NH	8.16 (br s)		126.1			8.17	

of 3-chloro-4-hydroxyarginine-3-hydroxy-3-methylproline-dehydrovaline. This was supported by NOEs of 2′-H/5″-H (4.11 ppm) and 2″-H/2‴-NH. Functionality of C-1‴ was supposed to be carboxylic acid because C-1‴ lacked correlations from any amide protons or α-protons, which met the molecular formula of **1** with assignments of 5-O and 3″-O to be free phenol and hydroxyl, respectively. With four protons from the guanidine and two protons from the amides, those assignments explain all of ten exchangeable protons of **1**. Thereby, the planar structure of **1** is completed as shown in Fig. 3.

The molecular formula of FR225656 (**2**) was determined to be $C_{37}H_{44}ClN_7O_8$ by HRESI-MS and HSQC data (calcd. for $C_{37}H_{45}ClN_7O_8$ 750.3018, found 750.3033). Major spectral difference between **1** and **2** is that **2** exhibits ethyl signals in stead of methyl ones at 4‴ in the ^1H and ^{13}C NMR spectra. As a consequence of similar NMR analysis with **1**, **2** was proven to possess a dehydroisoleucine in place of the dehydrovaline in **1**. The *E*-geometry of the dehydroisoleucine was shown by a NOE cross-peak of 2‴-NH/5‴-H_3. ^1H, ^{13}C and ^{15}N NMR assignments of **1** and **2** are depicted in Table 1.

The remaining problem in this structure determination is stereochemistry of those compounds. All attempts to obtain suitable crystals for X-ray crystallography were in vain. It proved difficult to elucidate their stereochemistry using NMR, partly because they showed rather broad signals. Thus, we embarked on degradative study of **1**. Acid hydrolysis (6 N HCl) followed by methyl esterification and acylation with a chiral phthalic acid[3] yielded **3** which gave fine crystals to allow X-ray crystallographic analysis. ORTEP drawing of **3** was shown in Fig. 4. Thus, the stereochemistry of the 3-hydroxy-3-methylproline was determined to be (2*S*,3*R*). We are currently seeking milder conditions to transform **1** into simpler structures to determine the stereochemistry at position 2′, 3′ and 4′.

Fig. 4. ORTEP drawing of **3**.



Conclusion

In conclusion, structure of **1** was determined to be [4-hydroxy-3-{2-(2-propenyl)-4-quinoline}benzoyl]-(3-chloro-4-hydroxyarginyl)-{(2*S*,3*R*)-3-hydroxy-3-methylprolyl}-dehydrovaline as shown in Fig. 1. The compound **2** was shown to own dehydroisoleucine in place of the dehydrovaline in **1**. Not only these compounds are novel natural products, but also all of their components are rare. As far as we know, the acyl moiety and 3-chloro-4-hydroxyarginine have never been encountered so far. With regard to arginine analogues in natural resources, 3,4-dihydroxyarginine was reported as a composite of eurypamide A by FAULKNER *et al.*[4], and there are several examples of 4-hydroxyarginine[5]. However, arginine analogues bearing chloride have never been reported at all. 3-Hydroxy-3-methylproline was reported as a composite of polyoxypeptins[6], and as a synthetic product in the course of total synthetic efforts on polyoxypeptins[7]. We believe the hydroxy group contributes to the high biological activity of **1** and **2** as well as their solubilities[2]. We are now focusing on further stereochemical elucidation of **1**, **2**, and their minor congeners, and also performing synthetic research of their analogues to pursue better biological properties. Those results and discussions will be reported in due course.

Experimental

^1H and ^{13}C NMR were measured on a Bruker DRX500 or a Varian Mercury300 NMR spectrometer. Standard pulse sequences were employed for COSY, HSQC, HMBC and NOESY. Phase-sensitive NOESY spectra were measured with a mixing time of 500 ms, while HMBC were optimized for $^nJ_{CH}$=8 Hz. Mass spectra were recorded on a Micromass Quattro, a Platform or an LCT mass spectrometer. Preparative thin-layer chromatography (TLC) was carried out on a Merck Silica gel F254 pre-coated

plate, Art 5744.

Preparation of **3**

A solution of **1** (100 mg) in 6 N-hydrochloric acid (5 ml) was heated to 110°C for 15 hours. After filtration, the filtrate was concentrated and purified on SP207 (7 ml) using water as eluent to give crude 3-hydroxy-3-methylproline (45 mg). A solution of the crude amino acid in methanol (1 ml) was then treated with a solution of 2 N-trimethylsilyldiazomethane in hexanes (0.5 ml) for 5 minutes, quenched with acetic acid, and evaporated. This esterification procedure was repeated twice. To a solution of the ester in dichloromethane (0.5 ml) was added *N*-(2-carboxybenzoyl)-(1*S*,2*R*, 4*R*)-2,10-camphorsultam[3] (34 mg), HOBt (1-hydroxybenzotriazole, 14 mg) and EDCI·EHCl (*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride, 20 mg). The mixture was stirred for 2 hours and purified on a preparative TLC using 4% methanol in chloroform as eluent to give 16 mg of **3** (26% in 3 steps) as a film. Crystallization from hot methanol gave colorless prismatic crystals suitable for X-ray crystallography: $[\alpha]_D^{21}-167°$ (*c* 0.17, CH_2Cl_2), m.p.>250°C, ESI-MS *m/z* 505 $(M+H)^+$, ^1H NMR (300 MHz, $CDCl_3$); δ 7.65~7.48 (4H, m), 4.46 (1H, s), 4.11 (1H, dd, 8 and 5 Hz), 3.96~3.70 (2H, m), 3.78 (3H, s), 3.45 (1H, d, 14 Hz), 3.38 (1H, d, 14 Hz), 2.25~1.80 (7H, m), 1.59 (3H, s), 1.57~1.24 (2H, m), 1.19 (3H, s), 0.97 (3H, s).

Acknowledgement

The authors appreciate Drs. M. HASHIMOTO for measuring HRESI-MS spectra, and T. KINOSHITA for performing X-ray crystallography.

References

1) OHTSU, Y.; H. SASAMURA, Y. TSURUMI, S. TAKASE, M. HASHIMOTO, T. SHIBATA, M. HINO & T. FUJII: The novel gluconeogenesis inhibitors FR225659 and related compounds that originate from *Helicomyces* sp. No. 19353. I. Taxonomy, fermentation, isolation and physicochemical properties. J. Antibiotics 56: 682~688, 2003

2) OHTSU, Y.; H. SASAMURA, T. SHIBATA, H. NAKAJIMA, M. HINO & T. FUJII: The novel gluconeogenesis inhibitors FR225659 and related compounds that originate from *Helicomyces* sp. No. 19353. II. Biological profiles. J. Antibiotics 56: 689~693, 2003

3) This chiral phthalic acid was reported as an useful auxiliary for a purpose of X-ray crystallography. See, HARADA, N.; T. NEHIRA, T. SOUTOME, N. HIYOSHI & F. KIDO: Chiral phthalic acid amide, a chiral auxiliary useful for enantiomer resolution and X-ray crystallographic determination of the absolute stereochemistry of alcohols. Enantiomer 1: 35~39, 1996

4) RAMI REDDY, M. V.; M. K. HARPER & D. J. FAULKNER: Eurypamide A, a cyclic tripeptide from the Palauan sponge *Microciona eurypa*. Tetrahedron 54: 10649~10656, 1998

5) a) BELL, E. A.; J. R. A. LYDDIERD, M. A. MALIK, M. MOTEVALLI, P. B. NUNN, P. O'BRIEN & K. P. W. C. PERERA: X-ray single-crystal structure of 2(*S*),4(*R*)-4-hydroxyarginine from *Len culinaris* seeds. Phytochemistry 50: 1201~1204, 1999
For an example of 4-hydroxy-NG-methyl-Arg, see b) IMAMURA, N.; I. MOTOIKE, M. NODA, K. ADACHI, A. KONNNO & H. FUKAMI: Argimicin A, a novel anticyanobacterial compound produced by an algae-lysing bacterium. J. Antibiotics 53: 1317~1319, 2000

6) UMEZAWA, K.; K. NAKAZAWA, Y. IKEDA, H. NAGANAWA & S. KONDO: Polyoxypeptins A and B produced by *Streptomyces*: Apotosis-inducing cyclic depsipeptides containing the novel amino acid (2*S*,3*R*)-3-hydroxy-3-methylproline. J. Org. Chem. 64: 3034~3038, 1999

7) a) NOGUCHI, Y.; H. UCHIRO, T. YAMADA & S. KOBAYASHI: Synthetic study of polyoxypeptin: stereoselective synthesis of (2*S*,3*R*)-3-hydroxy-3-methylproline. Tetrahedron Letters 42: 5253~5256, 2001
b) QIN, D.-G.; H.-Y. ZHA & Z.-J. YAO: Enantioselective synthesis of (2*S*,3*R*)-3-hydroxy-3-methylproline, a novel amino acid found in polyoxypeptins. J. Org. Chem. 67: 1038~1040, 2002
c) MAKINO, K.; A. KONDOH & Y. HAMADA: Synthetic studies on polyoxypeptins: stereoselective synthesis of (2*S*,3*R*)-3-hydroxy-3-methylproline using SmI_2-mediated cyclization. Tetrahedron Letters 43: 4695~4698, 2002