Tetrahedron Vol. 40, No. 13, pp. 2519 to 2527, 1984
Printed in Great Britain.

0040-4020/84 $3.00 + .00

STUDIES ON AI-77s, MICROBIAL PRODUCTS WITH GASTROPROTECTIVE ACTIVITY. STRUCTURES AND THE CHEMICAL NATURE OF AI-77s

YUKIJI SHIMOJIMA*, HIROSHI HAYASHI, TADAAKI OOKA and MITSURU SHIBUKAWA
Technical Research Laboratory, Asahi Chemical Industry Co Ltd, 2-1, Samejima, Fuji-shi, Shizuoka 416, Japan

and

YOICHI IITAKA
Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

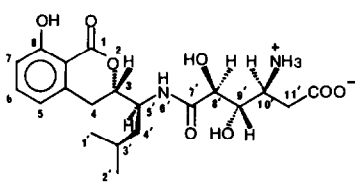
(*Received in Japan* 4 *July* 1983)

Abstract—The structures and the chemical nature of a novel gastroprotective substance AI-77-B (**1**) and its analogues AI-77-C (**2**), D (**3**), F (**4**) and G (**5**), which are produced by *Bacillus pumilus AI*-77, are described. The structure of **1** was confirmed to be 6-[[1(*S*)-(3(*S*),4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-yl)-3-methylbutyl]amino]-4(*S*),5(*S*)-dihydroxy-6-oxo-3(*S*)-aminohexanoic acid by X-ray in combination with chemical studies and the structures of **2**, **3**, **4** and **5** were determined by chemical syntheses from **1** and spectral analyses.

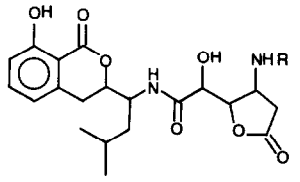
A novel gastroprotective substance AI-77-B **1** and its structural analogues with characteristic fluorescence AI-77-C **2**, D **3**, F **4**, and G **5** have been isolated from a culture broth of a bacteria which was newly isolated from a soil sample and was classified as *Bacillus pumilus* AI-77. During further studies on AI-77s, **1** was found to be a member of a unique drug class because it has non-central suppressive, non-anticholinergic and non-antihistaminergic properties in spite of its potent antiulcerogenicity action against stress ulcers induced in rats by restraint and water-immersion. The other AI-77s **2**, **3**, **4** and **5** were minor products with lower activities. Details of production isolation, primary characterization and pharmacological activity of each compound have been reported.[1] In order to clarify the relationship between the chemical structure and pharmacological activity, the structures and the chemical nature of five compounds have been studied. In our previous communication,[2] we preliminarily reported the absolute stereochemistry of **1** and proposed the structures of **2**, **3**, **4** and **5**. This paper concerns with details of the chemical nature and structure determination of these five compounds. The structure of **1** proposed by chemical studies and spectrometrical analyses was confirmed by a X-ray analysis and then the absolute stereochemistry was established by means of a chemical correlation. The structures of **2**, **3**, **4** and **5** were determined on the basis of spectrometrical analyses and chemical syntheses from **1**. The effects of minor structural modifications of **1** on the biological activity also were examined and structural requirements have been discussed in our preceding paper.[3]

AI-77-B **1**, m.p. 139.5 ~ 140° (dec), UV λ_{max}^{MeOH} nm (ϵ) 246 (6250) and 314 (4450), was shown to have the molecular formula $C_{20}H_{28}N_2O_8$ on the basis of its mass (FD) and ^{13}C NMR spectra in addition to the exact mass of its triacetylated derivative **8**. The color reactions of **1** with ninhydrin and with $FeCl_3$ reagent indicate the presence of an amino acid moiety and a phenolic hydroxyl function, respectively. The UV spectrum was similar to those of mellein[4], λ_{max}^{MeOH} nm (ϵ) 246 (6500) and 314 (4100), and bacipfelacin[5], λ_{max}^{MeOH} nm (ϵ) 245 (6050) and 314 (4900). These findings suggest that **1** contains a chromophore similar to the 3,4 - dihydro - 8 - hydroxy - 1*H* - 2 - benzopyran - 1 - one skeleton in its structure. The IR spectrum supports the presence of a lactone carbonyl group (1680 cm^{-1})[6] which is hydrogen bonded to a phenolic hydroxy function and a substituted phenyl group (810 ~ 777 cm^{-1}). The ^{13}C NMR spectrum confirmed the signals for twenty carbon atoms including three carbonyl carbons at δ 168.9, 172.7 and 174.6 (Table 2). The ^1H NMR spectrum indicated the presence of the following groups, two methyl, three methylene, one methine proton, five methine protons attached to 0 or N functions, three aromatic protons and one amide proton.

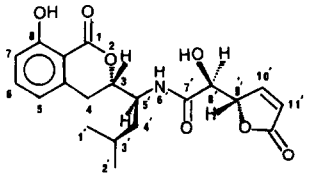
Extensive spin decoupling experiments of the ^1H NMR spectra of **1** allowed the formation of four partial structures. Decoupling from the broad multiplet at δ 1.67 converted two methyl doublets at δ 0.87 and 0.92 into two singlets, which indicated the presence of a $(CH_3)_2CH$– group (A part). The sequential decoupling from the methine proton was impossible to continue due to the nearly identical chemical shifts. The two protons at δ 2.22 and 2.38 appeared as two sets of double doublets. The larger coupling (J = 18 Hz) indicated that they were mutually coupled, forming the AB part of an ABX system. In addition to the chemical shifts of H_a and H_b, the enhanced geminal coupling ($J_{a,b}$ = 18 Hz) suggested that a carbonyl group was attached to this point. The X part was located at δ 3.34 as a multiplet, and was further coupled (J = 4 Hz) to the methine proton appearing as a double doublet at δ 3.73 (J = 4 and 7.5 Hz) which was coupled to another methine doublet proton at δ 3.99 (J = 7.5 Hz). The above findings



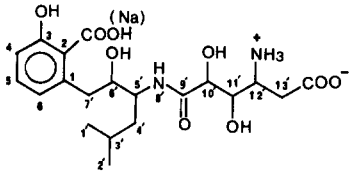
1



2 R=COCH3
3 R=COCH2CH3



4



5

Table 1. ^{1}H NMR chemical shifts (δ), multiplicities (J, Hz) and spin-decoupling study of **1** (400 MHz, DMSO-d_6 at 60°C)

Proton No.	8 (ppm)		J (Hz)	Spin-decoupling studies
C_3-H	4.68	(1H, m)		
C_4-H_a	2.88	(1H, dd)	$J_{4a,3}$ = 4.5, $J_{a,b}$ = 16	
C_4-H_b	3.06	(1H, dd)	$J_{4b,3}$ = 11, $J_{a,b}$ = 16	
C_5-H	6.84	(1H, d)	$J_{5,6}$ = 8	
C_6-H	7.48	(1H, dd)	$J_{6,5}$ = 8, $J_{6,7}$ = 8	
C_7-H	6.80	(1H, d)	$J_{7,6}$ = 8	
$C_{1'}$-H	0.89	(3H, d)	$J_{1',3}$ = 6.5	
$C_{2'}$-H	0.92	(3H, d)	$J_{2',3}$ = 6.5	
$C_{3'}$-H, $C_{4'}$-H_a	1.67 center	(2H, m)		
$C_{4'}$-H_b	1.38 center	(1H, m)		
$C_{5'}$-H	4.22	(1H, m)	$J_{5'3}$ = 4	
$C_{8'}$-H	3.99	(1H, d)	$J_{8',9'}$ = 7.5	
$C_{9'}$-H	3.73	(1H, dd)	$J_{9',10'}$ = 4, $J_{9',8'}$ = 7.5	
$C_{10'}$-H	3.34	(1H, m)		
$C_{11'}$-H_a	2.22	(1H, dd)	$J_{10',11'a}$ = 4.5, $J_{a,b}$ =18	
$C_{11'}$-H_b	2.38	(1H, dd)	$J_{10',11'b}$ = 9, $J_{a,b}$ = 18	
6'-NH	7.76	d J = 9		

Table 2. Assignments of ^{13}C NMR chemical shifts (δ)[1] of **1** and **4**

Carbon No	1[3]	4[4]
3	80.9	80.9
4	28.9	29.1
5	118.4	118.8
6	136.2	136.7
7	115.2	115.6
8	160.9	161.1
9	108.3	108.4
10	140.6	140.3
1'	21.5]2	21.2]2
2'	23.3]	23.2]
3'	38.6*	38.8*
4'	24.0	24.0
5'	48.1	48.5
8'	71.4]2	70.8
9'	71.6]	84.2
10'	50.4	154.7
11'	33.4	121.9
1	168.9]2	169.1]2
7'	172.7]	170.1]
12'	174.6]	173.2]

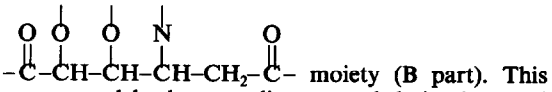
*40.1 ppm in CD_3OD

[1]Measured in DMSO-d_6 at room temperature.

[2]The assignments may be interchanged.

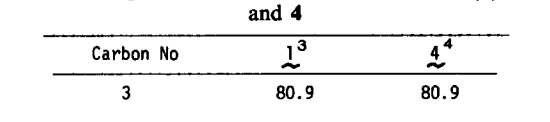
[3,4]Assignments were made based on chemical shifts and substituent effects as well as correlation of ^1H NMR and ^{13}C NMR spectra by the selective decoupling method or correlation to the ^{13}C NMR assignments of **1**.

and their chemical shifts suggested the presence of a



moiety (B part). This was proved by later studies on acyl derivatives and hydrolysates of **1**.

Decoupling from the methine multiplet at δ 4.22 simultaneously caused collapse of four parts of signals as follows; the methine multiplet at δ 4.68 was changed to a double doublet (J = 4.5 and 11 Hz), the amide doublet at 7.76 collapsed to a singlet and both multiplets at δ 1.38 and 1.67 simultaneously caused change in shape, indicating that H_a and H_b protons coupled mutually with a large geminal coupling resonated at above δ positions. It was not possible to continue the sequential decoupling from the geminal methylene protons due to the nearly identical chemical shifts. Decoupling from the methine multiplet at δ 4.68 caused substantial change in the shape of the methine multiplet at δ 4.22 and caused conversion of the two double doublets forming another AB part of an ABX system, δ 2.88 and 3.06, into two doublets (J = 16 Hz). These findings and their chemical shift considerations indicated the presence of a partial structure

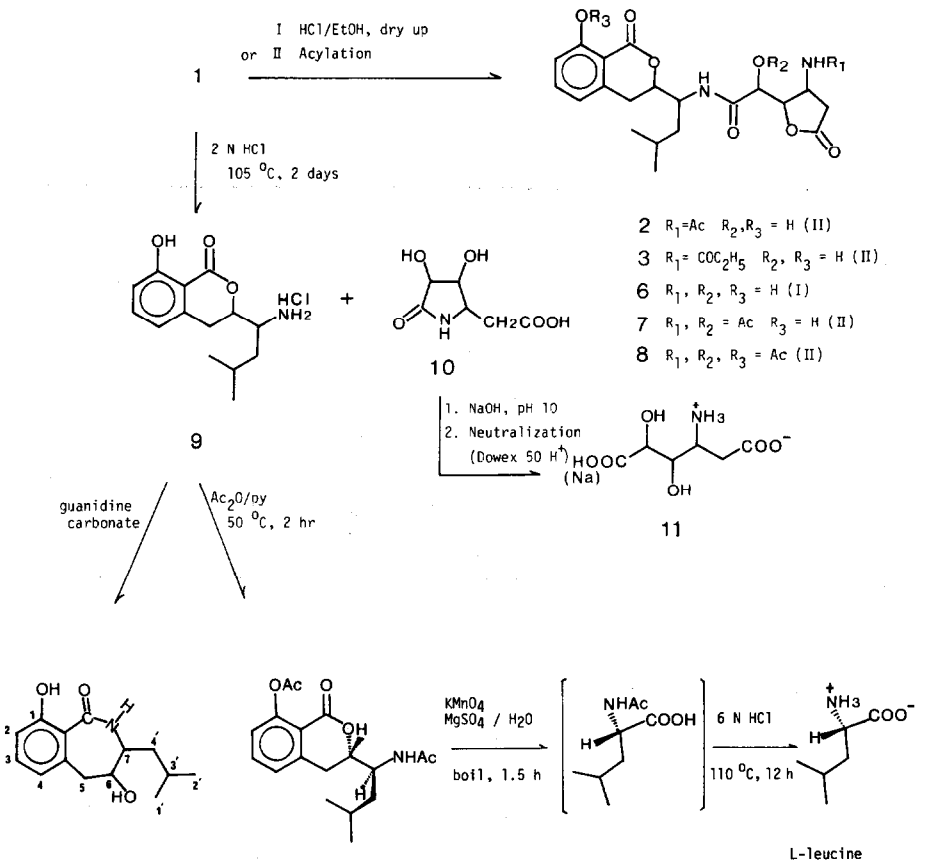


(C part). Possibly the methine of the isopropyl group (A part) is attached to the methylene terminal of this partial structure. In addition to the above, the presence of three adjacent aromatic protons were showed as two doublets at δ 6.80 and 6.84 (each J = 8 Hz) and a triplet like double doublet at δ 7.48 (J = 8 Hz) (D part). Decoupling from the proton at δ 7.48 converted the two doublets to two singlets. When both methylene protons at δ 2.88 and 3.06 were simultaneously irradiated, two doublets of aromatic protons revealed further splits by a meta coupling (J = 1 Hz) indicating that the methylene protons were also coupled to aromatic protons by long range couplings. This finding indicated that the terminal carbon of C part must be an aromatic carbon. The UV and IR spectra of **1** indicated the presence of a 3,4 - dihydro - 8 - hydroxy - 1*H* - 2 - benzopyran - 1 - one skeleton. Therefore the methylene protons coupled to aromatic protons could be assigned to those of C_4 of the skeleton and three adjacent aromatic protons could be assigned to those of C_5, C_6 and C_7.

^1H NMR and ^{13}C NMR spectral data and assignments are shown in Tables 1 and 2. ^{13}C NMR spectral data supported the presence of the above partial structures. In order to clarify which carbonyl group in B part forms an amide group with the –NH terminal group of C part and what functional groups having O or N exist in B part, following studies were carried out.

The γ-lactone derivative **6**, $C_{20}H_{26}N_2O_7$, was obtained by evaporating an ethanol solution of **1** with HCl to dryness.[3] The IR spectrum indicates the formation of a γ-lactone ring (1790 cm^{-1}). In the ^1H NMR spectrum (DMSO-d_6) of **6** the signal (δ 4.61) of a methine protone on $C_{9'}$ shifted downfield by 0.97 ppm compared with corresponding signal in the spectrum of **1**. The facts indicated that a secondary hydroxyl group attached to $C_{9'}$ and formed a γ-lactone ring with a carboxyl group located favorably to permit the ring formation.

Acetylation of **1** under appropriate conditions afforded three kinds of acetylated derivatives.[3] The reaction of **1** with acetic anhydride in pyridine at 0° gave exclusively a N-acetylated derivative **2**, $C_{22}H_{28}N_2O_8$. The ^1H NMR spectrum (DMSO-d_6) of **2** showed a methyl singlet of $-NHCOCH_3$ at δ 1.69 (3H, s) and two amide doublets at δ 7.85 (J = 8 Hz) and 8.36 (J = 7 Hz). Moreover, one methine multiplet at δ 3.40 ($C_{10'}$–H) in the spectrum of **6** shifted downfield to δ 4.32 in **2**. Treatment of **1** with $ZnCl_2$ in acetic anhydride at 50° afforded a diacetylated derivative **7**, $C_{24}H_{30}N_2O_9$. The ^1H NMR spectrum (DMSO-d_6) of **7** showed an additional signal of $-OCOCH_3$ at δ 2.08 (3H, s) with an accompanying shift of the methine proton ($C_{8'}$–H) by 0.91 ppm downfield compared with that of **2**. These findings indicate the presence of two secondary alcohol groups in **1**. Extensive acetylation of **1** with acetic anhydride in pyridine at 50° gave a triacetylated derivative **8**, $C_{26}H_{32}N_2O_{10}$. The UV spectrum of **8** revealed blue shifts [λ_{max}^{MeOH} nm (ϵ) 236 (7150) and 288 (1805)] with accompanying disappearance of the



fluorescence. The ^{1}H NMR spectrum (DMSO-d_6) of **8** revealed a methyl singlet due to $-OCOCH_3$ at δ 2.27 in addition to signals in the spectrum of **7**. Furthermore, the signal near δ 10.8 corresponding to the phenolic hydroxyl group in **2** or **7** was no longer detected in this spectrum. In the IR spectrum of **8** the absorption band due to the δ-lactone (1730 cm^{-1}) free from hydrogen bonding was found in addition to the three other ester bands [1790 (γ-lactone), 1775 and 1755 cm^{-1}].

The chromophoric hydrolysate **9** was obtained from **1** in an almost quantitative yield by conventional hydrolysis of the amide bond with 6 N HCl, but none of the corresponding side chain moiety was obtained from the hydrolysate due to a variety of side reactions.[3] Acid hydrolysis of **1** with 2 N HCl at 105° for two days afforded **9** and pyrrolidonecarboxylic acid[7] **10** predominately. Both **9** and **10** were separated by column chromatography (Amberlite XAD-2). White needles of **9** was obtained as a HCl salt from a methanol eluate of the column, $C_{14}H_{19}NO_3$, UVλ_{max}^{MeOH} nm (ϵ) 246 (6280) and 314 (4100). Compound **9** was identical with chromophoric moiety of baciphelacine.[5] In a methanol solution, the free base of **9** easily changed to a seven-membered lactam derivative **12** at room temperature. The UV spectrum of **12** revealed blue shifts (λ_{max}^{MeOH} 230 sh and 305 nm) and the IR absorption band shifted from 1680 to 1630 cm^{-1} (lactam). In the ^{1}H NMR spectrum of **12**, the chemical shift of C_3–H in **9** moved upfield by 0.93 ppm. These findings showed reasonable grounds to conclude **12** to be a seven-membered lactam derivative as shown in Fig. 2. The fraction eluted from the above Amberlite XAD-2 column with water gave **10**. It was subjected to hydrolysis with aqueous NaOH at pH 10, followed by desalting with Dowex 50 (H^+) to give **11**.[3] The completely salt-free sample of **11** could not be obtained due to facile formation of the pyrrolidone ring. Compound **10** and **11** were colored yellow and purple respectively with a ninhydrin reagent. The hydrolysate **11** showed two quasi molecular ion peaks at m/z 194 (M + 1) and 216 (M + Na) in the FD mass spectrum and the IR ν_{max}^{KBr} 3500–3000 (broad, NH_3^+, OH), 1710 (COOH), 1600 (COO^-) and 1570 sh (NH_3^+). The ^{1}H NMR spectrum (200 MHz D_2O) of **11** showed the following signals, three methine protons attached to O or N function at δ 4.25 (1H, d, J = 6 Hz, C_2–H), 4.15 (1H, dd, J = 4, and 6 Hz, C_3–H), and 3.92 (1H, m, C_4–H) and a methylene group showing the AB part of an ABX system at δ 2.96 (1H, dd, J = 4 and 17 Hz) and 2.79 (1H, dd, J = 8 and 17 Hz). The ^{13}C NMR spectrum showed six signals including those of two carbonyl carbons at δ 177.4, 175.9, 73.3, 71.7, 51.5 and 33.3 ppm. From these spectral data, **11** was concluded to be 4-amino-2,3-dihydroxyhexanedioic acid.

Based on the above studies and the ^{13}C NMR spectrum, the structure of **1** was proposed to be 6-[[1-(3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-

Table 3. ^{1}H NMR chemical shifts (δ) and multiplicities (J, Hz) of AI-77-C, D, F and G

Proton No	AI-77-C [1] 2	AI-77-D [1] 3	AI-77-F [1] 4	Proton No	AI-77-G [2] 5
C_3 H	4.66 (1H, m)	4.66 (1H, m)	4.68 (1H, m)	C_4 H	6.87 (1H, d) J=8
C_4 H	2.90 (2H, m)	2.90 (2H, m)	2.97 (2H, m)	C_5 H	7.33 (1H, dd) J=7, 8
C_5 H	6.77 [3] (1H. d) J=8	6.77 [3] (1H, d) J=8	6.80 [3] (1H, d) J=8	C_6 H	6.87 (1H, d) J=7
C_6 H	7.44 (1H, dd) J=8, 9	7.44 (1H, dd) J=8, 9	7.50 (1H, dd) J=8, 8	$C_{1'}$H	0.90 (3H, d) J=8
C_7 H	6.81 [4] (1H, d) J=9	6.81 [4] (1H, d) J=9	6.82 [4] (1H, d) J=8	$C_{2'}$H	0.90 (3H, d) J=8
$C_{1'}$H	0.82 [5] (3H, d) J=7	0.82 (3H, d) J=7	0.85 [5] (3H, d) J=6	$C_{3'}$H, $C_{4'}$H	1.25 - 1.8(3H, m)
$C_{2'}$H	0.89 [6] (3H, d) J=7	0.82 (3H, d) J=7	0.91 [6] (3H, d) J=6		
$C_{3'}$H, $C_{4'}$H	1.0 - 1.90 (3H, m)	1.0 - 1.90 (3H, m)	1.11 - 0.90 (3H, m)	$C_{5'}$H, $C_{6'}$H	3.64 - 4.2(2H, m)
$C_{5'}$H	4.18 (1H, m)	4.18 (1H, m)	4.18 (1H, m)	$C_{7'}$H	3.3 - 2.9 (2H, m)
$C_{8'}$H	4.28 (1H, dd) J=3, 6	4.28 (1H, dd) J=3,6	4.40 (1H, dd) J=3, 6	$C_{10'}$ H	4.35 (1H, d) J=6
$C_{9'}$H	4.61 (1H, m)	4.60 (1H, m)	5.35 (1H, dd) J=3, 2	$C_{11'}$H, $C_{12'}$H	3.64 - 4.2(2H, m)
$C_{10'}$H	4.32 (1H, m)	4.32 (1H, m)	6.24 (1H, dd) J=2, 6		
$C_{11'}$H	2.23 (1H, dd) J=2, 18 2.90 (1H, dd) J=9, 18	2.23 (1H, dd) J=2, 18 2.90 (1H, dd) J=9, 18	7.55 (1H, d) J=6	$C_{13'}$H	2.4 - 2.8 (2H, m)
6'-NH	8.36* d, J=7	8.36* d, J=7	7.78* d J=10.5		
C_8-OH	10.74* s	10.74* s	10.75* s		
-NHCOR	7.85* d, J=8	7.85* d, J=8			
-NHCOCH$_3$	1.69 (3H, s)				
-NHCOCH$_2$CH$_3$		1.94 (2H, q) J=8			
-NHCOCH$_2$CH$_3$		0.88 (3H, t) J=8			
$C_{8'}$-OH	6.18* d, J=6	6.18* d, J=6	6.17* d J=6		

*The signals disappeared on addition of D_2O.
[1]Recorded on 100 MHz spectrophotometer in DMSO-d_6.
[2]Recorded on 100 MHz spectrophotometer in D_2O.
[3,4,5,6]In the same column the assignments may be interchanged, respectively.

benzopyran - 3 - yl) - 3 - methylbutyl]amino] - 4,5-dihydroxy - 6 - oxo - 3 - aminohexanoic acid. This structure was subsequently confirmed by the single crystal X-ray analysis (Fig. 3) which also indicated that all the five asymmetric centers of **1** have the *S* configuration. The absolute configuration was assumed to be *S* by the X-ray anomalous dispersion method. In order to confirm the absolute configuration, leucine was obtained by the oxidative degradation of the diacetylated chromophore **13** with excess potassium permanganate in neutral aqueous solution,[8] followed by deprotection of the amino group by acid hydrolysis. After purification by column chromatography with Dowex 50 (H^+), the leucine was crystallized from water and shown to have L-(*S*)-configuration by $[\alpha]_D$ measurement. Therefore, the configuration at C-5′ was related to L-leucine and then all of the asymmetric centers of **1** were established to have *S* absolute stereochemistry.

AI-77-C, $C_{22}H_{28}N_2O_8$, was proved to be identical with the N-acetylated derivative **2** derived from **1**[3] by analyses of spectral data (exact mass, UV, IR and ^1H NMR) and melting point determination. The chemical shifts of the ^1H NMR spectrum are shown in Table 3.

AI-77-D, $C_{23}H_{30}N_2O_8$, showed almost similar UV and IR spectra to those of **2**. The ^1H NMR spectrum of AI-77-D **3** was almost identical with that of **2** except the presence of a methyl triplet at δ 0.88 (3H, J = 8 Hz) and a methylene quartet at δ 1.94 (2H, J = 8 Hz) which coupled each other in place of the methyl singlet at δ 1.69 due to $-NHCOCH_3$. AI-77-D was identified as the N-propionyl derivative **3** obtained by acylation of **1** by spectral analyses (exact mass, UV, IR and ^1H NMR).

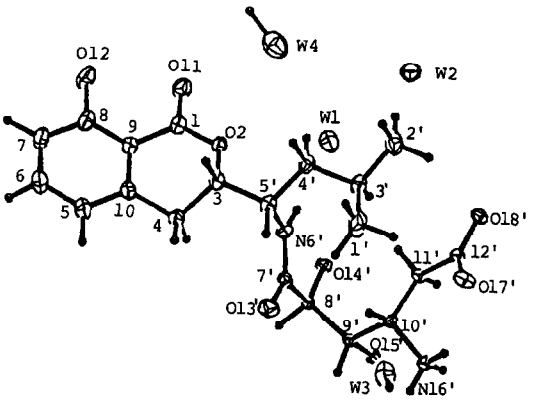


Fig. 3. The molecular structure of **1** drawn by ORTEP[9] program. Some of the hydrogen atoms bonded to solvent water molecules and hydroxyl groups were omitted because they were not shown by difference electron density maps.

AI-77-F **4**, $C_{20}H_{23}NO_7$ was indicated the presence of a butenolide ring in the IR spectrum (1790 and 1755 cm^{-1}). Unlike the ^1H NMR spectra of **2** or **3**, the spectrum of **4** was characterized by the appearance of signals of two olefinic protons at δ 6.24 (1H, dd) and 7.55 (1H, d) accompanied by the disappearance of signals for a methylene and a methine proton corresponding to C_{11}–H and C_{10}–H in **2**. Detailed spin decoupling experiments confirmed the structure of **4** as shown in Fig. 1. The ^{13}C NMR assignments of **4** are shown in Table 2. Compound **4**

Table 4. Comparison of the observed and calculated r^2 values to establish the absolute configuration

			Observed value		Calculated value
h	k	ℓ	r_o^2	e.s.d.	r_c^2
-18	1	0	1.05	0.05	1.02
-15	1	0	0.86	0.07	0.97
-14	1	0	0.97	0.05	0.97
-15	3	0	0.93	0.06	0.97
-3	3	0	1.01	0.04	1.02
-18	4	0	1.00	0.06	1.03
-7	4	0	0.94	0.04	0.97
-4	4	0	0.95	0.04	0.97
-4	11	0	0.87	0.84	0.93
-14	6	1	0.77	0.11	0.94
3	11	1	0.97	0.06	0.97
5	11	2	0.90	0.05	0.96
3	6	4	0.89	0.05	0.96
-8	10	4	0.78	0.10	0.94
-6	11	4	0.86	0.08	0.94
-10	9	5	0.74	0.15	0.91

$$r_o = |F_o(hk\ell)|/|F_o(h\bar{k}\ell)|, \quad r_c = |F_c(hk\ell)|/|F_c(h\bar{k}\ell)|$$

whose CD spectrum was identical with that of the natural product was produced concomitantly by methylation of the amino group on **1** with excess methyl iodide. Therefore, it is concluded that **4** have all *S* absolute stereochemistry as well as **1**. The ^{1}H NMR assignments of **4** are shown in Table 3.

AI-77-G **5** was obtained as a white powder with UV$\lambda_{max}^{H_2O}$ nm 245 sh and 300 and IRν_{max}^{KBr} 3500–3000 (broad), 1664 sh, 1620 sh and 1590 cm^{-1}. The UV spectrum of **5** agreed with that of **1** obtained after standing overnight in 0.1N NaOH solution. In comparison with the ^{1}H NMR spectrum (DMSO-d_6) of **1**, that of **5** revealed an upfield-shift (1.15 ppm) of the signal for a methine proton corresponding to C_3–H in the 1*H*-2-benzopyran-1-one skeleton. These findings suggest that hydrolysis of **1** at the ester bond of the δ-lactone ring leads to the formation of **5**. By evaporating a methanol solution of **5** with HCl to dryness, the γ-lactone derivative **6** was obtained in nearly 98% yield. Furthermore, **1** was obtained by hydrolysis of the γ-lactone derivative **6** with alkali under conditions such as pH 7–10. The molecular formula of **5** was determined to be $C_{20}H_{30}N_2O_9$ on the basis of the exact mass of **6** derived from **5** and its structure was elucidated as shown in Fig. 1. The completely salt-free sample of **5** could not be obtained due to the ease with which the δ-lactone ring formed. The ^{1}H NMR assignments of **5** are shown in Table 3.

EXPERIMENTAL

Melting points were determined in capillary tubes using a Yamato MP-21 silicone oil bath and are uncorrected. UV spectra were measured with a Shimadzu UV-210A spectrophotometer; CD spectra with a JASCO J-20 automatic recording spectropolarimeter; optical rotation with a JASCO DIP-4 automatic polarimeter; IR spectra with a Hitachi model 285 spectrophotometer and mass spectra with a JEOL model OISG-2 spectrometer. ^{1}H NMR spectra were recorded on a JEOL JNM-MH 100 or FX-200 or FX-400 spectrometer using Me_4Si as the internal standard and ^{13}C NMR spectra recorded on a JEOL FX-100 NMR spectrometer. TLC was carried out on plates coated with a 0.25 mm layer of silica gel 60 F_{254} (Merck). For amino acid analyses Avicel SF (Funakoshi) plates were used. For preparative separations, plates with a 2 mm layer thickness were used. Amino acids were analysed with a Hitachi 835 amino acid analyzer. Wacogel C-100 (40–100 mesh) was used for silicagel column chromatography.

AI-77-B **1**; white needles (CH_3OH), m.p. 139.5–140° (dec); $C_{20}H_{28}N_2O_8$, FD mass spectrum M + 1 425, showed UVλ_{max}^{MeOH} nm (ϵ) 246 (6250) and 314 (4450), CD (MeOH) $\Delta\epsilon_{325} = -0.42$, $\Delta\epsilon_{306} = -0.67$ and $\Delta\epsilon_{257} = -3.3$, Fluorescence excitation max 430 nm emission max 470 nm and IRν_{max}^{KBr} 3520 (sharp), ~3000 (broad), 2960, 2925, 1692 sh, 1680 sh, 1670 sh, 1662, 1625, 1590, 1570 sh, 1562 sh, 1520, 810, 795, 760, 705, 690 cm^{-1}. (Found: C, 56.28; H, 6.78; N, 6.58. Calc for $C_{20}H_{28}N_2O_8$: C, 56.60; H, 6.60; N, 6.60%.)

γ-Lactone derivative **6**, $C_{20}H_{26}N_2O_7$, (exact mass *m/z* 406.1736, calc 406.1733), showed UVλ_{max}^{MeOH} nm 246 and 314; IR(KBr) 1790, 1680 sh, 1660, 1625; ^{1}H NMR (CD_3OD) δ 7.46 (1H, dd, each J = 8 Hz, aromatic H), 6.84 and 6.81 (2H, 2d, each J = 8 Hz, aromatic H), 4.70 (1H, m, C_9–H), 4.56 (1H, m, C_3–H), 4.4–4.2 (2H, m, C_5–H and C_8–H), 3.40 (1H, m, C_{10}–H), 3.2–2.95 (3H, m, C_4–H and C_{11}–Ha), 2.70 (1H, dd, J = 4 and 18 Hz, C_{11}-Hb), 1.95–1.20 (3H, m, C_3-H and C_4-H), 0.96 and 0.92 (6H, 2d, each J = 8 Hz, $2CH_3$).

N-Acetylated derivative **2**; white needles (C_2H_5OH–H_2O), m.p. 210–211° (dec); $C_{22}H_{28}N_2O_8$ (exact mass *m/z* 448. 1830, calcd. 448. 1838); UVλ_{max}^{MeOH} 246 and 314 nm and IRν_{max}^{KRr} (carbonyl region) 1780 (γ-lactone), 1695, 1675, 1662, 1655 sh, 1638 sh, 1620 cm^{-1} (Found: C, 58.62; H, 6.58; N, 6.12. Calc for $C_{22}H_{28}N_2O_8$: C, 58.93; H, 6.25; N, 6.25%. ^{1}H NMR spectrum was identical with that of AI-77-C.

Diacetylated derivative **7**: white needles (C_2H_5OH–H_2O), m.p. 216–217° (dec); $C_{24}H_{30}N_2O_9$ (exact mass *m/z* 490.1939, calc 490.1943); UVλ_{max}^{MeOH} nm (ϵ) 246 (5410), 314 (3850; IRν_{max}^{KRr} (carbonyl region) 1805 and 1790 sh (γ-lactone), 1755 ester), 1685 sh, 1675 sh, 1660, 1655 sh, 1615 cm^{-1}; ^{1}H NMR (DMSO-d_6, 50°) δ 10.8 (s, phenolic OH), 8.38 (2d, J = 7 and 8 Hz, 2 CONH), 7.49 (1H, dd, each J = 8 Hz, aromatic H), 6.82 and 6.85 (each 1H, 2d, each J = 8 Hz, aromatic H), 5.18 (d, J = 4 Hz, C_8–H), 4.80–4.50 (2H, m, C_3–H and C_9–H), 4.55–4.00 (2H, m, C_5–H and C_{10}–H), 3.10–2.75 (3H, m, C_4H_{ab} and C_{11}–H_a), 2.30 (1H, dd, J = 18 Hz, C_{11}–H_b), 2.08 (3H, s, C_8–$OCOCH_3$), 1.58 (3H, s, C_{10}–$NHCOCH_3$), 1.85–1.10 (3H, m, C_4–H_{ab} and C_3–H), 0.90 and 0.80 (each 3H, 2d, each J = 6.5 Hz, C_1–H and C_2–H). (Found: C, 58.65; H, 6.46; N, 5.54. Calc for $C_{24}H_{30}N_2O_9$: C, 58.78; H, 6.12; N, 5.71%.)

Triacetylated derivative **8**; white needles (C_2H_5OH–H_2O), m.p. 170° (dec); $C_{26}H_{32}N_2O_{10}$ (exact mass *m/z* 532.2028, calc 532.2048); UVλ_{max}^{MeOH} nm (ϵ) 236 (7150) and 288 (1805); IRν_{max}^{KRr} (carbonyl region) 1790 (γ-lactone), 1775 (ester), 1755 (ester), 1730 (δ-lactone), 1690 sh, 1678, 1672, 1660 sh, 1650 sh, 1640 sh, 1613 cm^{-1}; ^{1}H NMR (DMSO-d_6, 50°C) δ 8.45 and 8.42 (2d, J = 7 Hz and J = 8 Hz, 2 CONH), 7.64 (1H, dd, J = 8 Hz, aromatic H), 7.27 and 7.12 (each 1H, 2d, each J = 8 Hz, aromatic H), 5.19 (1H, d, J = 4 Hz, C_8–H), 4.70–4.00 (4H, m, C_3–H, C_9–H, C_5–H and C_{10}–H), 3.12–2.80 (3H, m, C_4–H_{ab} and C_{11}–H_a), 2.40–2.20 (1H, m, C_{11}–H_b), 2.27 (3H, s, C_8–$OCOCH_3$), 2.09 (3H, s, C_8–$OCOCH_3$), 1.84–1.20 (3H, m, C_4–H_{ab} and C_3–H), 1.59 (3H, s, C_{10}–$NHCOCH_3$), 0.92 and 0.84 (each 3, 2d, each J = 6.5 Hz, C_1–H and C_2–H). (Found: C, 58.44; H, 6.39; N, 5.16. Calc for $C_{26}H_{32}N_2O_{10}$: C, 58.65; H, 6.02; N, 5.26%.)

AI-77-C was identical with N-acetylated derivative **2**.

AI-77-D; white needles (C_2H_5OH–H_2O); $C_{23}H_{30}N_2O_8$ (exact mass *m/z* 462.2007, calcd. 462.1999); UVλ_{max}^{MeOH} nm (ϵ) 246 (5700), 314 (4080); IRν_{max}^{KRr} (carbonyl region) 1790 (γ-lactone C=O), 1690 sh, 1680 sh, 1670 sh, 1665, 1650, 1635 cm^{-1}. (Found: C, 59.61; H, 6.40; N, 6.16. Calc for $C_{23}H_{30}N_2O_8$: C, 59.74; H, 6.49; N, 6.06%.)

AI-77-F **4**; white needles (C_2H_5OH–H_2O), m.p. 182–183°; $C_{20}H_{23}NO_7$ (exact mass *m/z* 389.1474, calc 389.1473): UVλ_{max}^{MeOH} nm (ϵ) 245 (6100) and 312 (4200); CD (dioxane) $\Delta\epsilon_{327} = -0.31$, $\Delta\epsilon_{303} = -0.47$ and $\Delta\epsilon_{258} = -4.5$; IR$\nu_{max}^{KBr}$ 3500–2900 (broad), 1790, 1755, 1735, 1685 sh, 1675 sh, 1670, 1655 sh, 1647 sh, 1635 sh, 1618, 805, 795 sh, 720 and 695 cm^{-1}. (Found: C, 61.73; H, 5.89; N, 3.62. Calc for $C_{20}H_{23}NO_7$: C, 61.70; H, 5.91; N, 3.60%.)

AI-77-G **5**; white powder, $C_{20}H_{30}N_2O_9$ (based on exact mass of **6**); UV$\lambda_{max}^{H_2O}$ 245 sh, 300 nm; IRν_{max}^{KBr} (carbonyl region) 1670 (sh), 1620 (sh), 1590 cm^{-1}; Na, 0.82 equivalent weight (atomic absorption analysis).

Conversion from **9** *to* **12**. A suspension of **9** (230 mg, 0.8 mmol) and guanidine carbonate (90 mg, 0.5 mmol) in ethanol (10 ml) was stirred overnight at room temp and then the solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, the insoluble guanidine hydrochloride were filtered and then filtrate was successively extracted with 0.01 N aqueous HCl (10 ml) and water (10 ml). After drying over Na_2SO_4, the organic layer was evaporated to dryness to give 160 mg (yield 80%) of **12**: UVλ_{max}^{MeOH} nm (ϵ) 230 sh (5740), 305 (3860); IRν_{max}^{KBr} 3600–3050 (broad), 1645 sh 1630, 1610 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.34 (s, phenolic OH), 9.6 (d, J = 6.5 Hz, amide), 7.21 (1H, dd, J = 7 and 8 Hz, C_3–H), 6.74 and 6.72 (2H, 2d, J = 7 and 8 Hz, C_2–H and C_4–H), 4.85 (1H, d, J = 6 Hz, C_6–OH), 3.90 (1H, m, C_6–H), 3.06–2.86 (2H, m, C_{5a}–H and C_7–H), 2.56 (1H, dd, J = 10 and 12 Hz, C_{5b}–H), 1.90–1.04 (3H, m, C_3–H and C_4–H), 0.80 and 0.69 (each 3H, each d, J = 6.5 and 7 Hz, C_1–H and C_2–H).

Acetylation of **9**. To a soln of **9** (2.0 g, 7 mmol) in pyridine (10 ml) was added acetic anhydride (2.14 g, 21 mmol) the soln was stirred for 2 h at 50°C. After evaporating the pyridine *in vacuo*, the residue was dissolved in 5 ml of

methanol and passed through a column packed with 50 ml of Amberlite XAD-2. The column was washed with 150 ml of 20% methanol and then with 150 ml of 50% methanol, followed by elution with 80% methanol. Fractions containing **13** were evaporated to dryness to give 2.30 g (91%) of **13** as a white powder: UVλ_{max}^{MeOH} 236 nm (ϵ 3300) and 290 (ϵ 1650); IRν_{max}^{KBr} 3430 (broad), 1770 (ester), 1735 (δ-lactone), 1660, 1610 cm^{-1}; ^1H NMR (CD_3OD) δ 7.56 (1H, dd, each J = 8 Hz, aromatic H), 7.21 and 7.04 (2H, 2d, each J = 8 Hz aromatic H), 4.50 (1H, m, C_3–H), 4.26 (1H, m, C_5–H), 2.98 (2H, m, C_4–H), 2.29 and 1.97 (each 3H, each s, each-$OCOCH_3$), 1.90–2.00 (3H, m, C_3–H and C_4–H), 0.97 and 0.92 (each 3H, 2d, each J = 6 Hz, C_1–H and C_2–H).

Oxidative degradation of **13** *(L-leucine from* **13**). Magnesium sulfate (4.32 g, 36 mmol), potassium permanganate (5.70 g, 36 mmol), water (180 ml) and **13** (1.00 g, 3 mmol) were added in this order to a round-bottomed flask fitted with a reflux condenser and the mixture was heated on an oil bath (105–110°) with stirring. After 1.5 h, the manganese dioxide was filtered off and washed with 100 ml of 50% aqueous acetone previously warmed to about 50°. The pale yellow filtrate was concentrated *in vacuo* to about 50 ml. The concentrate containing **14** was extracted twice with 50 ml of ethyl ether (50 ml × 2) and the aqueous layer was concentrated *in vacuo* to about 5 ml, which was heated in a sealed tube with 6 N HCl at 120°C for 16 h. The yield of leucine **15** in the hydrolysate was 23.3% by amino acid autoanalysis. The hydrolysate was evaporated *in vacuo* to dryness and dissolved in 5 ml of water. The solution was passed through a column packed with 15 ml of Dowex 50 (H^+). After washing successively with 30 ml of water and 100 ml of buffer-1*, the column was eluted with 100 ml of buffer-2*. The eluted fraction was monitored on TLC (Avicel SF plates) by spraying with a ninhydrin reagent. This treatment separated the objective compound **15** from the other ninhydrin positive minor product. Fractions containing **15** were collected and passed through another column packed with 15 ml of Dowex 50 (H^+). The column was washed successively with 100 ml each of water, 0.1 N HCl and 0.5 N HCl, followed by elution with 1N HCl.

Fractions containing only **15** were combined and neutralized with Dowex 2 (OH^-), filtered and evaporated to dryness to give 85 mg of crude **15** (purity 87% by amino acid autoanalysis, yield 19%). The crude sample was crystallized from water to give 31 mg of **15** as colorless glossy crystals: (Found; C 54.90, H 10.05, N 10.66. Calc for $C_6H_{13}NO_2$; C 54.90, H 10.00, N 10.68%): $[\alpha]_D^{23} = +15.4$ (c 1.57, 6 N HCl). The optical rotation of authentic L-leucine, + 15.2 (c 1.58, 6 N HCl), was obtained under the same conditions as for **15**. Other analytical data (m.p., IR and ^1H NMR) of **15** agreed with those of authentic L-leucine.

composition	*buffer-1	**buffer-2
distilled water	700 ml	700 ml
lithium citrate ($4H_2O$)	9.80 g	9.80 g
lithium chloride	2.12 g	6.36 g
citric acid (H_2O)	34.00 g	12.00 g
ethanol	40 ml	30 ml
thiodiglycol	5 ml	5 ml
caproic acid	0.1 ml	0.1 ml
Total	1000 ml	1000 ml
pH	3.00	3.70

Synthesis of **3** *from* **1**. A stirred, ice-cooled soln of **1** (4.24 g, 10 mmol) in pyridine (25 ml) was treated with propionic anhydride (2.04 g, 20 mmol) for 2 h. After evaporating *in vacuo*, the residue was crystallized from ethanol/water to give 2.6 g (56%) in **3** of the form of white needles. All the analytical data (UV, IR and ^1H NMR) were identical with those of AI-77-D **3**.

Synthesis of **4** *and N-methylated* **6** *from* **1**. **1** (4.24 g 10 mmol) and N,N-dimethylformamide (20 ml) were placec in 100 ml glass pressure container. Methyl iodide (6 ml 96 mmol) and triethylamine (4.5 ml, 30 mmol) were addec to it and then the container was closed and shaken vigorously for 2 h at room temperature. Two portions of 6 m each of methyl iodide were added to the container at 2 h intervals. After vigorous shaking of the container for 24 h the mixture was evaporated. The residue was dissolved ir 50 ml of tetrahydrofuran and the solution was filtered anc evaporated to dryness. The residue was dissolved again ir 30 ml of tetrahydrofuran containing 0.5 ml HCl and the solution was evaporated to dryness to complete the formation of the butenolide and γ-lactone rings. The resulting powder was dissolved in 5 ml of methanol and applied to a column packed with 200 g of silica gel slurried in chloroform. The column was washed with 200 ml of chloroform and eluted with $CHCl_3$/MeOH (15:1). Fractions containing only **4** were collected, and evaporation to give 350 mg of 4 (yield 9.0%) in a form of white needles, which was recrystallized from ethanol/water. (Found: C, 61.73; H, 5.89 N, 3.62. Calc for $C_{20}H_{23}NO_7$; C, 61.70; H, 5.91; N, 3.60%) All the analytical data (m.p., UV, CD, IR and ^1H NMR) agreed with those of **4** isolated from the culture broth of *Bacillus pumilus AI-77*. The column was then eluted with $CHCl_3$/MeOH (10:1) and fractions containing only **16** were evaporated to dryness. The resulting powder was dissolved in 50 ml of 0.01 N HCl. The solution was adjusted to pH 7.5 with 0.5 M $NaHCO_3$ and extracted with ethyl acetate (30 ml × 2). The organic layer was dried over Na_2SO_4 and evaporated *in vacuo* to give 410 mg of N-methylated **6** (yield 9.8%) in salt-free form: UVλ_{max}^{MeOH} 247 and 315 nm; IRν_{max}^{KBr} (carbonyl region) 1780, 1690 sh, 1675, 1655 sh and 1620 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.40 (1H, dd, J = 8 Hz, aromatic H), 6.79 and 6.74 (2H, 2d, each J = 8 Hz aromatic H), 4.70 (1H, m, C_9–H), 4.64 (1H, m, C_3–H), 4.38 (1H, d, J = 2 Hz, C_8–H), 4.32 (1H, m, C_5–H), 3.30(1H, m C_{10}–H), 3.10–2.80 (3H, m, $C_{11'a}$–H and C_4–H), 2.35 and 2.20 (1H, overlapping with $-NHCH_3$, $C_{11'b}$–H), 2.28 (3H, s, $-NHCH_3$), 2.05 – 1.10 (3H, m, C_3–H and C_4–H), 0.98 and 0.94 (each 3H, 2d, each J = 6 Hz, C_1–H and C_2–H). (Found: C, 60.08; H, 6.64; N, 6.62. Calc. for $C_{21}H_{28}N_2O_7$: C; 60.00 H, 6.71; N, 6.67%.)

Conversion of **5** *to* **1**. An aqueous solution (20 ml) of **5** (500 mg, 1.1 mmol), was evaporated with HCl (1 ml) *in vacuo* to dryness and the residue was dissolved in methanol (20 ml), and then re-evaporated to give **6**. To the dried residue dissolved in methanol (20 ml), was added dropwise 0.1 N aqueous NaOH solution with stirring to a pH of 9.0. The pH was held at 9.0 by the addition of alkali until the spot of **6** could no longer be detected on TLC to open the γ-lactone ring, and then the pH was adjusted to 6.5 with 0.1 N HCl. The resulting solution was passed through a column (Amberlite XAD-2, 50 ml). The column was washed with methanol/water (1:4, 100 ml) and eluted with methanol (100 ml). The eluate was concentrated *in vacuo* to about 20 ml to give 220 mg of **1** (47%) in a form of white needles: m.p. 139–140° (dec). All the spectral data (UV, IR and ^1H NMR) were identical with those of the authentic sample of **1**.

X-Ray crystallographic study of AI-77-B **1**. Crystals grown in aqueous solutions were used for X-ray diffraction study using Cukα radiation. The crystal data are: AI-77-B tetrahydrate, $C_{20}H_{28}N_2O_8 \cdot 4H_2O$, FW = 496.5, monoclinic, space group $P2_1$, $Z = 2$, $a = 15.902(7)$, $b = 10.161(5)$, $c = 7.571(4)$ Å, $\beta = 95.28(5)°$, $V = 1218$ Å^3, $D_m = 1.356$ Mgm^{-3}, $D_c = 1.354$ Mgm^{-3}. The structure shown in Fig. 3 was determined and refined on the basis of 2655 observed structure factors (90% of those involved within $2\theta 165$) to an R value of 0.054 including 27 hydrogen atoms. Nine of those bonded to the oxygen atoms of crystal water and hydroxyl groups were not included. To determine the absolute configuration, the values of $r_c = |F_c(hkl)|/|F_c(h\bar{k}\bar{l})$ were calculated by taking into account the anomalous dispersion effect of C, N and O atoms for Cukα radiation.

Table 4 shows a comparison of r_c^2 with the observed r_0^2 values for all the 11 Friedel pairs giving the largest or smallest r_c which indicates the configuration presented in Fig. 3.

Supplementary material available. Crystallographic data including positional and thermal parameters as well as bond distance and angle calculation have been deposited with the Cambridge Crystallographic Data Centre (CCDC) in England.

Acknowledgements—The authors are indebted to Prof. T. Goto (Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University) and Prof. Y. Yamada (Tokyo College of Pharmacy) for valuable suggestions. We thank Mr. I. Nagoya (NMR spectra) and Mr. K. Tamura (Mass spectra) in our laboratory.

REFERENCES

[1]Y. Shimojima, H. Hayashi, T. Ooka and M. Shibukawa, *Agric. Biol. Chem.* **46**, 1823 (1982).

[2]Y. Shimojima, H. Hayashi, T. Ooka, M. Shibukawa and Y. Iitaka, *Tetrahedron Letters* **23**, 5435 (1982).

[3]Y. Shimojima and H. Hayashi, *J. Med. Chem.* **26**, 1370 (1983).

[4]J. Blair and G. T. Newbold, *J. Chem. Soc.* 2871 (1955); M. Sasaki, Y. Kaneko, K. Oshita, H. Takamatsu, Y. Asao and T. Yokotsuka, *Agric. Biol. Chem.* **34**, 1296 (1970).

[5]H. Okazaki, T. Kishi, T. Beppu and K. Arima, *J. Antibiotics* **28**, 719 (1975).

[6]M. A. W. Eaton and D. W. Hutchinson, *Tetrahedron Letters* 1337 (1971).

[7]A. I. Virtanen and T. Ettala, *Acta. Chem. Scand.* **11**, 182 (1957).

[8]J. P. English, R. C. Clapp, Q. P. Cole, I. F. Halverstadt, J. O. Lampen and R. O. Roblin, *J. Am. Chem. Soc.* **67**, 295 (1945).

[9]C. K. Johnson, ORTEP. Report ORNL-3794, Oak Ridge National Laboratory, Oak Ridge, Tennessee. (1965).