5-*N*-ACETYLARDEEMIN, A NOVEL HETEROCYCLIC COMPOUND WHICH REVERSES MULTIPLE DRUG RESISTANCE IN TUMOR CELLS

II. ISOLATION AND ELUCIDATION OF THE STRUCTURE OF 5-*N*-ACETYLARDEEMIN AND TWO CONGENERS

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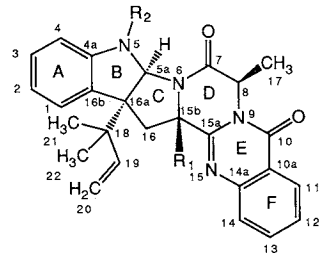
A family of novel compounds has been detected and isolated following an assay for the attenuation of multiple drug resistance in tumor cells from the fermentation broth and mycelia of a strain of *Aspergillus fischeri* which we have designated var. *brasiliensis*. The structures of three components were determined employing 1-D and 2-D homonuclear and heteronuclear NMR spectroscopy and mass spectrometry. The structure of 5-*N*-acetylardeemin was confirmed by single crystal X-ray diffraction. These compounds are most closely structurally related to asperlicin E[1].

In the course of screening for compounds which reverse multi-drug resistance to antitumor antibiotics, the ardeemins were isolated from a new strain of *Aspergillus fischeri* (designated var. *brasiliensis*). A companion paper[2] describes the taxonomy and fermentation of the producing organism and the biological activities of ardeemin (**1**) and 5-*N*-acetylardeemin (**2**). This paper will describe the isolation and structures of these novel heterocyclic compounds.

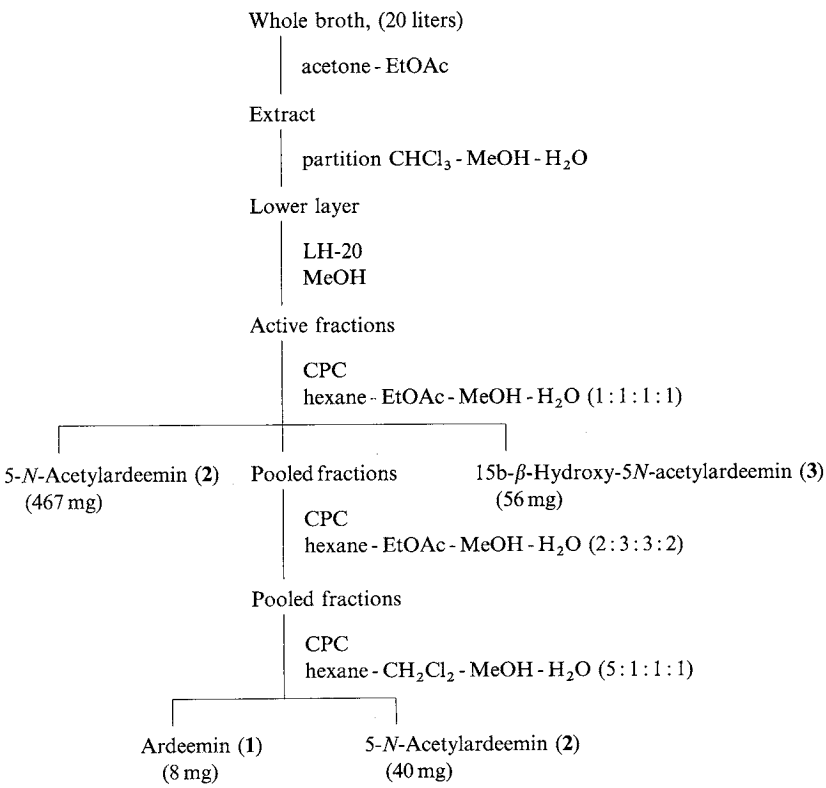
Isolation of the Ardeemins

Upon completion of the fermentation, 8 liters of acetone was added to 20 liters of whole broth and the mixture was stirred for 3 hours (See Fig. 1). To this was added 16 liters of ethyl acetate and the resultant mixture was stirred for 1 hour at which time the organic layer was removed. An additional 16 liters of ethyl acetate was added to the aqueous acetone and the mixture was stirred for 1 hour. The second ethyl acetate layer was removed and added to the first, and the combined ethyl acetate extracts were concentrated to an aqueous suspension which was partitioned between equal volumes of water - chloroform - methanol (1.5 liters of each). The upper layer from this partition was extracted twice with further volumes of chloroform (0.7 liters each time) and these chloroform extracts were combined with the original lower layer and concentrated to an oil. This oil was chromatographed over a Sephadex LH-20 column (8 cm × 80 cm) eluted with methanol. Active fractions from this column were combined and concentrated to an oil. This oil was



1 Ardeemin $R_1=H$, $R_2=H$
2 5-*N*-Acetylardeemin $R_1=H$, $R_2=Ac$
3 15b-β-Hydroxy-5-*N*-acetylardeemin $R_1=OH$, $R_2=Ac$

Fig. 1. Isolation of the ardeemins.



subjected to countercurrent chromatography on an Ito multi-layered coil planet centrifuge (CPC) in a solvent system of hexane - ethyl acetate - methanol - water (1 : 1 : 1 : 1) with the upper phase stationary. Active fractions were combined based upon their thin layer chromatographic behavior to yield pure 5-*N*-acetylardeemin (**2**) (467 mg), 15b-β-hydroxy-5-*N*-acetylardeemin (**3**) (56 mg) and a pool of mixed fractions. This pool was subjected to countercurrent chromatography on an Ito multi-layered coil planet centrifuge in a solvent system of hexane - ethyl acetate - methanol - water (2 : 3 : 3 : 2) with the lower phase stationary. Active fractions from this chromatography were combined and subjected to countercurrent chromatography in a solvent system of hexane - methylene chloride - methanol - water (5 : 1 : 1 : 1) with the lower phase stationary. Fractions were combined based upon their behavior in thin layer chromatography and fraction pools were concentrated to yield pure **1** (8 mg) and **2** (40 mg).

Structure Elucidation of the Ardeemins

Structure Elucidation of 5-*N*-Acetylardeemin (**2**)

A high resolution electron impact mass spectrum of **2** (5-*N*-acetylardeemin) gave an exact mass of $m/z=468.2168$ for a molecular formula of $C_{28}H_{28}N_4O_3$ (calculated exact mass: $m/z=468.2161$). A ^{13}C NMR spectrum and DEPT[3] contained 28 carbon signals with 28 attached protons demonstrating that there were no exchangeable protons in the structure of **2**.

A modified COSY experiment indicated the presence of two isolated aromatic spin systems with coupled protons at δ 7.38, 7.17, 7.34 and 8.00 (positions 1 ~ 4, respectively, in structure **2**), and δ 8.24, 7.47, 7.76 and 7.65 (positions 11 ~ 14, respectively). An HMQC experiment[4] (See Table 1) allowed the

extension of these to two 1,2-disubstituted benzene systems. The first, with proton signals at δ 7.34 and δ 7.38 (See Table 1) on carbons at δ 129.1 (C-3) and δ 124.4 (C-1), respectively, long range coupled to a quaternary carbon signal at δ 142.9 (C-4a) whose chemical shift suggests the attachment of a N atom at this position, and a proton signal at δ 7.17 on a carbon at δ 124.5 (C-2) long range coupled to a quaternary carbon signal at δ 132.2 (C-16b). The second 1,2-disubstituted benzene is defined by proton signals at δ 8.24 and δ 7.76 on carbons at δ 126.8 (C-11) and δ 134.6 (C-13), respectively, long range coupled to a quaternary carbon signal at δ 147.0 (C-14a) whose chemical shift suggests the attachment of a N atom at this position, and proton signals at δ 7.47 and δ 7.65 on carbons at δ 127.2 (C-12) and δ 127.1 (C-14), respectively each long range coupled to a quaternary carbon signal at δ 120.4 (C-10a). Further, the proton signal at δ 8.24 shows long range coupling to a quaternary carbon signal at δ 159.6 (C-10) indicating the attachment of an amide carbonyl through the carbon at the second substituted position of this benzene ring.

A monosubstituted olefin system with coupled proton signals at δ 5.11 and δ 5.15 on a carbon at δ 114.5 (C-20) and the third proton at δ 5.84 on a carbon at δ 143.0 (C-19) show long range coupling to a quaternary carbon signal at δ 40.3 (C-18). Two singlet methyl signals at δ 1.01 and δ 1.20 are long range coupled to this same quaternary carbon signal thus defining an isoprene moiety C-18 through C-22 as shown in **2**. In addition, the methyl singlet signals at δ 1.01 and δ 1.20 show long range coupling to a quaternary carbon signal at δ 60.9 (C-16a) to which a methylene with proton signals at δ 3.01 and δ 2.67 (position 16) are also long range coupled. This methylene is part of an isolated methylene-methine spin system (positions 15b, 16) with the methine proton and carbon signals at δ 4.41 and δ 58.2, respectively. This methine proton shows long range coupling to a quaternary carbon signal at δ 150.2 whose chemical shift suggests an amidine functionality. Further, the methylene proton signals at δ 3.01 and δ 2.67 in this spin system show long range coupling to the quarternary carbon signal at δ 132.2 (C-16b) in one of the 1,2-disubstituted benzenes and to carbon signal at δ 58.2 (C-15b) and δ 79.4 (C-5a).

The proton and carbon signals of a coupled methyl-methine pair at δ 1.42 and δ 17.0 (C-17) and δ 5.37 and δ 53.4 (C-8), respectively, shows long range coupling to an amide carbonyl with a chemical shift of δ 165.9 (C-7). The methine signal at δ 5.37 further shows coupling to the amide carbon signal at δ 159.6 (C-10) which was previously established as connected to one of the 1,2-disubstituted benzenes (C-10a through C-14a).

Finally, an isolated acetate group with long range coupling (see Table 1) observed only between the acetate proton signal at δ 2.63 and carbon signals at δ 23.5 (N-5 Acetate C-2) and δ 169.9 (N-5 Acetate C-1), respectively, is positioned at N-5.

The molecular formula of 5-*N*-acetylardeemin (**2**), $C_{28}H_{28}N_4O_3$, requires 17 units of unsaturation, eight of which are accomodated in the two substituted benzene rings. Three more are accounted for in carbonyl functions and one in the olefin of the isoprenoid unit. The remaining five are accounted for by formulating 5-*N*-acetylardeemin with an amidine function and four more rings. This reasoning, the extensive NMR coupling data described above and biosynthetic considerations, led to the proposed formula **2** for 5-*N*-acetylardeemin.

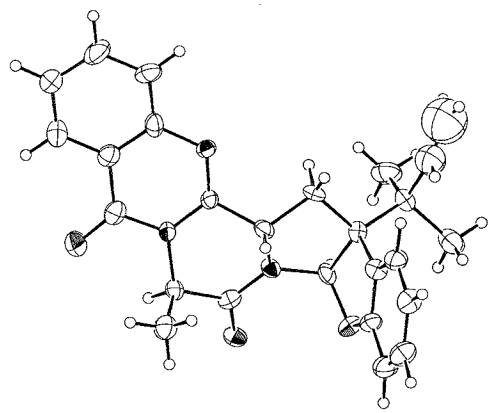
An X-ray structure determination of **2** assigned the relative configuration for 5-*N*-acetylardeemin and confirmed the structure. The compound was crystallized from methanol. The crystallographic data are summarized as follows: Orthorhombic, $P2_12_12_1$, $a=16.50(2)$, $b=23.19(1)$, $c=6.21(2)$Å, $V=2377(2)$Å^3, $Z=4$; $D_{calc}=1.28$ g/cm^3. The structure was solved with SHELXS76[5]. The structure was

Table 1. ^{13}C and ^{1}H NMR assignments for 5-*N*-acetylardeemin (**2**) (in $CDCl_3$).

Carbon No.	C shift	Attached proton	Long range coupled	Carbon No.	C shift	Attached proton	Long range coupled
1	124.4	7.38	7.34	15a	150.2		4.41, 2.67, 5.37
2	124.5	7.17		15b	58.2	4.41	3.01, 2.67
3	129.1	7.34	7.38	16	37.3	3.01, 2.67	4.41
4	119.4	8.00	7.17				
4a	142.9		7.34, 7.38	16a	60.9		3.01, 2.67, 1.01, 1.20
5a	79.4	6.08	3.01	16b	132.2		7.17, 3.01, 2.67
7	165.9		1.42, 5.37	17	17.0	1.42	5.37
8	53.4	5.37	1.42	18	40.3		5.84, 5.15, 5.11, 1.01, 1.20
10	159.6		8.24, 5.37	19	143.0	5.84	5.15, 1.01, 1.20
10a	120.4		7.65, 7.47	20	114.5	5.15, 5.11	
11	126.8	8.24	7.76				
12	127.2	7.47	7.65	21	22.4	1.20	5.84
13	134.6	7.76	8.24	22	23.1	1.01	5.84
14	127.1	7.65	7.47	N-5 Ac-C1	169.9		2.63
14a	147.0		8.24, 7.76	N-5 Ac-C2	23.5	2.63	

refined by the full-matrix least-squares method[6]. After isotropic refinement, the Fourier difference map showed one molecule of solvent (methanol). The final full-matrix least squares (anisotropic for non-hydrogen atoms and calculated hydrogen atoms) converged with a R factor of 0.065 (Rw= 0.072, S=1.98). Fig. 2 is an ORTEP[7] drawing of **2** with thermal ellipsoids scaled at the 30% probability level for non-hydrogen atoms. Hydrogen atoms are located at calculated positions and are drawn with β set to 1 Å^3. The solvent molecule (methanol) is not shown.

Fig. 2. ORTEP structure of 5-*N*-acetylardeemin.



Structure Elucidation of Ardeemin (**1**)

A desorption chemical ionization (DCI) mass spectrum of **1**, using ammonium ions, indicated a molecular weight for the parent molecule of m/z=426. A ^{13}C NMR spectrum and DEPT indicated the presence of 26 carbon atoms with 25 attached protons. The most notable difference between the ^{13}C NMR spectra of **1** and **2** (see Table 2) is the absence in **1** of two carbon signals assigned as the N-5 acetate group in **2**. (The acetate methyl signal which apears at δ 2.63 in the ^{1}H NMR (see Table 3) of **1** is also absent in the ^{1}H NMR spectrum of **2**.) Additional differences between the ^{13}C NMR spectra of these two compounds appear for carbons assigned in **2** at C-4, C-4a, C-2 and C-16b. These data can be explained by an assignment for **1** as shown in which the *ortho*, *para* and C-1 points of attachment for N-5 would be expected to shift from their position in the spectrum of **2** as a result of deacetylation at the 5-N position.

Structure Elucidation of 15b-β-Hydroxy-5-*N*-acetylardeemin (**3**)

The DCI (NH_4^+) mass spectrum of **3** indicated a molecular weight of m/z=484. A ^{13}C NMR

Table 2. ^{13}C NMR spectra of the ardeemins (in $CDCl_3$).

Carbon No.	1	2	3
1	125.1	124.4	124.9
2	118.8	124.5	124.6
3	129.0	129.1	129.8
4	109.2	119.4	120.6
4a	149.7	142.9	142.5
5a	77.7	79.4	80.0
7	166.6	165.9	168.2
8	53.2	53.4	54.6
10	160.0	159.6	159.9
10a	120.6	120.4	120.8
11	126.9	126.8	126.9
12	127.1	127.2	127.7
13	134.6	134.6	134.6
14	127.1	127.1	127.7
14a	147.1	147.0	146.9
15a	150.8	150.2	150.3
15b	58.0	58.2	89.3
16	38.1	37.3	44.2
16a	61.7	60.9	59.3
16b	128.9	132.2	133.5
17	16.8	17.0	18.6
18	41.0	40.3	40.6
19	143.5	143.0	142.8
20	114.5	114.5	115.1
21	22.5	22.4	22.0
22	22.8	23.1	22.4
N-5 Ac-C1		169.9	170.3
N-5 Ac-C2		23.5	23.6

Table 3. ^1H NMR spectra of the ardeemins (in $CDCl_3$).

H on C No.	1	2	3
1	7.27	7.38 (br d, 1H, J=7.6 Hz)	7.43
2	6.80	7.17 (br t, 1H, J=7.6 Hz)	7.25
3	7.13	7.34 (dd, 1H, J=8.1, 7.6 Hz)	7.45
4	6.66	8.00 (very broad signal, 1H)	8.09
5a	5.60	6.08 (very broad signal, 1H)	6.10
8	5.44	5.37 (q, 1H, J=7.1 Hz)	5.38
11	8.28	8.24 (dd, 1H, J=8.1, 1.5 Hz)	8.29
12	7.49	7.47 (br dd, 1H, J=8.1, 7.1 Hz)	7.53
13	7.76	7.76 (ddd, 1H, J=8.1, 7.1, 1.5 Hz)	7.80
14	7.68	7.65 (br d, 1H, J=8.1 Hz)	7.74
15b	4.52	4.41 (dd, 1H, J=10.7, 5.6 Hz)	
16α	2.92	3.01 (dd, 1H, J=12.7, 5.6 Hz)	3.18
16β	2.75	2.67 (dd, 1H, J=12.7, 10.7 Hz)	3.11
17	1.49	1.42 (d, 3H, J=7.1 Hz)	1.68
19	6.03	5.84 (dd, 1H, J=17.3, 10.7 Hz)	5.83
20α	5.12	5.11 (br d, 1H, J=10.7 Hz)	5.16
20β	5.15	5.15 (br d, 1H, J=17.3 Hz)	5.18
21	1.19	1.20 (s, 3H)	1.22
22	1.04	1.01 (s, 3H)	1.03
N-5 Acetate		2.63 (br s, 3H)	2.68

spectrum and DEPT of **3** indicated the presence of 28 carbon atoms with 27 attached protons. The most notable difference between the ^1H NMR spectra of **2** and **3** is the absence in **3** of the proton signal at δ 4.41 in **2** which was assigned to the proton on position 15b. Additionally, signals for the two protons on carbon 16 which appear as two doublets of doublets in the spectrum of **2** (δ 3.01 (dd, 1H, J=12.7, 5.6 Hz) and δ 2.67 (dd, 1H, J=12.7, 10.7 Hz)), show in the spectrum of **3** (δ 3.18 (d, 1H, J=14.2 Hz) and δ 3.11 (d, 1H, J=14.2 Hz)) as a pair of doublets with only the large geminal coupling constants to one another. These data are most consistent with an hydroxylation at position 15b in **3** which would yield a molecular formula of $C_{28}H_{28}N_4O_4$ consistent with the deduced molecular weight of m/z=484. The relative stereochemistry at C-15b was determined by an analysis of NMR data in DMSO. Assignment of ^1H NMR shifts for **3** were determined *via* a COSY experiment

Table 4. ^1H NMR spectrum of 15b-β-hydroxy-5-*N*-acetylardeemin (in DMSO).

Carbon No.	Proton chemical shift
1	7.41 (dd, 1H, J=7.8, 1.2 Hz)
2	7.13 (td, 1H, J=7.8, 0.8 Hz)
3	7.24 (td, 1H, J=7.8, 1.2 Hz)
4	7.80 (very broad signal, 1H)
5a	6.04 (very broad signal, 1H)
8	5.03 (q, 1H, J=7.0 Hz)
11	8.18 (dd, 1H, J=8.2, 1.6 Hz)
12	7.58 (br dd, 1H, J=8.2, 7.1 Hz)
13	7.87 (ddd, 1H, J=8.1, 7.1, 1.6 Hz)
14	7.76 (br d, 1H, J=8.1 Hz)
16α	3.09 (d, 1H, J=13.7 Hz)
16β	2.97 (d, 1H, J=13.7 Hz)
17	1.51 (d, 3H, J=7.0 Hz)
19	5.83 (dd, 1H, J=17.6, 11.0 Hz)
20α	5.08 (br d, 1H, J=11.0 Hz)
20β	5.05 (br d, 1H, J=17.6 Hz)
21	0.91 (s, 3H)
22	1.08 (s, 3H)
N-5 Acetate	2.55 (br s, 3H)

and are assigned as in Table 4. A phase sensitive CAMEL[8] experiment defined the relative stereochemistry at C-15b. An NOE was observed between the C-15b hydroxyl proton at δ 6.44 ppm and the methyl proton signal at δ 1.51 (position 17) indicating that this C-8 methyl and the C-15b hydroxyl group must be *cis* to one another.

The ardeemins are structurally related to several other fungal metabolites which can be formally regarded as being derived from tryptophan and other amino acids. Perhaps the closest structural analog is asperlicin E with a similar skeleton derived from tryptophan and two molecules of anthranilic acid[9]. In contrast, the ring system of the ardeemins would appear to be derived from one molecule each of tryptophan, anthranilic acid and alanine leading to a six membered ring D rather than seven membered ring as in asperlicin E. The ardeemins also have an attached isoprenyl group as is found in oxaline and neooxaline[10], amauromine[11] and aszonalenin and LL-S490β[12]. The structural difference in these last two compounds is analogous to that between **1** and **2**. The position of oxygenation in **3** (15b-β-hydroxy-5-*N*-acetylardeemin) is novel but some modest precedence for oxygenation at this carbon is supplied by fumitremorgin B[13].

Experimental

General Procedures

Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter in a 10 cm cell. Melting points were determined on a Hoover Unimelt and are reported uncorrected. Rf values reported were acquired on Merck Kieselgel 60 F_{254} TLC plates and were visualized using ceric sulfate spray reagent[14]. Mass spectra were measured on a Kratos MS-50 mass spectrometer. Ultraviolet spectra were recorded on a Perkin-Elmer Lambda 3B UV-visible spectrophotometer and infrared spectra on a Nicolet model 60SX FT-IR attached to a Nicolet computer. NMR spectra were acquired on a General Electric GN500 spectrometer.

Characterization of the Compounds

Ardeemin (**1**), $[\alpha]_D^{25} = -92°$ (c=0.24, MeOH) having a Rf value of 0.69 in EtOAc (Rf=0.56 in $CHCl_3$-MeOH (97:3) and Rf=0.56 in toluene-acetone (2:1) was a white amorphous solid. UV (MeOH) λ_{max} (nm) 210 (ε=12,600), 224 (ε=7,400), 268 (ε=2,700), 274 (ε=2,600), 302 (ε=1,700), and 314 (ε=2,600). These bands were unchanged with the addition of acid or base. IR ($CDCl_3$) ν_{max}: 2970, 2930, 2860, 1672, 1606, 1511, 1467, 1397, 1305, 1289, 1305, 1288, 1247, 1227, 1215, 1176, 1149, 1114, 1084, 1060, 1026 cm^{-1}.

5-*N*-Acetylardeemin (**2**), $[\alpha]_D^{25} = -33°$ (c=0.78, MeOH) having a Rf value of 0.69 in EtOAc (Rf=0.56 in $CHCl_3$-MeOH (97:3) and Rf=0.56 in toluene-acetone (2:1) was a white solid with a melting point of 226~228 (uncorrected). UV (MeOH) λ_{max} (nm) 210 (ε=10,700), 224 (ε=10,700), 264 (ε=4,300), 274 (ε=4,000), 302 (ε=1,700) and 314 (ε=1,360). These bands were unchanged with the addition of acid or base. IR ($CDCl_3$) ν_{max}: 2978, 1684, 1605, 1477, 1468, 1433, 1402, 1388, 1335, 1307, 1287, 1246, 1160, 1150, 1048, 1035 cm^{-1}.

15b-β-Hydroxy-5-*N*-acetylardeemin (**3**), $[\alpha]_D^{25} = -245°$ (c=0.21, MeOH) having a Rf value of 0.64 in EtOAc (Rf=0.50 in $CHCl_3$-MeOH (97:3) and Rf=0.39 in toluene-acetone (2:1) was a white amorphous solid. UV (MeOH) λ_{max} (nm) 210 (ε=13,000), 224 (ε=8,000), 268 (ε=2,600), 274 (ε=2,400), 302 (ε=1,200) and 314 (ε=1,200). IR ($CDCl_3$) ν_{max}: 3490, 2978, 2930, 1678, 1605, 1475, 1465, 1400, 1382, 1338, 1302, 1242, 1160, 1118, 1085, 1010 cm^{-1}.

References

1) LIESCH, J. M.; O. D. HENSENS, D. L. ZINK & M. A. GOETZ: Novel cholecystokinin antagonists from *Aspergillus alliaceus*. II. Structure determination of asperlicins B, C, D, and E. J. Antibiotics 41: 878~881, 1988

2) KARWOWSKI, J. P.; M. JACKSON, S. K. KADAM, R. R. RASMUSSEN, P. E. HUMPHREY, M. L. MAUS & W. L. KOHL: 5-*N*-Acetylardeemin, a novel heterocyclic compound which reverses multiple drug resistance in tumor cells. I. Taxonomy and fermentation of the producing organism and biological activity. J. Antibiotics 46: 374~379, 1993
3) MÜLLER, L.: Sensitivity enhanced detection of weak nuclei using heteronuclear multiple quantum coherence. J. Am. Chem. Soc. 101: 4481~4484, 1979
4) BAX, A. & M. F. SUMMERS: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986
5) SHELDRICK, G. M. (*Ed.*): Program for Crystal Structure Determination. University of Cambridge, 1976
6) TEXSAN: TEXSAN Structure analysis package. Molecular Structure Corporation, 1985
7) JOHNSON C. K. (*Ed.*): ORTEPII. Oak Ridge National Laboratory, USA, 1976
8) BOTHNER-BY, A. A.; R. L. STEPHENS, J. LEE, C. D. WARREN & R. W. JEANLOZ: Structure determination of a tetrasaccharide: Transient nuclear Overhauser effects in the rotating frame. J. Am. Chem. Soc. 106: 811~813, 1984
9) HOUCK, D. R.; J. ONDEYKA, D. L. ZINK, E. INAMINE, M. A. GOETZ & O. D. HENSENS: On the biosynthesis of asperlicin and the directed biosynthesis of analogs in *Aspergillus alliaceus*. J. Antibiotics 41: 882~891, 1988
10) KONDA, Y.; M. ONDA, A. Hirano & S. ŌMURA: Oxaline and neoxaline. Chem. Pharm. Bull. 28: 2987~2993, 1980
11) TAKASE, S.; Y. KAWAI, I. UCHIDA, H. TANAKA & H. AOKI: Structure of amauromine, a new hypotensive vasodilator produced by *Amauroascus* sp. Tetrahedron 41: 3037~3048, 1985
12) KIMURA, Y.; T. HAMASAKI & H. NAKAJIMA: Structure of aszonalenin, a new metabolite of *Aspergillus zonatus*. Tetrahedron Lett. 23: 225~228, 1982
13) YAMAZAKI, M.; K. SASAGO & K. MIYAKI: The structure of fumitremorgin B (FTB), a tremorgenic toxin from *Aspergillus fumigatus* Fres. J. Chem. Soc. Chem. Comm. 1974: 408~409, 1974
14) KREBS, K. G.; D. HEUSSER & H. WIMMER: Spray Reagents in Thin Layer Chromatography. *Ed.*, E. STAHL, pp. 861, New York: Springer-Verlag