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Ammocidin, a New Apoptosis Inducer in Ras-dependent Cells from *Saccharothrix* sp.

II. Physico-chemical Properties and Structure Elucidation

RYO MURAKAMI, TAIJIRO TOMIKAWA, KAZUO SHIN-YA, JUNKO SHINOZAKI†, TAKAYUKI KAJIURA†, HARUO SETO and YOICHI HAYAKAWA*

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan
†Pharmaceutical Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-shi, Kanagawa 210-8681, Japan

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The structure of ammocidin, a new apoptosis inducer in Ras-dependent cells from *Saccharothrix* sp. AJ9571, was elucidated to be as shown in Fig. 1 by NMR and degradation studies. Ammocidin consists of a 20-membered macrolide ring and three deoxy sugars identified as 6-deoxy-L-glucose, D-digitoxose and D-olivomycose.

In the preceding paper[1], we have described the production, isolation and biological activity of ammocidin, a new apoptosis inducer in Ras-dependent cells from *Saccharothrix* sp. We report herein the physico-chemical properties and structure elucidation of ammocidin.

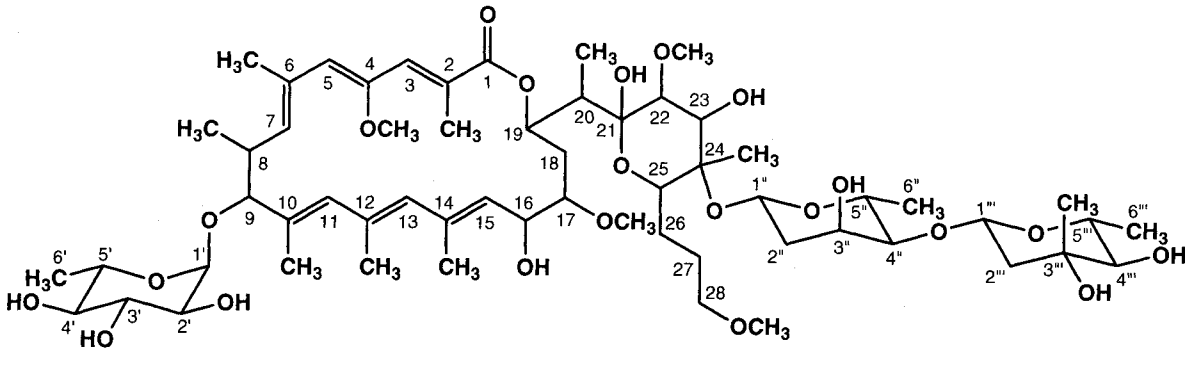
Physico-chemical Properties

The physico-chemical properties of ammocidin are summarized in Table 1. The molecular formula was established to be $C_{59}H_{96}O_{22}$ by high-resolution FAB-MS. The IR spectrum indicated the presence of hydroxyl (3450 cm^{-1}) and conjugated carbonyl groups (1700 cm^{-1} and 1680 cm^{-1}).

Structure Elucidation

The ^{13}C NMR spectrum of ammocidin confirmed the presence of 59 carbons. A heteronuclear multiple-quantum coherency (HMQC)[2] experiment established all one-bond ^{1}H-^{13}C connectivities as shown in Table 2. A COSY experiment revealed eight spin networks to generate partial

Fig. 1. Structure of ammocidin.



* Corresponding author: hayakawa@iam.u-tokyo.ac.jp

Table 1. Physico-chemical properties of ammocidin.

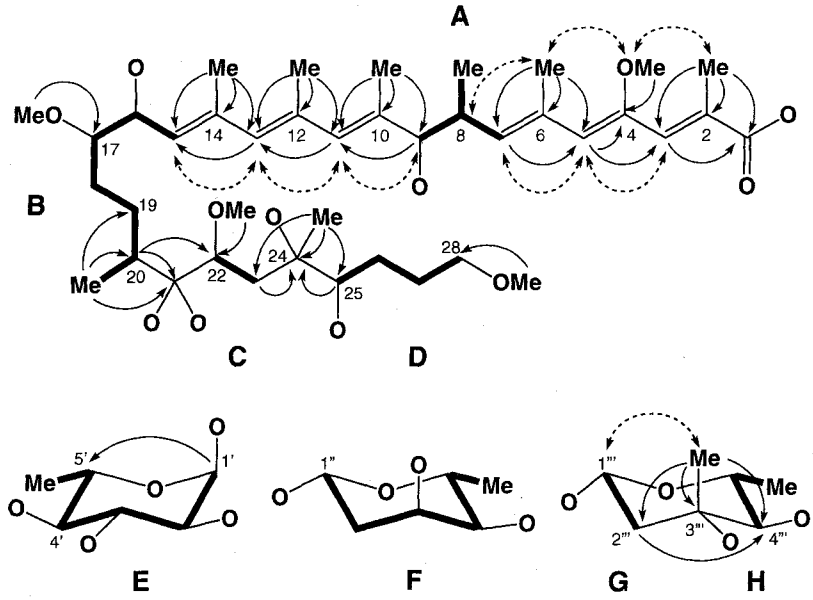
Appearance	Colorless powder
MP	154 ~156 °C
$[\alpha]_D^{25}$	+80 ° (*c* 0.50, MeOH)
Molecular formula	$C_{59}H_{96}O_{22}$
HRFAB-MS Found:	1179.6318 $(M+Na)^+$
Calcd.:	1179.6291
UV λ_{max} nm (ε) in MeOH	270 (22,300), 326 (26,600)
IR ν_{max} (KBr) cm^{-1}	3450, 1700, 1680

Table 2. ^{13}C and ^{1}H NMR data for ammocidin in CD_3OD.

No.	δ_C	δ_H	No.	δ_C	δ_H (J = Hz)
1	171.2		10-Me	12.0	1.62
2	125.4		12-Me	18.1	1.91
3	138.2	7.00	14-Me	17.6	1.76
4	153.6		20-Me	9.3	1.14
5	134.2	5.79	24-Me	10.7	1.16
6	131.8		4-OMe	61.4	3.54
7	142.0	5.35	17-OMe	57.6	3.38
8	36.8	2.88	22-OMe	61.4	3.57
9	87.9	3.85	28-OMe	58.7	3.27
10	134.8		1'	96.1	4.69 (3.5)
11	136.3	5.94	2'	73.6	3.43 (9.5, 3.5)
12	134.1		3'	74.8	3.70 (9.5, 9.5)
13	133.5	5.61	4'	77.5	3.04 (9.5, 9.5)
14	136.2		5'	69.1	3.79 (9.5, 6.5)
15	129.4	5.25	6'	18.5	1.29 (6.5)
16	67.9	4.83	1"	92.6	5.11 (10.0, 1.5)
17	82.8	2.70	2"	38.9	1.97 (13.0, 3.0, 1.5)
18	35.8	1.93, 1.67			1.76 (13.0, 3.0, 10.0)
19	72.3	5.54	3"	68.3	4.28 (3.0, 3.0, 3.0)
20	44.9	2.07	4"	83.3	3.31 (10.0, 3.0)
21	99.9		5"	69.5	3.92 (10.0, 6.5)
22	81.9	3.08	6"	18.3	1.27 (6.5)
23	76.8	3.95	1"'	101.8	4.72 (10.0, 2.0)
24	82.4		2"'	46.6	1.97 (13.0, 2.0)
25	73.9	3.66			1.74 (13.0, 10.0)
26	25.8	1.60, 1.24	3"'	72.3	
27	27.8	1.53, 1.34	4"'	80.2	3.13 (10.0)
28	73.9	3.30	5"'	72.6	3.41 (10.0, 6.5)
2-Me	13.8	2.12	6"'	19.0	1.30 (6.5)
6-Me	13.1	2.09	3"'-Me	20.4	1.27
8-Me	18.2	1.20			

Fig. 2. Partial structures of ammocidin.

Bold lines show proton spin networks and arrows indicate ^{1}H-^{13}C long-range couplings (solid) and NOEs (dashed).



structures **A** to **H** (Fig. 2). The heteronuclear multiple-bond correlation (HMBC)[3] spectrum displayed ^{1}H-^{13}C long-range couplings from 2-CH_3 to C-1, C-2 and C-3, from 6-CH_3 to C-5, C-6 and C-7, from 3-H to C-1, from 5-H to C-3 and C-4, and from 7-H to C-5, thereby showing that a triene moiety joined the ester carbonyl carbon (C-1) to partial structure **A** (Fig. 2). ^{1}H-^{13}C long-range correlations from 10-CH_3 to C-9, C-10 and C-11, from 12-CH_3 to C-11, C-12 and C-13, and from 14-CH_3 to C-13, C-14 and C-15 established the other triene moiety, which connected partial structures **A** and **B**. A ketal carbon (C-21) was located between partial structures **B** and **C** based on long-range correlations from 20-CH_3 to C-19, C-20 and C-21, and from 20-H to C-21 and C-22. A tertiary methyl group (24-CH_3) revealed ^{1}H-^{13}C long-range couplings to C-23, C-24 and C-25, indicating the connection between partial structures **C** and **D** *via* C-24. ^{1}H-^{13}C long-range correlations from 3‴-CH_3 to C-2‴, C-3‴ and C-4‴ constructed a hexose moiety containing partial structures **G** and **H**. Four methoxyl groups exhibited ^{1}H-^{13}C couplings to their adjacent carbons (C-4, C-17, C-22 and C-28).

Vicinal proton coupling constants (Table 1) and an NOE between 1‴-H and 3‴-CH_3 identified three sugar residues as 6-deoxy-α-glucoside (**E**), β-digitoxoside (**F**) and β-olivomycoside (**G**+**H**). The three glycosidic linkages were formed on the basis of long-range couplings between 1′-H and C-9, 1″-H and C-24, and 1‴-H and C-4″. Finally, ^{1}H-^{13}C long-range correlations from 19-H to C-1 and from 25-H to C-21 constructed 20-membered macrolide and 6-membered hemiketal rings.

The geometrical configurations of the two triene moieties proved to be all *E* based on upfield chemical shifts for the five allylic methyl carbons (Table 1) and NOE correlations as shown in Fig. 2. The absolute stereochemistry for the sugar moieties was established by methanolysis of ammocidin, which yielded methyl 6-deoxy-L-glucoside, methyl D-digitoxoside and methyl D-olivomycoside. From these results, the structure of ammocidin was elucidated to be a new 20-membered macrolide related to apoptolidin[4,5] as shown in Fig. 1. Stereochemical studies on the ammocidin aglycone are in progress.

Experimental

General

UV and IR spectra were measured on Hitachi U-3210 and JASCO A-102 spectrometers, respectively. Mass spectra were obtained on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and

polyethylene glycol as internal standard. Optical rotations were recorded on a JASCO DIP-1000 spectropolarimeter. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM-A500 spectrometer with ^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Methanolysis of Ammocidin

Ammocidin (132 mg) was treated with 5% HCl-MeOH at 50°C for 10 minutes and the reaction mixture was applied to a silica gel column. Development of the column with $CHCl_3$-MeOH (20:1) gave methyl olivomycoside and methyl digitoxoside fractions. The former fraction was purified by HPLC using a Senshu Pak PEGASL ODS column with 10% MeOH to yield methyl α-olivomycoside (6.4 mg) and methyl β-olivomycoside (4.4 mg). The optical rotations of the methyl glycosides (α: $[\alpha]_D^{24}$ +145° (*c* 0.32, EtOH), β: $[\alpha]_D^{26}$ −40° (*c* 0.23, EtOH)) gave an agreement with the literature values for methyl D-olivomycoside (α: +147°, β: −50°)[6]. The methyl digitoxoside fraction was purified by ODS-HPLC with 10% MeOH to give methyl β-digitoxofuranoside (1.5 mg). Its optical rotation ($[\alpha]_D^{25}$ −97° (*c* 0.075, $CHCl_3$)) gave an agreement with the literature value for methyl β-D-digitoxofuranoside (−106°)[7]. The residual fraction recovered from the silica gel column was stirred in 10% HCl-MeOH at reflux for 10 hours. The reaction mixture was subjected to silica gel chromatography with $CHCl_3$-MeOH (10:1). Further purification was carried out using ODS-HPLC with 5% MeOH to yield methyl 6-deoxy-α-glucoside ($[\alpha]_D^{29}$ −166° (*c* 0.085, H_2O)), which was identified as methyl 6-deoxy-α-L-glucoside by comparison with the optical rotation of its authentic D-enantiomer ($[\alpha]_D^{25}$ +158° (*c* 1.0, H_2O)).

Acknowledgements

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