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Arisostatins A and B, New Members of Tetrocarcin Class of Antibiotics from *Micromonospora* sp. TP-A0316

II. Structure Determination

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Structures of arisostatins A and B were determined by spectroscopic analyses. Arisostatins were found to be new analogs of tetrocarcin A and possess an *iso*-butanoyldigitoxose unit instead of the acetyldigitoxose one. NMR analyses of arisostatins and tetrocarcin A led to the revision of the anomeric configurations in the tetrasaccharide moiety of tetrocarcin A.

Arisostatins A and B have been found from a culture broth of *Micromonospora* sp. TP-A0316 isolated from the seawater sample collected in Toyama Bay, Japan. They showed potent *in vitro* activity against Gram-positive bacteria and some solid tumor cell lines. In the preceding paper, taxonomy of the producing organism, fermentation, isolation and biological properties of arisostatins have been reported[1]. We herein describe the structure determination of arisostatins.

Results and Discussion

Physico-chemical properties of arisostatins are summarized in Table 1. Their UV spectra showed absorptions characteristic of antlermicin A[2] and tetrocarcins[3~5]. One of the bioactive components isolated from the fermentation broth was identified as a sodium salt of tetrocarcin A, AC6D·Na[6] by comparing the NMR data. The final confirmation was obtained by the NMR analyses of the equimolar mixture of the isolated and authentic tetrocarcin A. Tetrocarcins possess an acidic dissociative proton in the tetronic acid moiety and its *p*Ka value is expected to be near 5[7]. Although arisostatins could be isolated as a salt-form and acid-form by extracting at pH 7 and 3.5, respectively, they were often unstable and decomposed under acidic conditions. For example, the aldehyde function reacted with CD_3OD to yield a dimethyl acetal due to the acidity of the free tetronic acid residue. Hence, further NMR experiments were performed with the samples extracted at pH 7 as a sodium salt.

Arisostatin A

The molecular formula of arisostatin A (**1**) was determined as $C_{69}H_{100}N_2O_{24}$ by HRFAB-MS, which gave a $(M+Na)^+$ ion at *m/z* 1363.6554 (calcd for $C_{69}H_{100}N_2O_{24}Na$ 1363.6564) and by the NMR analyses. As the NMR spectra of **1** were similar to those of tetrocarcin A (TCA) and acid hydrolysis of **1** afforded a mixture of tetrocarcin F-1[8] and tetronolide[8], **1** was proposed to be a tetrocarcin analog different in the structure of tetrasaccharide moiety. In the ^1H NMR spectrum, **1** showed two doublet signals at 1.18 and 1.20 ppm due to methyl groups and a septet methine

Table 1. Physico-chemical properties of arisostatins A (**1**) and B (**2**).

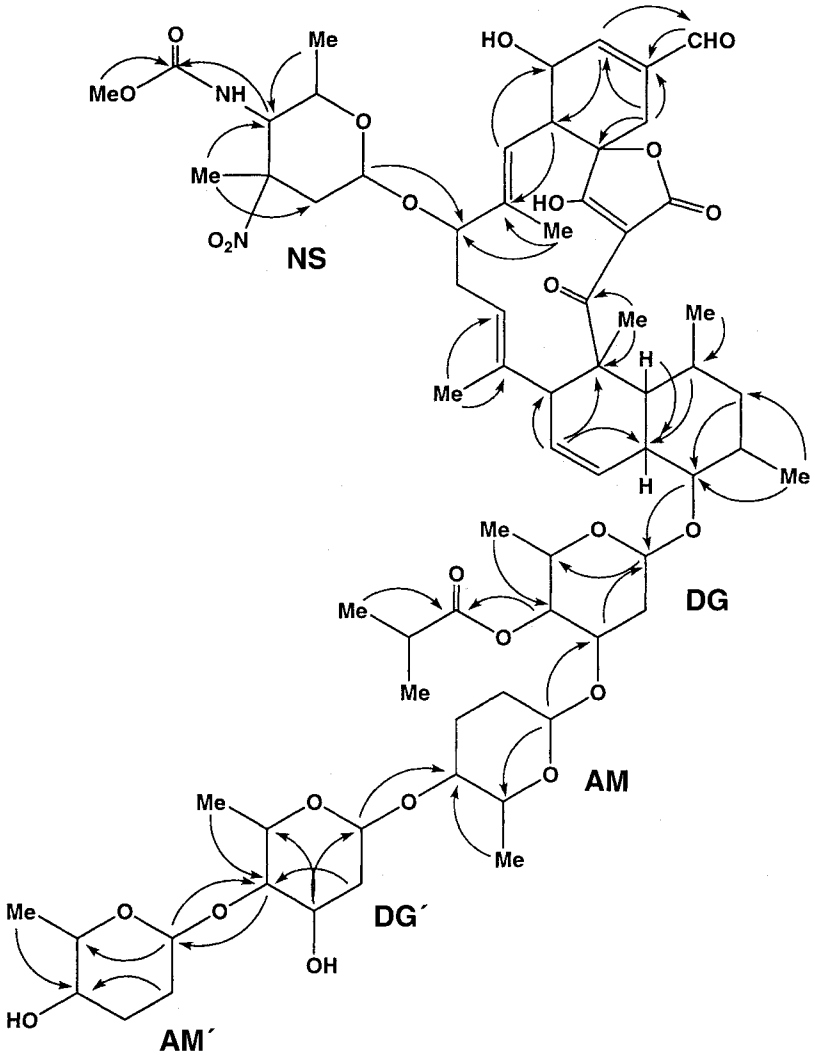
	1	**2**
Appearance	Colorless powder	Colorless powder
MP	>220°C (dec)	>210°C (dec)
$[\alpha]_D^{28}$	-93.8 (*c*=1.17, MeOH, 25°C)	-119.7 (*c*=0.54, MeOH, 26°C)
HRFAB-MS		
Found:	1363.6554 $(M+Na)^+$	1311.7048 $(M+H)^+$
Calcd:	1363.6564 (for $C_{69}H_{100}N_2O_{24}Na$)	1311.7002 (for $C_{69}H_{103}N_2O_{22}$)
Molecular formula	$C_{69}H_{100}N_2O_{24}$	$C_{69}H_{102}N_2O_{22}$
UV λ_{max}^{MeOH} nm (log ε)		
in MeOH	211 (4.23), 235 (4.07), 265 (3.92)	205 (4.48), 235 (4.33), 266 (4.11)
in 0.01N HCl-MeOH (1:9)	207 (4.22), 264 (3.77)	207 (4.42), 263 (3.98)
in 0.01N NaOH-MeOH (1:9)	210 (4.17), 236 (4.07), 265 (3.94)	206 (4.41), 237 (4.31), 266 (4.10)
IR ν_{max} (cm^{-1})	3440, 1720, 1640, 1550	3440, 1720, 1635
Solubility		
soluble in	MeOH, pyridine	MeOH, pyridine
slightly soluble in	$CHCl_3$, acetone	$CHCl_3$, acetone
TLC (Rf)[a]	0.35	0.07
HPLC (Rt)[b]	19.5 min	5.0 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=10:1)
[b] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm, i.d.), Mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (75:25), Flow rate: 0.7 ml/min, Detection: UV-230 nm.

signal at 2.61 ppm instead of a signal at 2.08 ppm due to the acetyl group on a digitoxose (DG) of TCA. In the ^{13}C NMR spectrum, three signals at 19.19, 19.68 and 35.38 ppm appeared instead of a signal at 21.04 ppm and a signal due to the acetyl carbonyl carbon at 172.06 ppm was downfield shifted to 178.08 ppm. The C-H connectivities of these signals were confirmed by the HMQC spectrum. The coupling of the two methyl groups to the methine residue was confirmed by ^1H-^1H COSY and proton decoupling experiments and the long range couplings from the methine and the two methyl protons to the carbonyl carbon at 178.08 ppm revealed the presence of *iso*-butanoyl substituent. The position was confirmed by the long range coupling from H-DG4 to the carbonyl carbon (Fig. 1).

NMR analyses also allowed the confirmation of the presence of tetronitrose (NS), two amicetoses (AM, AM′) and two digitoxoses (DG, DG′), one of which was acylated with *iso*-butanoate. The position where sugars were linked and the sequence of the deoxysugars were established by the HMBC experiments. The long range couplings from H-9 to C-DG1 and H-NS1 to C-17 confirmed the attachment of DG and NS at C-9 and C-17, respectively. HMBC correlations between H-AM1 and C-DG3, H-DG′1 and C-AM4, and H-AM′1 and C-DG′4 established the sequence of the deoxysugars and the positions of their glycosidic linkages. The coupling constants $J_{1,2}$ of the anomeric protons were analyzed to determine the anomeric configurations. In the ^1H NMR spectrum, a signal at 4.64 ppm ($J_{1,2}$=2.1 and 9.7 Hz, NS1) indicated that NS has a β-glycosidic linkage to the aglycon. Four other anomeric protons at 4.82 ($J_{1,2}$=4.2 Hz, DG1), 4.98 ($J_{1,2}$=2.7 Hz, AM1), 4.91 ($J_{1,2}$=1.7 and 9.7 Hz, DG′1) and 4.92 ($J_{1,2}$= ~0 Hz, AM′1) confirmed that the configurations were α, α, β and α, respectively. The structure of the tetrasaccharide moiety was finally determined as α-amicetosyl-(1→4)-β-digitoxosyl-(1→4)-α-amicetosyl-(1→3)-α-4-*O*-*iso*-butanoyldigitoxosyl. The gross structure of **1** was thus established as shown in Fig. 4. The absolute configuration of the aglycon part is unknown[9]. Deoxysugars in **1** are expected to have the same absolute configurations as those of TCA in consideration of the biosynthetic consistency among the congeners. **1** posseses an *iso*-

Fig. 1. Significant ^{1}H-^{13}C long-range correlations observed in the HMBC spectrum of **1**.



butanoyldigitoxosyl unit instead of the acetyldigitoxosyl one in TCA. Additionally, two anomeric configurations are inverted: α-AM and β-DG′ for **1** and β-AM and α-DG′ for TCA. As arisostatins and TCA were considered to be generated by a common biosynthetic sequence, it was hardly acceptable that the microorganism employed a set of glycosyltransferases for α- and β-glycosylation depending on the congener. The stereochemistry of the anomeric positions was thus reexamined with TCA.

Anomeric Configurations of Tetrocarcin A

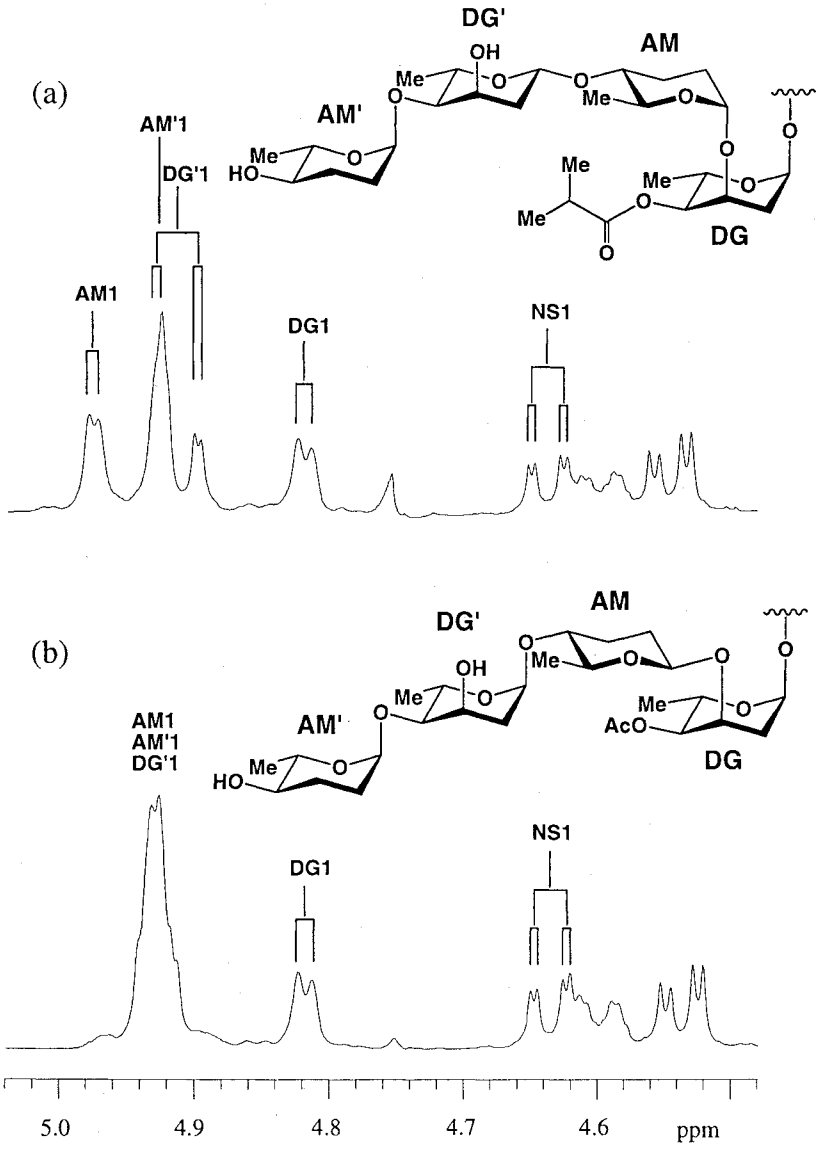
Anomeric regions of the ^{1}H NMR spectra of **1** and TCA are shown in Fig. 2. In the spectrum of **1**, the all anomeric proton signals were separated from each other and unambiguously assignable (Fig. 2a). In contrast, the spectrum of TCA showed the overlapping of three anomeric signals due to AM1, DG′1 and AM′1 between 4.91 and 4.94 ppm except for the two isolated signals at 4.63 ppm (NS1) and 4.82 ppm (DG1) (Fig. 2b). To extract each anomeric proton signal, TCA was subjected to selective 1D-TOCSY experiments at 750 MHz with the mixing time of 80 msec. H-AM4 at 3.21 ppm, H-DG′3 at 4.26 ppm and H-AM′4 at 3.13 ppm were irradiated independently and the subspectra corresponding to AM1, DG′1 and AM′1 were obtained as shown in Fig. 3. Fig. 3b and 3d show that AM and AM′ have an α-configuration with $J_{1,2}$ values of less than 3 Hz whereas $J_{1,2}$ value of *ca.* 9 Hz in Fig. 3c suggests the β-glycosidic linkage at DG′1. Consequently, TCA was confirmed to have the same anomeric configurations as

Table 2. ^{1}H (400 MHz) NMR data for arisostatins A (**1**) and B (**2**) and tetrocarcin A (TCA).

Position	1	2	TCA
5	2.06 (m)	2.07 (m)	2.04 (m)
6	1.52 (m)	1.48 (m)	1.49 (m)
7	1.46 (m), 1.60 (m)	1.46 (m), 1.60 (m)	1.47 (m), 1.59 (m)
8	2.21 (m)	2.20 (m)	2.20 (m)
9	3.42 (dd, 5.1, 10.5)	3.43 (dd, 5.1, 10.5)	3.42 (dd, 5.2, 10.9)
10	2.08 (m)	2.10 (t, 9.8)	2.10 (t, 9.7)
11	5.39 (ddd, 2.2, 5.1, 10.0)	5.39 (ddd, 2.2, 5.1, 10.0)	5.39 (ddd, 2.4, 5.1, 10.0)
12	5.74 (d, 10.2)	5.74 (d, 10.2)	5.73 (d, 10.4)
13	3.64 (m)	3.65 (m)	3.64 (m)
15	5.25 (m)	5.22 (m)	5.23 (m)
16	2.25 (m)	2.22 (m), 2.31 (m)	2.22 (m), 2.29 (m)
17	4.20 (br.s)	4.20 (br.s)	4.20 (br.s)
19	5.25 (m)	5.20 (m)	5.23 (m)
20	2.81 (t, 9.5)	2.79 (t, 9.5)	2.81 (t, 9.5)
21	4.60 (dm, 9.3)	4.54 (m)	4.58 (dm, 9.3)
22	6.87 (s)	6.84 (s)	6.87 (s)
24	2.25 (m), 2.75 (dt, 2.5, 18.6)	2.23 (m), 2.75 (dt, 2.5, 18.9)	2.24 (d, 18.5), 2.75 (dd, 2.6, 18.7)
27	1.49 (s)	1.48 (s)	1.48 (s)
28	0.64 (d, 7.3)	0.64 (d, 5.8)	0.64 (d, 5.9)
29	1.11 (d, 7.3)	1.11 (d, 7.2)	1.10 (d, 7.0)
30	1.36 (s)	1.37 (s)	1.36 (s)
31	1.47 (s)	1.48 (s)	1.47 (s)
32	9.52 (s)	9.51 (s)	9.52 (s)
NS1	4.64 (dd, 2.1, 9.7)	4.75 (dd, 2.2, 9.8)	4.63 (dd, 2.0, 9.6)
NS2	1.76 (m), 2.73 (dm, 14.5)	1.47 (m), 1.62 (m)	1.74 (m), 2.72 (dm, 14.9)
NS4	4.34 (br.s)	3.17 (br.s)	4.34 (br.s)
NS5	3.56 (m)	4.10 (dq, 1.4, 6.3)	3.57 (m)
NS6	1.12 (d, 6.3)	1.09 (d, 6.4)	1.12 (d, 6.1)
NS3-CH_3	1.52 (s)	1.11 (s)	1.52 (s)
NS4-NHCOOCH_3	3.69 (s)	3.64 (s)	3.68 (s)
DG1	4.82 (d, 4.2)	4.82 (d, 3.7)	4.82 (d, 4.4)
DG2	1.80 (m), 2.35 (dd, 2.4, 15.2)	1.80 (m), 2.35 (dd, 2.4, 15.2)	1.75 (m), 2.30 (dd, 2.5, 15.1)
DG3	4.15 (dt, 2.5, 3.2)	4.15 (dt, 2.9, 3.2)	4.16 (dt, 3.0, 3.1)
DG4	4.54 (dd, 3.2, 9.8)	4.54 (dd, 3.2, 9.5)	4.53 (dd, 3.1, 9.6)
DG5	4.40 (dq, 6.4, 9.5)	4.40 (dq, 6.4, 9.5)	4.39 (dq, 6.4, 9.6)
DG6	1.13 (d, 6.4)	1.13 (d, 6.8)	1.13 (d, 6.4)
DG4-O_2CCH_3			2.08 (s)
DG4-$O_2CCH(CH_3)_2$	2.61 (sept, 7.1)	2.61 (sept, 7.1)	
DG4-$O_2CCH(CH_3)_2$	1.18 (d, 7.1), 1.20 (d, 7.1)	1.18 (d, 7.1), 1.20 (d, 7.1)	
AM1	4.98 (d, 2.7)	4.97 (d, 2.9)	4.92 (d, 3.5)
AM2	1.75 (m)	1.72 (m), 1.79 (m)	1.73 (m), 1.80 (m)
AM3	1.87 (m), 1.95 (m)	1.87 (m), 1.90 (m)	1.86 (m), 1.89 (m)
AM4	3.20 (ddd, 4.4, 9.5)	3.20 (ddd, 4.4, 9.5)	3.21 (ddd, 4.5, 9.4)
AM5	3.60 (m)	3.59 (m)	3.66 (m)
AM6	1.11 (d, 6.3)	1.11 (d, 6.3)	1.11 (d, 6.3)
DG' 1	4.91 (dd, 1.7, 9.7)	4.91 (dd, 1.9, 9.5)	4.93 (d, 9.0)
DG' 2	1.60 (m), 2.00 (m)	1.60 (m), 2.00 (m)	1.62 (m), 2.01 (m)
DG' 3	4.26 (dt, 2.7, 3.2)	4.26 (dt, 2.9, 3.2)	4.26 (dt, 2.9, 3.1)
DG' 4	3.27 (dd, 2.8, 9.4)	3.27 (dd, 3.0, 9.5)	3.27 (dd, 2.7, 9.5)
DG' 5	3.90 (dq, 6.3, 9.5)	3.90 (dq, 6.3, 9.5)	3.91 (dq, 6.2, 9.5)
DG' 6	1.29 (d, 6.4)	1.28 (d, 6.1)	1.29 (d, 6.1)
AM' 1	4.92 (br.s)	4.92 (br.s)	4.93 (br.s)
AM' 2	1.75 (m), 1.90 (m)	1.86 (m), 1.98 (m)	1.87 (m), 1.99 (m)
AM' 3	1.73 (m), 1.77 (m)	1.73 (m), 1.77 (m)	1.72 (m), 1.75 (m)
AM' 4	3.14 (ddd, 4.5, 9.5)	3.14 (ddd, 4.5, 9.5)	3.13 (ddd, 4.5, 9.8)
AM' 5	3.59 (m)	3.59 (m)	3.59 (m)
AM' 6	1.19 (d, 6.3)	1.19 (d, 6.3)	1.19 (d, 6.1)

The CD_3OD signal (3.30 ppm) was used as a reference.
The multiplicities and the coupling constants (Hz) are in parentheses.

Fig. 2. ^{1}H NMR spectra of **1** (a) and tetrocarcin A (b) in the anomeric proton region.



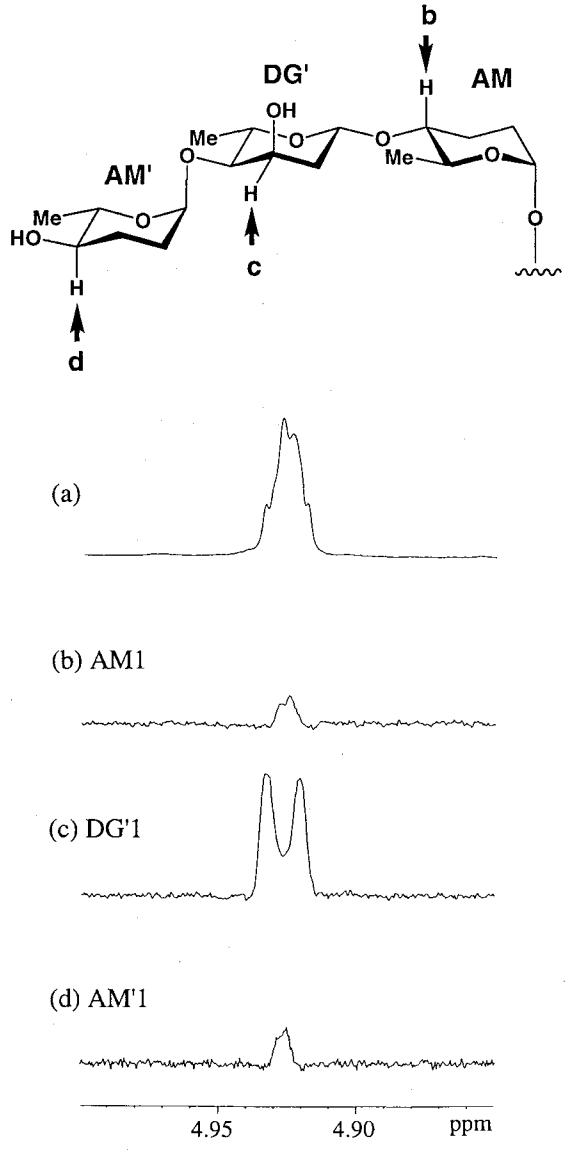
1. The same conclusion was obtained with authentic tetrocarcin A on selective 1D-TOCSY experiments. Therefore, the proposed structure for the tetrasaccharide moiety of TCA shown in Fig. 2b should be revised as shown in Fig. 4.

Arisostatin B

FAB-MS of arisostatin B (**2**) gave a molecular ion peak $(M+H)^+$ at *m/z* 1331. Its molecular formula was determined as $C_{69}H_{102}N_2O_{22}$ based on the HRFAB-MS, ^{1}H and ^{13}C NMR spectra. The NMR spectra of **2** suggested that it is related to **1**, although several distinct differences could be noted in the proton and carbon chemical shifts attributed to NS. Especially noteworthy was the large upfield shift of C-NS3, a quarternary carbon bonded to a nitro group, from 92.24 ppm to 53.72 ppm. A similar change of chemical shifts has been observed between TCA and AC6H[6], a tetrocarcin analog in which the nitro group of tetronitrose (NS) is reduced to an amino group. Taking into account the difference in 30 mass units between **1** and **2** and the shorter retention time of **2** on HPLC developed with acetonitrile - pH 3.5 phosphate buffer, the structure of **2** was determined as shown in Fig. 4.

In conclusion, the structures of arisostatins were determined by spectroscopic analyses. Arisostatins A (**1**)

Fig. 3. Selective 1D-TOCSY spectra of tetrocarcin A in the anomeric proton region.



(a) 1D-NMR spectrum, (b) irradiated at 3.21 ppm (AM4), (c) irradiated a 4.26 ppm (DG′3), (d) irradiated at 3.14 ppm (AM′4)

and B (**2**) were determined to be new members of tetrocarcin class of antibiotics. The proposed structure for tetrocarcin A was revised with regard to the anomeric configurations in the tetrasaccharide moiety.

Experimental

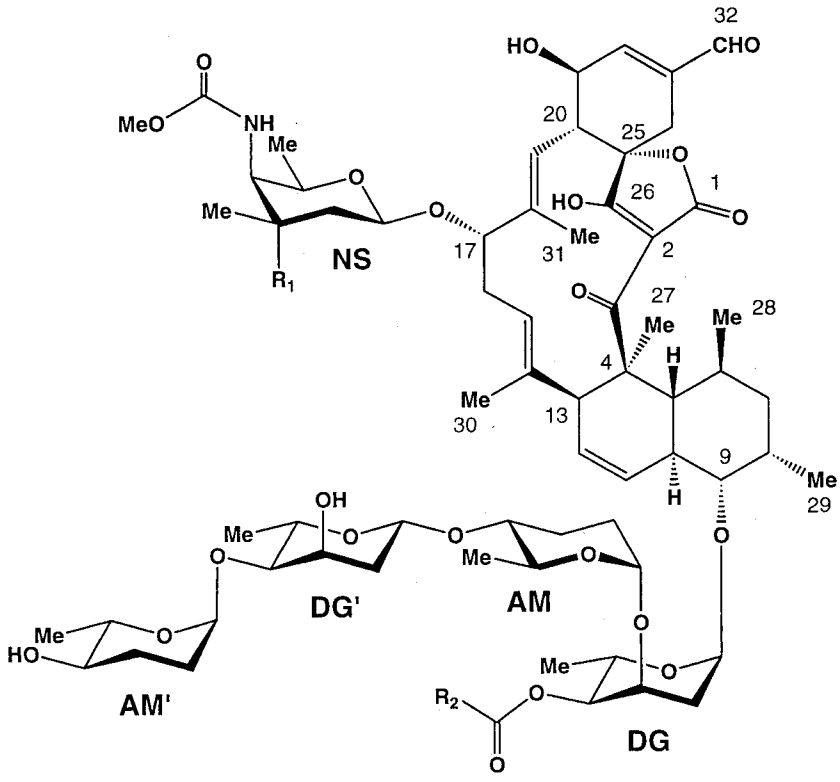
Melting points were determined on a Yanagimoto apparatus and are uncorrected. NMR experiments were performed on JEOL JNM-LA400, JNM-A600 and DMX-750 NMR spectrometers in CD_3OD at 35 °C. The MS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were recorded on a BECKMAN DU 640 spectrophotometer. IR spectra were recorded on a SHIMADZU FT IR-300 spectrophotometer. Optical rotations were measured on a HORIBA SEPA-300 polarimeter.

Table 3. ^{13}C (100 MHz) NMR data for arisostatins A (**1**) and B (**2**) and tetrocarcin A (TCA).

Position	1	2	TCA	Position	1	2	TCA
1	177.37	177.42	177.30	NS4	55.20	59.26	55.18
2	98.81	98.69	98.82	NS5	69.96	69.26	69.96
3	199.98	199.52	200.17	NS6	17.92	17.63	17.85
4	52.85	52.88	52.83	NS3-CH_3	25.97	28.60	25.96
5	40.30	40.35	40.30	NS4-NHCOOCH$_3$	160.09	160.02	160.07
6	32.64	32.68	32.64	NS4-NHCOO*C*H$_3$	53.03	52.67	53.02
7	43.22	43.27	43.18	DG1	99.96	99.96	99.89
8	36.17	36.18	36.15	DG2	31.64	31.64	32.15
9	86.83	86.82	86.65	DG3	67.43	67.46	67.83
10	45.54	45.55	45.50	DG4	76.07	76.08	76.12
11	129.03	129.09	128.97	DG5	63.66	63.66	63.46
12	126.89	126.87	126.89	DG6	18.42	17.91	18.24
13	52.90	52.77	52.90	DG4-O_2C-	178.08	178.08	172.06
14	138.22	138.24	138.15	DG4-O_2CCH_3			21.04
15	123.22	123.14	123.22	DG4-$O_2CCH(CH_3)_2$	35.38	35.38	
16	32.17	32.23	32.15	DG4-$O_2CCH(CH_3)_2$	19.19	19.19	
17	79.98	80.44	79.88		19.68	19.68	
18	139.24	139.37	139.21	AM1	92.88	93.32	92.85
19	122.02	122.25	122.00	AM2	28.28	28.28	28.26
20	46.57	46.55	46.53	AM3	30.42	30.42	30.40
21	71.37	71.38	71.35	AM4	82.04	82.07	82.13
22	151.85	152.48	151.85	AM5	69.26	69.26	69.27
23	139.37	139.82	139.34	AM6	17.27	18.42	17.26
24	30.70	30.78	30.71	DG'1	100.63	100.63	100.58
25	85.39	85.42	85.36	DG'2	39.19	39.21	39.18
26	200.73	200.57	200.77	DG'3	64.00	64.01	63.99
27	15.57	15.51	15.57	DG'4	76.41	76.41	76.37
28	23.04	23.08	23.01	DG'5	69.50	69.51	69.51
29	14.73	14.73	14.66	DG'6	19.26	19.25	19.26
30	15.05	15.10	15.02	AM'1	93.30	92.88	93.71
31	16.32	16.45	16.33	AM'2	27.50	27.52	27.45
32	195.31	195.21	195.33	AM'3	30.42	30.42	30.40
NS1	98.08	98.74	98.05	AM'4	72.62	72.64	72.60
NS2	36.74	40.83	36.73	AM'5	71.50	71.38	71.41
NS3	92.24	53.72	92.23	AM'6	18.24	18.24	18.38

The CD_3OD signal (49.0 ppm) was used as a reference.

Fig. 4. Structure of arisostatins A and B and tetrocarcin A.



Arisostatin A: $R_1=NO_2$, $R_2=CH(CH_3)_2$
B: $R_1=NH_2$, $R_2=CH(CH_3)_2$
Tetrocarcin A: $R_1=NO_2$, $R_2=CH_3$

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