DUNAIMYCINS, A NEW COMPLEX OF SPIROKETAL 24-MEMBERED MACROLIDES WITH IMMUNOSUPPRESSIVE ACTIVITY

II. ISOLATION AND ELUCIDATION OF STRUCTURES

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A novel complex of antifungal and immunosuppressant compounds has been isolated from the fermentation broth and mycelia of two strains of *Streptomyces diastatochromogenes*. The structures of eight related components were determined employing 1D and 2D homonuclear and heteronuclear NMR spectroscopy and mass spectrometry. These structures represent the first reported spiroketal 24-membered macrolide natural products related to the common 26-membered oligomycins.

In the course of screening for antifungal antibiotics, two strains of *Streptomyces diastatochromogenes* were isolated which produced a complex of novel spiroketal 24-membered macrolides. Two companion papers[1,2] describe the taxonomy and fermentation of the producing organisms and the biological activities of the dunaimycins.

Isolation

As described in Part I of this series, two strains of *S. diastatochromogenes* were discovered which produced different members of the dunaimycin complex. Strain AB 1691Q-321 produced dunaimycins C1, C2, D2, D2S, D3 and D4S. Strain AB 1711J-452 under different conditions produced either dunaimycins A1 and D2S or dunaimycins D2S and D3S.

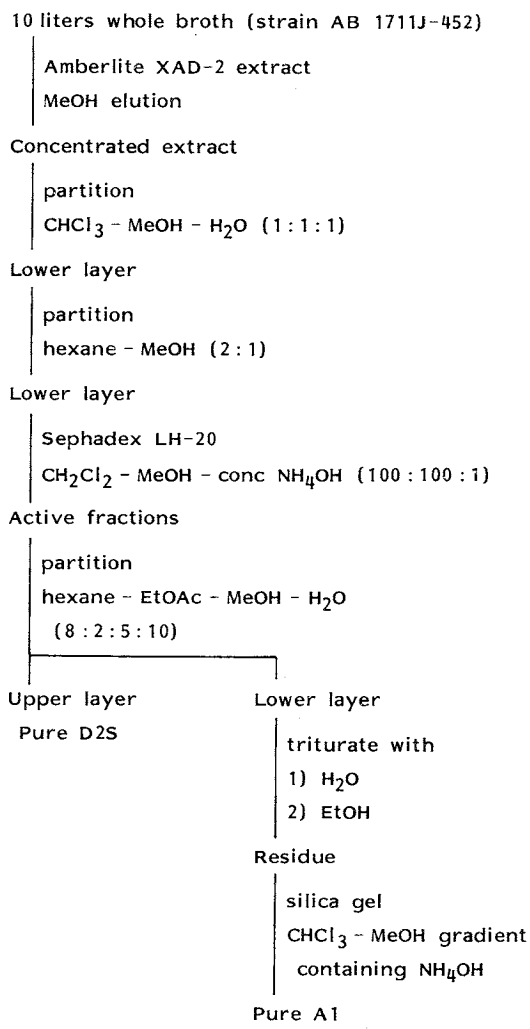
The dunaimycins are lipophilic antibiotics and tend to be mainly associated with the mycelia of the producing culture, although some activity can be detected in the filtered broth. Two different approaches have been taken to recovery of the antibiotics from the fermentation mix. Either whole broth was steeped with a solid phase polystyrene resin such as Amberlite XAD-2, a mixture of mycelia and resin was then filtered from the broth and this mixture was washed with water and then extracted with methanol to yield the crude antibiotic complex. Alternatively, the mycelia was removed from the fermentation broth by centrifugation and decanting. The mycelial residue was then extracted with acetone to give the crude antibiotic complex. In this latter method, any antibiotic in the beer and not associated with the mycelia was discarded. This represented only a small fraction of the total antifungal activity. Initial purification was achieved by partitioning of the crude complex in a multicomponent two phase solvent system. Final purification and separation of components of the complex was achieved using a variety of chromatographic methods including Sephadex LH-20 gel exclusion columns, normal phase silica gel columns and high speed countercurrent chromatography on an Ito multi-layered coil planet centrifuge. This yielded eight biogenetically related congeners as chromatographically and spectrally pure compounds with somewhat broad melting ranges. To date none has been obtained crystalline. The detailed purification schemes are outlined in Figs. 1, 2, and 3.

Structure Determination of Dunaimycin C1

Dunaimycin C1 has a molecular formula of $C_{42}H_{72}O_{10}$ as established by HRFAB positive ion MS (Table 1). Proton decoupled and distortionless enhancement by polarization transfer (DEPT) carbon NMR spectra (Table 2) confirmed the presence of 42 unique carbon atoms with 67 attached protons, suggesting a total of five exchangeable protons in the molecule.

A heteronuclear multiple-quantum correlation (HMQC)[3] map, heteronuclear multiple-bond correlation (HMBC)[4] map and proton correlation spectrum (COSY) of dunaimycin C1 suggested structural components as follows: A *trans* a,b-unsaturated olefin was defined by coupled protons at δ 5.80 (d, J=15.6 Hz) and δ 6.62 (dd, J=15.6 and 10.0 Hz) long range coupled to an ester carbon at δ 164.6. Coupling information from the COSY spectrum further expanded this fragment to carbons 1~9 as in **1**. A quaternary carbon at δ 74.8 was placed next in this sequence as it was long range coupled to the proton at δ 3.66 on carbon 9 and to a singlet methyl proton signal at δ 1.14. This δ 1.14 methyl also showed long range coupling to the C-9 carbon signal at δ 79.2 as well as to a methylene carbon at δ 39.0, thus extending the fragment through carbon 11 as in **1**.

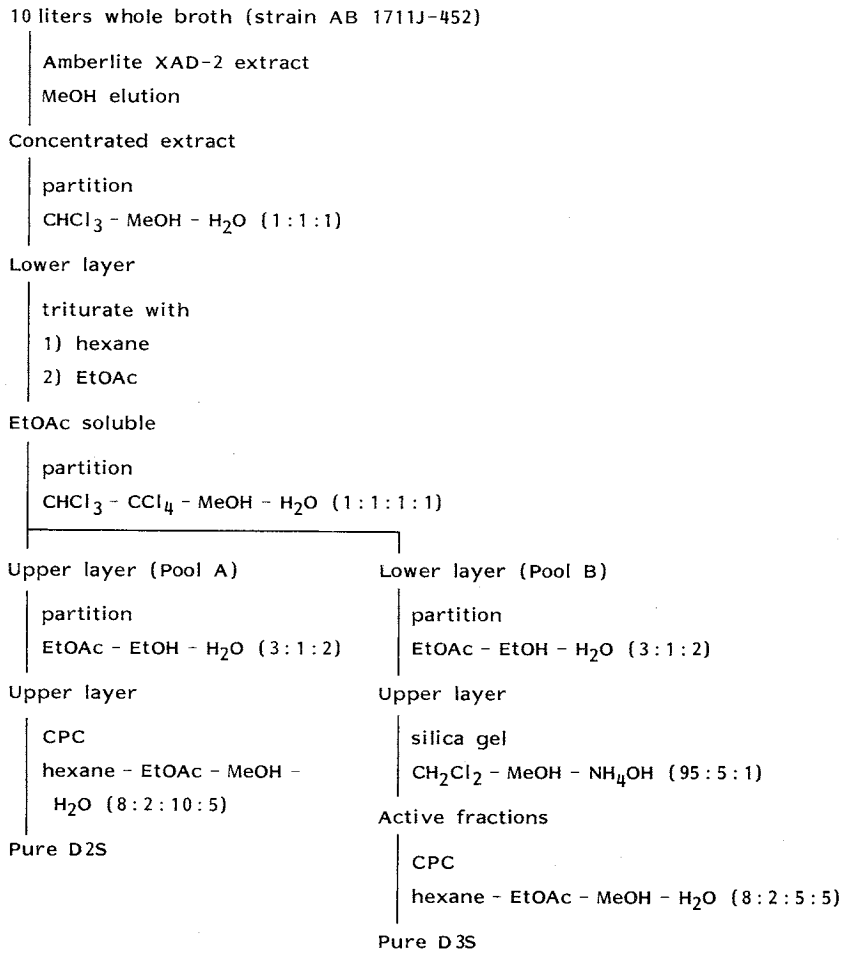
Fig. 1. Isolation of dunaimycins A1 and D2S from strain AB 1711J-452.



A second olefinic system with proton signals at δ 5.58 and δ 5.27 was determined to be *trans* based upon an observed 15.6 Hz coupling constant between these protons. The olefinic proton at δ 5.27 was coupled to a methine at δ 2.99 and further couplings defined a system encompassing carbons 37~40. This same δ 2.99 proton signal was coupled to a ketone carbon at δ 210.9, as were the two protons of a methine at δ 3.02 and δ 2.13. Therefore a ketone bridged the groups as shown in **1** for carbons 18~20. The δ 2.99 methine proton signal was further coupled to two methylene proton signals at δ 1.57 and δ 1.34, each of which was coupled to a methine at δ 1.55. This methine at δ 1.55 was coupled to each of two methyl signals at δ 0.82 and δ 0.89 of a *gem*-dimethyl group as shown for carbons 37~40.

The α-keto methylene signals at δ 3.02 and δ 2.13 (C-20) were coupled to a methine proton signal at δ 4.37, the further coupling from which extended this structural fragment to carbons 20~24. One proton from the methylene at C-24 had a chemical shift of δ 1.80 and was long range coupled to a quaternary carbon signal at δ 97.4. As this same (δ 1.80) proton was coupled only to the geminal methylene partner at δ 1.63 and to the δ 5.34 methine signal at C-23, it was determined that C-25 must be a quaternary.

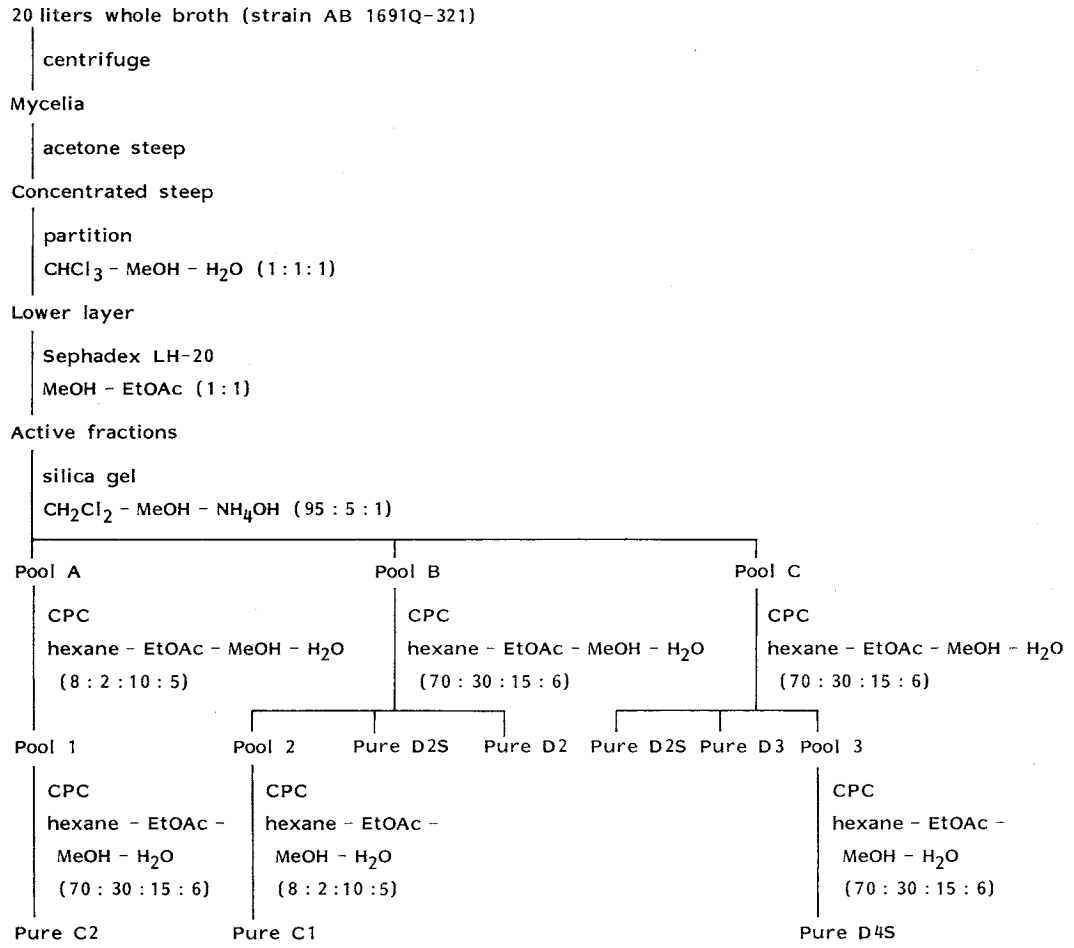
Fig. 2. Isolation of dunaimycins D2S and D3S from strain AB 1711J-452.



Although in dunaimycin C1 the ketal carbon signal at δ 97.4 assigned to C-25 showed no long range coupling in the HMBC spectrum to protons on C-26 or C-27, other dunaimycins to be discussed in this paper established the fact that these two methylene bearing carbons were next in sequence from the ketal. In dunaimycin C1 these methylene proton signals on C-26 and C-27 appear at δ 1.62, δ 1.41 and δ 1.95, δ 1.39, respectively. Both signals at δ 1.95 and δ 1.39 are further coupled to a methine whose proton signal at δ 1.58 is also coupled to a methyl signal at δ 0.91 and to a methine at δ 4.06 (29-H). The proton coupling from 29-H can then be followed out through the C-30 methylene (δ 1.25 and δ 1.62), the C-31 oxymethine (δ 3.93) and the C-32 methylene (δ 1.55, 2H) to the terminal methyl group, C-33, at δ 1.03 to define the fragment 26~33 as in **1**.

There remained to be placed into the structure of dunaimycin C1, four methylene carbons. The olefinic proton signal at δ 5.58 for carbon 16 was coupled to a methylene with proton signals at δ 2.20 and δ 2.08. This methylene then coupled into the methylene envelope of protons with chemical shifts of δ 1.25~1.75. Additionally, the methylene carbon at δ 39.0 assigned to C-11 correlated to proton signals at δ 1.45~1.50. Each of these sets of methylene proton signals has additional coupling only into the methylene envelope and therefore a five carbon long chain of methylenes from C-11 to C-15 is assigned.

Fig. 3. Isolation of dunaimycins from strain AB 1691Q-321.



Structure Determination of Dunaimycin A1

Dunaimycin A1 has a molecular formula of $C_{42}H_{72}O_9$ as established by HRFAB negative ion MS (Table 1). This is an oxygen atom lower than the molecular formula of dunaimycin C1. A comparison of the ^{13}C NMR and ^{1}H NMR spectra of dunaimycins C1 and A1 (Table 3) suggested that the oxidation difference between these two was localized at C-10. The chemical shifts for C-10 and its attached methyl C-36, which appear at δ 74.8 (q) and δ 20.2 (CH_3) in dunaimycin C1, are replaced in dunaimycin A1 by signals at δ 28.7 (CH) and δ 12.8 (CH_3), respectively. These assignments are supported by following the vicinal proton-proton and the one and three-bond proton-carbon coupling patterns from C-2 to C-11 in an analogous manner to that presented for dunaimycin C1 above. These data and close comparison of other spectral features between dunaimycins A1 and C1 led to the formulation of the latter as 10-deoxydunaimycin C1.

Structure Determination of Dunaimycin C2

Dunaimycin C2 has a molecular formula of $C_{42}H_{72}O_{11}$. This contains an oxygen atom more than the molecular formula for dunaimycin C1. A number of proton and carbon chemical shift differences were

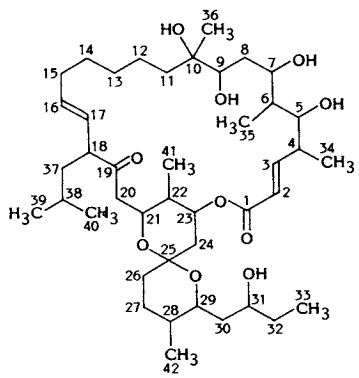
Table 1. HR mass spectral data (FAB positive ion) for the dunaimycins.

Compound	Molecular formula	Ion observed	Observed MW	Calcd MW (for observed ion)
A1	$C_{42}H_{72}O_9$	$C_{42}H_{72}O_9Na$	743.5071	743.5074
C1	$C_{42}H_{72}O_{10}$	$C_{42}H_{72}O_{10}Na$	759.5022	759.5023
C2	$C_{42}H_{72}O_{11}$	$C_{42}H_{72}O_{11}Na$	775.4975	775.4973
D2	$C_{42}H_{72}O_{12}$	$C_{42}H_{72}O_{12}Na$	791.4920	791.4921
D2S	$C_{50}H_{87}NO_{14}$	$C_{50}H_{87}NO_{14}Na$	948.6022	948.6024
D3	$C_{42}H_{70}O_{11}$	$C_{42}H_{70}O_{11}Na$	773.4819	773.4819
D3S	$C_{50}H_{85}NO_{13}$	$C_{50}H_{86}NO_{13}$	908.6103	908.6099
D4S	$C_{51}H_{89}NO_{14}$	$C_{51}H_{90}NO_{14}$	940.6363	940.6361

Table 2. NMR assignments for dunaimycin C1 ($CDCl_3$).

Carbon	Chemical shift (multiplicity)	Attached proton chemical shift (multiplicity, coupling constant(s) Hz)	Carbon	Chemical shift (multiplicity)	Attached proton chemical shift (multiplicity, coupling constant(s) Hz)
1	164.6 (s)	—	23	69.7 (d)	5.34 (1H, ddd, J=12.2, 5.1, 4.9)
2	121.7 (d)	5.80 (1H, d, J=15.6)	24	35.4 (t)	1.80 (1H, dd, J=12.8, 5.1),
3	150.5 (d)	6.62 (1H, dd, J=15.6, 10.0)			1.63 (1H, m)
4	40.8 (d)	2.42 (1H, br dq, J=10.0, 6.4)	25	97.4 (s)	—
5	81.0 (d)	3.65 (1H, br d, J=10.3)	26	29.7 (t)*	1.62 (1H, m), 1.41 (1H, m)
6	40.7 (d)	1.40 (1H, m)	27	26.5 (t)	1.95 (1H, tt, J=13.4, 4.3),
7	78.8 (d)	4.08 (1H, br d, J=9.5)			1.39 (1H, m)
8	35.9 (t)	1.62 (1H, m), 1.22 (1H, m)	28	30.9 (d)	1.58 (1H, m)
9	79.2 (d)	3.66 (1H, br d, J=10.3)	29	67.4 (d)	4.06 (1H, dt, J=11.9, 1.8)
10	74.8 (s)	—	30	41.7 (t)	1.62 (1H, m), 1.25 (1H, m)
11	39.0 (t)	Proton envelope 1.25~1.75	31	68.6 (d)	3.93 (1H, br m)
12	29.8 (t)*	Proton envelope 1.25~1.75	32	30.4 (t)	1.55 (2H, br m)
13	23.4 (t)*	Proton envelope 1.25~1.75	33	10.1 (q)	1.03 (3H, t, J=7.3)
14	29.2 (t)*	Proton envelope 1.25~1.75	34	17.1 (q)	1.19 (3H, d, J=6.4)
15	32.5 (t)	2.20 (1H, br m), 2.08 (1H, br m)	35	3.9 (q)	0.90 (3H, d, J=7.0)
16	135.5 (d)	5.58 (1H, ddd, J=15.6, 9.2, 4.9)	36	20.2 (q)	1.14 (3H, s)
17	128.4 (d)	5.27 (1H, br dd, J=15.6, 9.1)	37	38.3 (t)	1.57 (1H, m), 1.34 (1H, m)
18	55.4 (d)	2.99 (1H, ddd, J=14.3, 9.1, 5.5)	38	25.0 (d)	1.55 (1H, m)
19	210.9 (s)	—	39	21.7 (q)	0.82 (3H, d, J=6.4)
20	42.4 (t)	3.02 (1H, dd, J=17.4, 10.1),	40	23.1 (q)	0.89 (3H, d, J=6.4)
		2.13 (1H, dd, J=17.4, 2.8)	41	5.3 (q)	0.72 (3H, d, J=7.0)
21	65.0 (d)	4.37 (1H, dt, J=10.1, 2.8)	42	11.0 (q)	0.91 (3H, d, J=6.7)
22	34.6 (d)	2.05 (1H, m)			

* Assignments may be interchanged.



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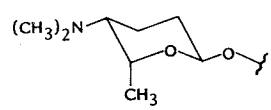

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Table 3. ^{13}C NMR assignments for the dunaimycins ($CDCl_3$).

Carbon	A1	C1	C2	D2	D2S	D3	D3S	D4S
1	164.6	164.6	164.2	164.3	164.7	164.9	165.5	165.1
2	121.6	121.7	121.4	118.9	119.3	120.2	119.8	120.0
3	150.4	150.5	150.8	150.1	148.7	149.1	148.9	149.0
4	40.7	40.8	41.0	75.0	74.9	75.3	75.3	74.8
5	81.1	81.0	80.7	81.7	79.5	80.2	79.5	79.8
6	40.4	40.7	40.9	40.7	35.6	40.0	35.5	35.3
7	78.8	78.8	78.6	78.6	77.3	78.7	77.5	77.6
8	34.7	35.9	35.9	35.6	86.0	35.5	84.8	85.6
9	77.2	79.2	79.8	79.8	74.4	79.3	74.3	72.1
10	28.7[b]	74.8	74.8	74.9	74.6	74.8	74.3	75.1
11	34.8[a]	39.0	38.6	38.7	39.9	38.2	39.3	40.0
12	34.2[a]	29.8[a]	29.5[a]	29.7[a]	29.7[a]	29.6[a]	30.0[a]	30.8[a]
13	29.7[a]	23.4[a]	23.9[a]	23.5[a]	23.6[a]	22.1[a]	22.3[a]	24.1[a]
14	30.2[a]	29.2[a]	29.0[a]	29.0[a]	29.1[a]	29.2[a]	28.9[a]	28.6[a]
15	32.9	32.5	32.5	32.5	32.5	33.3[a]	32.8[a]	32.8
16	135.7	135.5	133.6	133.5	133.7	126.4	125.5	132.0
17	128.2	128.4	129.0	129.1	129.2	122.7	123.8	129.8
18	55.3	55.4	54.0	54.0	54.2	110.6	110.5	48.7
19	211.1	210.9	106.0	106.0	106.1	147.5	147.6	111.3
20	42.3	42.4	38.2	38.3	38.2	28.2	28.5	37.5
21	65.0	65.0	67.9	67.9	67.8	66.2	66.3	66.7
22	34.6	34.6	34.6	34.2	34.5	32.7	32.8[b]	37.0
23	69.7	69.7	69.0	68.8	69.6	70.4	71.7	70.5
24	35.4	35.4	35.4	35.6	35.5	35.8	35.8[a]	35.6
25	97.3	97.4	98.2	98.1	98.2	97.9	98.0	97.6
26	30.1[a]	29.7[a]	29.3[a]	29.2[a]	29.0[a]	29.4[a]	29.7[a]	29.7[a]
27	26.4	26.5	26.8	26.8	26.8	26.6[a]	26.7[a]	26.6[a]
28	30.8[b]	30.9	30.5	30.5	30.6	30.7	30.7[b]	31.5
29	67.3	67.4	67.1	67.2	67.1	67.0	67.0	67.4
30	41.7	41.7	41.3	41.4	41.4	40.5	40.5	43.4
31	68.6	68.6	68.1	68.2	68.2	68.3	68.3	69.1
32	30.1	30.4	30.2	30.2	30.2	31.3	28.6[a]	30.6
33	10.1	10.1	10.1	10.1	10.1	10.5	10.6	10.5
34	17.0	17.1	17.2	27.3	28.6	28.5	28.3	27.9
35	3.8	3.9	3.8	5.0	6.0	5.6	5.8	6.0
36	12.8	20.2	19.0	18.9	22.2	19.6	22.2	23.4
37	38.2	38.3	43.5	43.5	43.2	43.5	43.4	42.9
38	24.9	25.0	81.7	81.7	81.7	84.0	84.1	81.3
39	21.6	21.7	28.6	28.8	28.8	27.7	28.3	30.2
40	23.1	23.1	30.3	30.2	30.3	28.4	28.5	31.0
41	5.3	5.3	5.0	5.0	5.6	4.7	4.3	5.1
42	10.9	11.0	11.4	11.4	11.4	11.1	11.1	10.9
1′					98.4		98.3	98.3
2′					29.2[a]		29.4[a]	30.1
3′					20.0		20.0	19.8
4′					61.9		61.6	61.8
5′					72.3		72.2	72.0
6′					13.4		13.5	13.6
4′-$N(CH_3)_2$					43.3		43.3	43.3
19-OCH_3								48.4

[a,b] Assignments may be interchanged.

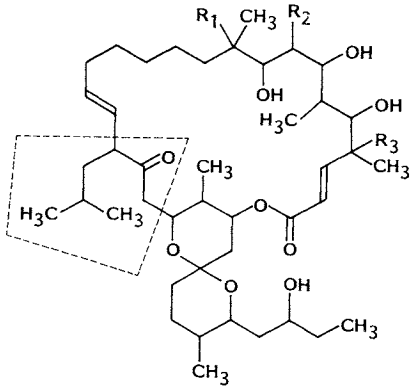
observed between dunaimycins C1 and C2, but were localized about the region of C-16 through C-20 and C-37 through C-40 (Tables 3 and 4). In particular, the ketone carbon whose signal appears at δ 210.9 in dunaimycin C1 is replaced in dunaimycin C2 with a ketal carbon with a signal at δ 106.0. Further, the

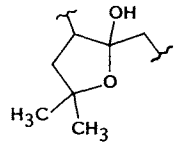
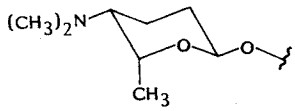
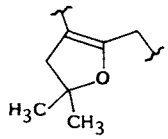
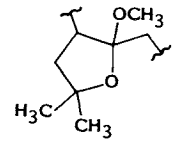
Table 4. ^{1}H NMR assignments of the dunaimycins ($CDCl_3$).

H on C No.	A1	C1	C2	D2	D2S	D3	D3S	D4S
2	5.80	5.80	5.76	6.13	6.17	6.10	6.11	6.13
3	6.62	6.62	6.60	6.78	6.84	6.74	6.80	6.82
4	2.42	2.42	2.40					
5	3.64	3.65	3.66	3.80	3.82	3.78	3.80	3.78
6	1.40	1.40	1.36	1.44	1.75	1.51	1.82	1.83
7	4.06	4.08	4.07	4.40	4.04	4.05	3.95	3.96
8	1.60,	1.62,	1.55,	1.56,	3.77	1.53,	3.67	3.69
	1.18	1.22	1.18	1.18		1.31		
9	3.80	3.66	3.59	3.58	2.99	3.64	3.10	3.1
10～14	env	env	env	env	env	env	env	env
15	2.20,	2.20,	2.29,	2.30,	2.30,	2.13,		2.09,
	2.05	2.08	1.99	1.99	1.98	1.95		1.97
16	5.60	5.58	5.33	5.34	5.35	5.24	5.17	5.42
17	5.28	5.27	5.46	5.46	5.45	6.08	6.09	5.32
18	3.01	2.99	2.42	2.44	2.40			3.10
20	3.08,	3.02,	2.06,	2.03,	2.06,	2.77,	2.89,	1.90,
	2.11	2.13	1.38	1.39	1.38	2.32	2.32	1.62
21	4.39	4.37	4.36	4.37	4.35	4.08	4.12	3.99
22	2.06	2.05	2.09	2.11	2.30	2.03	2.02	2.01
23	5.35	5.34	5.35	5.37	5.28	5.37	5.24	5.30
24	1.82,	1.80,	1.79,	1.83,	1.69	1.76,	1.70	1.76,
	1.64	1.63	1.72	1.74		1.68		1.66
26	1.68,	1.62,	env	env	env	env	env	env
	1.48	1.41	env	env	env	env	env	env
27	1.95,	1.95,	1.98,	env	1.94,	env	env	env
	1.36	1.39	1.50	env	1.48	env	env	env
28	1.61	1.58	1.59	1.62	1.63	1.59	1.57	1.54
29	4.04	4.06	4.19	4.20	4.19	4.12	4.13	4.12
30	1.60,	1.62,	1.62,	1.66,	1.52,	1.57,	1.62,	1.59,
	1.22	1.25	1.23	1.20	1.22	1.21	1.22	1.22
31	3.94	3.93	3.94	3.95	3.93	3.78	3.81	3.69
32	1.52	1.55	1.52	1.53	1.48	1.49	1.46	1.48
33	1.03	1.03	1.00	1.01	0.98	1.03	1.02	1.00
34	1.19	1.19	1.20	1.40	1.34	1.44	1.35	1.40
35	0.90	0.90	0.88	0.96	0.88	1.02	0.90	0.92
36	0.91	1.14	1.10	1.11	1.15	1.10	1.10	1.14
37	1.55,	1.57,	2.03,	2.04,	2.02,	2.50	2.52	2.18,
	1.35	1.34	1.82	1.78	1.68			1.72
38	1.52	1.55						
39	0.84	0.82	1.26	1.27	1.26	1.45	1.40	1.36
40	0.90	0.89	1.42	1.43	1.40	1.46	1.40	1.42
41	0.75	0.72	0.71	0.72	0.78	0.85	0.82	0.85
42	0.92	0.91	0.94	0.94	0.92	0.93	0.94	0.90
1′					4.84		4.81	4.84
2′					2.02,		1.95,	1.96,
					1.58		1.68	1.60
3′					1.85,		1.88,	1.86,
					1.64		1.65	1.65
4′					2.35		2.35	2.35
5′					4.42		4.43	4.42
6′					1.29		1.30	1.30
4′-$N(CH_3)_2$					2.25		2.36	2.25
19-OCH_3								3.35

env: Indicates that these protons cannot be individually identified in the proton envelope from δ 1.25～1.75.

Fig. 4. Structures of the dunaimycins.



Systematic suffix[a]	R_1	R_2	R_3	Insert
A1	H	H	H	As shown
C1	OH	H	H	As in A1
C2	OH	H	H	
D2	OH	H	OH	As in C2
D2S	OH		OH	As in C2
D3	OH	H	OH	
D3S	OH	As in D2S	OH	As in D3
D4S	OH	As in D2S	OH	

[a] Hydroxylation at 4 and 10 determines initial designation A = −,−; B = +,−; C = −,+; D = +,+. Elaboration of the 19, 38 ketone acetal system determines the second designation 1 = open chain, 2 = hemiacetal, 3 = enol ether, 4 = methyl acetal. Third designation is for sugar attachment through an oxidized 8-position.

methine carbon signal at δ 25.0 for C-38 in the spectrum of dunaimycin C1 is replaced by a quaternary carbon signal at δ 81.7 in dunaimycin C2. The carbon signals for the methyl groups attached to C-38 in the spectrum of dunaimycin C2 are approximately 7 ppm downfield from the position of the corresponding carbon signals in the spectrum of dunaimycin C1. Moreover they show one bond couplings to proton signals (also downfield by approximately 0.5 ppm in the spectrum of dunaimycin C2 relative to their positions in the spectrum of dunaimycin C1). These methyl proton signals are singlets in the spectrum of

dunaimycin C2 as opposed to the doublet methyl signals in the spectrum of dunaimycin C1. These data suggest a tetrahydrofuran ring in dunaimycin C2 encompassing carbons 19, 18, 37 and 38 as shown in Fig. 4.

Structure Determination of Dunaimycin D2

Dunaimycin D2 has a molecular formula of $C_{42}H_{72}O_{12}$, containing an oxygen atom more than the molecular formula of dunaimycin C2. A comparison of the ^{13}C NMR spectra of these two compounds suggested that the difference between them was localized at C-4. The chemical shifts for C-4 and its attached methyl C-34 which appear at δ 41.0 (CH) and δ 17.2 (CH_3) in the spectrum of dunaimycin C2 are replaced in the spectrum of dunaimycin D2 with signals at δ 75.0 (q) and δ 27.3 (CH_3), respectively. These data are explained by formulating dunaimycin D2 as 4-hydroxydunaimycin C2 as shown in Fig. 4.

Structure Determination of Dunaimycin D2S

Dunaimycin D2S has a molecular formula of $C_{50}H_{87}NO_{14}$ as established by HR mass matching of the $(M+Na)^+$ ion in FAB positive ion mass spectrum. This spectrum also contains a signal at *m/z* 767 for the loss of a $C_8H_{16}NO_2Na$ (by comparison the molecular weight of dunaimycin D2 is 768). This observation and analysis of the ^{13}C NMR and ^1H NMR data for dunaimycin D2S (Tables 3 and 4) indicated the presence of an amino sugar (**2**). An axial anomeric proton at δ 4.84 (1H, dd, $J=8.5$ and 3.0 Hz) was coupled to each of two methylene protons at δ 2.02 and δ 1.58. These methylene protons were each further coupled to an adjacent set of methylene protons at δ 1.64 and δ 1.85, which were each coupled to a methine at δ 2.35. This 4′ methine proton signal was coupled to the 5′ proton signal at δ 4.42 by a 4.8 Hz coupling constant and to one of the 3′ methylene proton signals by 9.7 Hz indicating that the 4′ proton must be axial and the 5′ proton in the equatorial orientation. As further evidence of this, a NOE was observed between the 6′ methyl proton signal at δ 1.29 and the anomeric proton signal at δ 4.84 indicating a 1,3-diaxial relationship between these two protons. A comparison of the ^1H NMR and ^{13}C NMR data between dunaimycins D2 and D2S indicates the site of sugar attachment in dunaimycin D2S as C-8. The C-8 methylene carbon in dunaimycin D2 with proton signals at δ 1.56 and δ 1.18 and carbon signal at δ 35.6, is replaced in the spectrum of dunaimycin D2S by a methine group with proton and carbon signals at δ 3.77 and δ 86.0, respectively. A long range coupling is observed in the HMBC spectrum between the δ 3.77 methine and the anomeric carbon at δ 98.4 supporting this site of sugar attachment.

Structure Determination of Dunaimycins D3 and D3S

Dunaimycin D3 has a molecular formula of $C_{42}H_{70}O_{11}$, which corresponds to the molecular formula of dunaimycin D2 minus the elements of a water molecule. This difference in molecular formulae is attributed to dehydration in dunaimycin D3 at C-18, C-19. The hemiketal carbon at δ 106.0 in the carbon spectrum of dunaimycin D2 is absent in the spectrum of D3, whereas an extra olefin is evidenced by carbon signals at δ 110.6 (q) and δ 147.5 (q) in the spectrum of dunaimycin D3. Each of these two olefinic carbon signals show long range coupling in the HMBC experiments to a methylene two-proton signal at δ 2.50 which shows one bond coupling to a carbon signal at δ 43.5 (C-37). This allows for formulation of dunaimycin D3 as the dihydrofuran containing structure shown in Fig. 4.

The same spectral comparisons as have been made between dunaimycins D2 and D3 can also be made between dunaimycins D2S and D3S with the olefinic carbon signals occurring at δ 110.5 (q) and δ 147.6 (q) each showing long range coupling to the methylene proton pair at δ 2.52 — which in turn have one bond coupling to C-37 at δ 43.4. The proton and carbon signals assigned to the sugar moiety in the spectra of dunaimycin D3S are virtually coincident with those similarly assigned in the spectra of dunaimycin

D2S — allowing for the formulation of dunaimycin D3S as shown in Fig. 4.

Structure Determination of Dunaimycin D4S

HRFAB positive ion MS established the molecular formula of dunaimycin D4S as $C_{51}H_{89}NO_{14}$, *i.e.* it contains CH_2 more than the molecular formula of dunaimycin D2S. The ^1H NMR spectrum of dunaimycin D4S was similar to that of dunaimycin D2S except for the addition of a methoxyl signal at δ 3.35 (3H, s). These methoxyl protons showed one bond coupling to a carbon at δ 48.4 and long range coupling in the HMBC spectrum to the C-19 carbon signal at δ 111.3 (shifted downfield from its (δ 106.1) position in the spectrum of dunaimycin D2S). Significant differences in the spectra of dunaimycins D2S and D4S also occur in the chemical shifts of the proton and carbon signals assigned to the C-18 methine. They occur at δ 2.40 and δ 54.2 in the spectra of dunaimycin D2S and at δ 3.10 and δ 48.7 in that of dunaimycin D4S. These data are explained by the formulation of dunaimycin D4S as the methyl hemiketal of dunaimycin D2S at C-19.

Discussion

The dunaimycins comprise a novel complex of spiroketal macrolides related to the 26-membered oligomycins and the 22-membered cytovaricin[5], phthoramycin[6] and kaimonolide A[7]. The large family of avermectins and milbemycins may also be viewed as spiroketal 20-membered macrolides modified by an intramolecular Diels Alder reaction. Interestingly, there is no previous report of spiroketal 24-membered macrolides. The oligomycins, phthoramycin and kaimonolide A were discovered as antifungal agents, although with different assay organisms. The dunaimycins were discovered and purified following their activity against *Aspergillus niger*, albeit of relatively low potency. They were however potent in the mixed lymphocyte reaction as potential immunosuppressants. Many of the biosynthetic features of the dunaimycins seem to more closely resemble the 22-, rather than the 26-, membered spiroketal macrolides. The putative starter to the polyketide chain is "propionate" whereas this unit is "acetate" in the oligomycins. They also have the very unusual "4-methylpentanoate" as the eighth unit in the polyketide as seen in the 22-membered series, but unlike that series where the presumed post-polyketide oxidation occurs on the terminal methyl group, in the dunaimycins it occurs on the adjacent methine. Furthermore, among the known antibiotics of these families, only cytovaricin is glycosylated[5], whereas three of the dunaimycins bear a "sugar" moiety. Although the sugars are quite different, the points of attachment to the macrolide ring are analogous. In addition to the presence or absence of the sugar moiety, the dunaimycins differ by the extent of, presumably post-polyketide, hydroxylation at C-4, C-10 and C-38. Hydroxylation at C-38 is accompanied by hemiketal formation with the strategically positioned C-19 ketone. Dehydration of the hemiketal leads to the enol ether structure as in dunaimycins D3 and D3S which probably arise as artifacts of isolation rather than enzymatically. This is presumably also the case for the mixed ketal, dunaimycin D4S.

The sugar which is present in dunaimycins D2S, D3S and D4S is unusual in that the 6′ methyl group exists in the axial position. This fact appears well established by coupling constant and NOE data as outlined in the text. This sugar is epimeric at C-5 with forosamine (2,3,4,6-tetradeoxy-4-(dimethylamino)-D-*erythro*-hexose) which is present in the spiramycins[8]. The *threo* isomer of the dunaimycins is identical to, or enantiomeric with, ossamine (2,3,4,6-tetradeoxy-4-(dimethylamino)-D-*threo*-hexose), the sugar present in the antibiotic ossamycin[9,10]. A comparison of NMR data between the dunaimycins and a synthetic sugar (methyl α-L-ossamidine)[11] to that found in ossamycin supports this conclusion.

The structures were determined by mass spectrometry and numerous NMR experiments and these have allowed for definition of the stereochemistry at both olefins and the relative stereochemistry of the sugar moiety. From 14 to 16 asymmetric centers in the various dunaimycins are, as yet, of undefined stereochemistry.

Experimental

Isolation of Dunaimycins A1 and D2S from Strain AB 1711J-452

As outlined in Fig. 1, to whole broth of strain AB 1711J-452 (10 liters) was added 1 liter of Amberlite

XAD-2 and the mix agitated for 4 hours at room temperature. The Amberlite XAD-2 was then recovered by filtration, washed with distilled water (4 liters) and eluted with metanol (4 liters). The methanol was concentrated to dryness, then partitioned between 500 ml each of chloroform - methanol - water. The lower layer was concentrated to an orange oily residue. This residue was partitioned between 1 liter of hexane and 0.5 liter of methanol. The methonal layer was concentrated to an oily residue. This residue was loaded onto a Sephadex LH-20 column which was eluted with dichloromethane - methanol (1 : 1) containing 1% conc ammonia. Active fractions were concentrated to dryness and their residue partitioned between hexane - ethyl acetate - methanol - water (8 : 2 : 5 : 10). The upper layer of this partition was concentrated to dryness to yield pure compound D2S (4 mg). The lower layer from the above partition was triturated with distilled water (500 ml) and then ethanol (500 ml). The active residue remaining after trituration was loaded onto a silica gel column which was eluted with 5% ~ 10% methanol in chloroform containing 1% conc ammonia. Active fractions were combined and evaporated under reduced pressure to yield pure compound A1 (2 mg).

Isolation of Dunaimycins D2S and D3S from Strain AB 1711J-452

As outlined in Fig. 2, to whole broth of strain AB 1711J-452 (10 liters) was added 1 liter of Amberlite XAD-2 and the mix agitated for 16 hours. The Amberlite XAD-2 was then recovered by filtration, washed with water (5 liters) and eluted with methanol (4 liters). The methanol was concentrated to dryness and the residue was partitioned in a mixture of 600 ml each of chloroform - methanol - water. The lower layer was concentrated to dryness and triturated sequentially with 500 ml each of hexane and ethyl acetate. The ethyl acetate soluble material was concentrated to dryness and partitioned in a mixture consisting of 500 ml each of chloroform - carbon tetrachloride - methanol - water. The upper layer (Pool A) was concentrated to dryness and the residue was partitioned between ethyl acetate - ethanol - distilled water (3 : 1 : 2, total volume 1,200 ml). The upper layer from this partition was chromatographed on an Ito multi-layered coil planet centrifuge (CPC) in a solvent system of hexane - ethyl acetate - methanol - water (8 : 2 : 10 : 5) with the lower layer as stationary. Active fractions were pooled and concentrated to yield pure compound D2S (2 mg). The lower layer (Pool B) was concentrated to dryness and partitioned between ethyl acetate - ethanol - water (3 : 1 : 2, total volume 1,200 ml). The upper layer from this partition was loaded onto a silica gel column eluted with dichloromethane - methanol - conc ammonia (95 : 5 : 1). Active fractions were pooled and concentrated to give a white powder. This powder was chromatographed on a CPC in a solvent system of hexane - ethyl acetate - methanol - water (8 : 2 : 5 : 5) with the lower layer as stationary. Active fractions were pooled and concentrated to yield pure compound D3S (2 mg).

Isolation of Dunaimycins C1, C2, D2, D2S, D3 and D4S from Strain AB 1691Q-321

As outlined in Fig. 3, whole broth of strain AB 1691Q-321 (20 liters) was centrifuged to remove the mycelial mass, which was then steeped with acetone (2 times, 5 liters). Acetone extracts were combined, concentrated to dryness and the residue was partitioned between chloroform - methanol - water (1 liter of each). The lower layer from this partition was concentrated to dryness and chromatographed over a Sephadex LH-20 column eluted with ethyl acetate - methanol (1 : 1). Active fractions were pooled, concentrated to dryness and rechromatographed on a silica gel column eluted with dichloromethane - methanol - conc ammonia (95 : 5 : 1). Fractions were analyzed by TLC, and combined into three pools and each was concentrated to a residue, labeled A, B and C. Residue A was chromatographed on a CPC in a solvent system consisting of hexane - ethyl acetate - methanol - water (8 : 2 : 10 : 5) with the lower layer as stationary. Active fractions were combined into two pools which were each concentrated to dryness. The residue originating from the earlier eluting fractions was chromatographed on a CPC in a solvent system consisting of hexane - ethyl acetate - methanol - water (70 : 30 : 15 : 6) with the lower layer as stationary. Active fractions were combined and concentrated to yield pure compound C2. The residue from Pool 2 was chromatographed on a CPC in a solvent system consisting of hexane - ethyl acetate - methanol (8 : 2 : 10 : 5) with the lower layer as stationary. Active fractions were combined and concentrated to yield pure compound C1. Pool B was chromatographed on a CPC in a solvent system of hexane - ethyl acetate - methanol - water (70 : 30 : 15 : 6) with the lower layer as stationary. Fractions were analyzed by TLC and combined accordingly into three fraction pools, the first two of which yielded pure compounds D2S and D2.

Residue C was chromatographed over a CPC in a solvent system consisting of hexane-ethyl acetate-methanol-water (70:30:15:6) with the lower layer as stationary. Fractions were monitored by TLC, combined accordingly and concentrated to yield pure compound D2S and pure compound D3. The third pool was chromatographed on a CPC in a solvent system of hexane-ethyl acetate-methanol-water (8:2:5:5) with the lower layer as stationary. Active fractions were combined and concentrated to yield pure compound D4S.

General Procedures

Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter in a 10-cm cell. MP's 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[12]. Mass spectra were measured on a Kratos MS-50 mass spectrometer. UV spectra were recorded on a Perkin-Elmer Lambda 3B UV-visible spectrophotometer and IR spectra on a Nicolet model 60SX FT-IR attached to a Nicolet computer. NMR spectra were acquired on a General Electric GN500 spectrometer. ^{13}C and ^{1}H NMR spectral data for the dunaimycins are reported in tables within the text.

Dunaimycin A1: $[\alpha]_D^{25}$ +13° (*c* 0.40, MeOH), white solid, mp 67~72°C and Rf 0.34 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.73 in EtOAc-MeOH (1:1) and Rf 0.48 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 214 (1,500) unchanged upon addition of acid or base. IR ($CDCl_3$) cm^{-1} 3524, 2961, 2928, 2871, 2858, 1712, 1464, 1386, 1307, 1278, 1229, 1186, 1151, 1133, 1102, 1082, 1060, 1019, 987.

Dunaimycin C1: $[\alpha]_D^{25}$ +2° (*c* 0.06, MeOH), white solid, mp 89~100°C and Rf 0.20 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.78 in EtOAc-MeOH (1:1) and Rf 0.33 in EtOAc. IR ($CDCl_3$) cm^{-1} 3517, 2963, 2935, 2871, 2857, 1712, 1463, 1440, 1419, 1386, 1364, 1308, 1278, 1229, 1186, 1169, 1152, 1133, 1105, 1081, 1060, 1020, 987, 965.

Dunaimycin C2: $[\alpha]_D^{25}$ +7° (*c* 0.07, MeOH), white solid, mp 104~109°C and Rf 0.17 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.85 in EtOAc-MeOH (1:1) and Rf 0.37 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 206 (7,740) unchanged upon addition of acid or base. IR ($CDCl_3$) cm^{-1} 3491, 2971, 2936, 2876, 2858, 1711, 1460, 1435, 1421, 1385, 1368, 1309, 1278, 1231, 1188, 1164, 1133, 1116, 1098, 1078, 1061, 1020, 1002.

Dunaimycin D2: $[\alpha]_D^{25}$ −34° (*c* 0.07, MeOH), white solid, mp 132~137°C and Rf 0.17 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.76 in EtOAc-MeOH (1:1) and Rf 0.37 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 208 (6,800) unchanged upon addition of acid or base. IR ($CDCl_3$) cm^{-1} 3494, 2972, 2937, 2879, 2857, 1715, 1463, 1455, 1440, 1420, 1385, 1369, 1310, 1286, 1272, 1230, 1187, 1167, 1148, 1117, 1097, 1079, 1060, 1020, 1001, 979, 963.

Dunaimycin D2S: $[\alpha]_D^{25}$ −22° (*c* 0.37, MeOH), white solid, mp 117~123°C and Rf 0.30 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.47 in EtOAc-MeOH (1:1) and Rf 0.07 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 206 (7,200) unchanged upon addition of acid or base. IR ($CDCl_3$) cm^{-1} 3490, 2971, 2934, 2877, 1711, 1464, 1455, 1440, 1385, 1368, 1310, 1286, 1273, 1264, 1231, 1186, 1170, 1151, 1131, 1119, 1081, 1053, 1038, 1018, 1003.

Dunaimycin D3: $[\alpha]_D^{25}$ −39° (*c* 0.23, MeOH), white solid, mp 122~128°C and Rf 0.12 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.93 in EtOAc-MeOH (1:1) and Rf 0.26 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 216 (12,000), 252 (18,000), unchanged upon addition of acid or base. IR ($CDCl_3$) cm^{-1} 3529, 2972, 2935, 2878, 2857, 1714, 1672, 1462, 1436, 1420, 1386, 1370, 1309, 1285, 1279, 1244, 1227, 1198, 1182, 1148, 1133, 1097, 1075, 1063, 1033, 1012, 984.

Dunaimycin D3S: $[\alpha]_D^{25}$ −33° (*c* 0.15, MeOH), white solid, mp 118~122°C and Rf 0.18 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.45 in EtOAc-MeOH (1:1) and Rf 0.00 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 212 (12,000), 254 (12,000) unchanged upon addition of acid or base. IR ($CDCl_3$) cm^{-1} 3526, 3382, 2972, 2933, 2877, 2856, 1712, 1464, 1440, 1385, 1370, 1308, 1287, 1274, 1244, 1228, 1184, 1170, 1150, 1133, 1094, 1082, 1054, 1036, 1009, 979.

Dunaimycin D4S: $[\alpha]_D^{25}$ −53° (*c* 0.36, MeOH), white solid, mp 133~140°C and Rf 0.31 in CH_2Cl_2-MeOH-NH_4OH (95:5:1), Rf 0.42 in EtOAc-MeOH (1:1) and Rf 0.00 in EtOAc. UV λ_{max}^{MeOH} nm (ε) 210 (sh, 11,700) unchanged upon addition of base. Upon addition of acid, changed irreversibly to λ_{max}^{MeOH} nm (ε) 210 (11,000), 254 (>20,000). IR ($CDCl_3$) cm^{-1} 3458, 2938, 2978, 2973, 1712, 1464, 1456, 1440, 1422, 1409, 1384, 1368, 1309, 1286, 1273, 1230, 1217, 1186, 1170, 1151, 1131, 1119, 1081, 1053, 1039,

1015, 979.

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