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Structure Elucidation of New Ascomycins Produced by Genetic Engineering

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Abstract Three new ascomycins produced by genetic engineering of *Streptomyces hygroscopicus* ATCC 14891 have been purified and characterized. Replacement of the 13-methoxyl group of ascomycin was accomplished by substitution of the corresponding acyltransferase domain of the polyketide synthase with a domain specific for either malonyl-CoA or methylmalonyl-CoA. The strain containing the methylmalonyl-specific acyltransferase domain produced a compound with properties consistent with those expected for 13-demethoxy-13-methylascomycin. NMR analysis revealed this material to be predominantly the *cis* amide rotamer, similar to ascomycin. The strain containing the malonyl-specific acyltransferase domain produced a mixture of two compounds, 13-demethoxyascomycin and the 9,14-hemiacetal isomer of 13-demethoxyascomycin, in nearly equal amounts. NMR analysis revealed both compounds to be predominantly the *trans* amide rotamers.

Keywords ascomycin, FK520, polyketide, macrolide, *Streptomyces hygroscopicus*, genetic engineering

Introduction

Ascomycin (FK520, **1**) is a macrolide immunosuppressant produced by *Streptomyces hygroscopicus* var. *ascomyceticus*. Ascomycin and the related macrolide, FK506, have methoxyl substituents at positions 13 and 15, which in addition to being primary sites of metabolism by cytochrome P450 oxidases [1], also interact strongly with calcineurin when the compounds form FK506/520-FKBP12-calcineurin ternary complexes [2]. Removal or replacement of these methoxyl substituents might therefore provide analogues of ascomycin and FK506 having improved pharmacological and/or calcineurin-independent neuroregenerative properties [3].

We have recently demonstrated that the 13- and 15-methoxyl groups of ascomycin derive from incorporation of unusual extender units by the polyketide synthase (PKS) rather than through hydroxylation and methylation of a canonical malonyl-CoA extender unit [4]. Replacement of the acyltransferase domain of the ascomycin PKS responsible for introducing the 13-methoxyl substitution by domains with more standard specificities (*e.g.*, malonyl- and methylmalonyl-CoA) was found to produce novel ascomycin analogues. We report here the characterization of three new ascomycin analogues obtained in this manner, 13-demethoxy-13-methylascomycin (**2**), 13-demethoxy-ascomycin (**3**), and the 9,14-hemiacetal isomer of 13-demethoxyascomycin (**4**).

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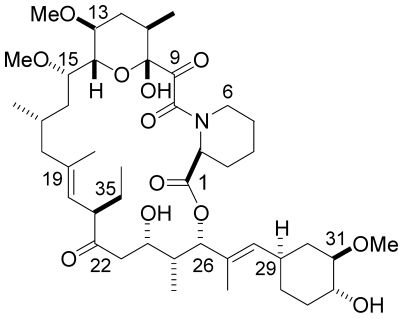
Materials and Methods

Mass Spectrometry

Detection of ascomycin analogues by LC-MS using a PE-Sciex API-100LC mass spectrometer has been described elsewhere [3]. HR-MS spectra were measured by flow inject analysis on an Applied Biosystems Mariner time-of-flight mass spectrometer equipped with a turbo-ionspray source and operated in positive ion mode.

NMR Spectroscopy

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data were recorded in $CDCl_3$ solution at 300 K with a Bruker DRX 400 spectrometer equipped with either a Bruker QNP or a



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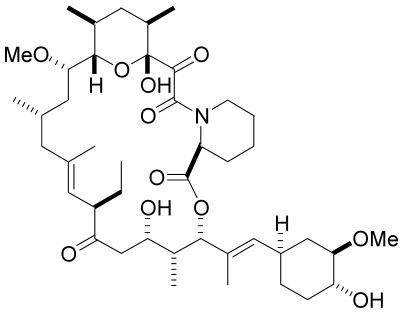

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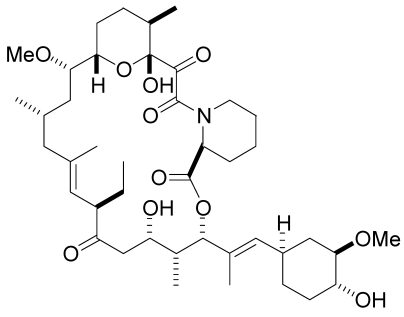

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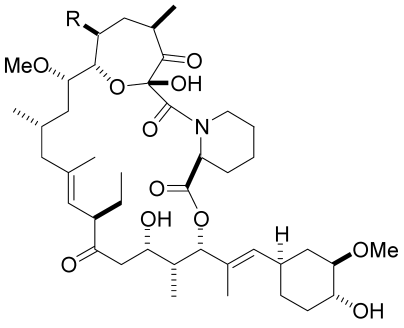

4 R = H
5 R = OMe

Nalorac IDG400-5B indirect detection z-axis gradient probe head. Chemical shifts in $CDCl_3$ solution were referenced to δ 7.26 (residual $CHCl_3$) and 77.0 (center line of $CDCl_3$) for ^1H and ^{13}C spectra, respectively.

13-Demethoxy-13-methylascomycin (2)

Compound **2** was prepared and isolated as described elsewhere [3～5]. HR-MS $(M+Na)^+$ *m/z* 798.4785 (required for $C_{43}H_{69}NO_{11}Na$, 798.4763).

13-Demethoxyascomycin (3) and Oxepane (4)

13-Demethoxyascomycin was prepared as described elsewhere [4, 5]. A typical isolation of **3** and **4** from a 100-liter fermentation follows. Celite Hyflo (10 kg) and MeOH (100 liters) were added to the fermentation broth (100 liters) and mixed. After standing for 1 hour, the methanolic suspension was filtered and the cake was washed with MeOH. The filtrate and cake wash were combined and diluted to 50% (v/v) MeOH - H_2O. The diluted solution was filtered and loaded onto an HP20 column (15 liters). The column was washed with 50% (v/v) MeOH - H_2O (30 liters) and eluted with 100% MeOH (80 liters). Fractions were analyzed by HPLC, and those containing **3** and **4** were pooled. The product pool was diluted to 50% (v/v) MeOH - H_2O and loaded onto a pre-equilibrated column packed with HP20SS resin (11 liters). The column was washed with 50% (v/v) MeOH - H_2O (7 liters) and eluted with increasing concentrations of MeOH in water. Fractions containing **3** and **4** were pooled and diluted to 50% (v/v) MeOH - H_2O. The solution was loaded onto a C18 column (2.1 liters) that was pre-conditioned with 50% (v/v) MeOH - H_2O, and eluted first with 70% (v/v) MeOH - H_2O, followed by 73% (v/v) MeOH - H_2O. Fractions containing **3** and **4** were passed through a solvent-exchange column (C18, 500 ml) and concentrated to yield a brown solid (4.04 g). This material was further purified by preparative HPLC on a Metachem Polaris C18A column (10 μm, 21×250 mm) using 55% (v/v) CH_3CN - H_2O as the mobile phase at a flow rate of 20 ml/minute. Fractions containing **3** and **4** were combined, passed through a solvent-exchange column, and evaporated to solids to afford 2.73 g (95% chromatographic purity). The isomeric forms were separated by reversed-phase HPLC as follows. A 100-mg sample of the partially-purified mixed isomers was dissolved in 1 ml of CH_3CN and centrifuged to remove insolubles. The supernatant was injected in 4 batches onto a 20×50 mm HPLC column (5 μm MetaChem Inertsil ODS-3) equilibrated in 50% (v/v) CH_3CN - H_2O containing 0.1% AcOH running at 8 ml/minute. A linear gradient to 100% CH_3CN was run over 10 minutes, with UV detection using a photodiode array detector. Fractions corresponding to the two peaks were collected on ice. Some loss of the later-

eluting compound was accepted in order to minimize tailing from the earlier-eluting compound. Corresponding fractions from the 4 runs were pooled and evaporated quickly to dryness on a rotary evaporator connected to an oil pump, keeping the contents of the flask just above the freezing point. The residues were dissolved in dry CH_3CN and re-evaporated, then placed in a vacuum desiccator over KOH pellets for 16 hours to remove traces of AcOH. This process yielded 35 mg of the earlier-eluting **3**, and 10 mg of the later-eluting **4**. HR-MS for **3** $(M+Na)^+$: *m/z* 784.4625 (required for $C_{42}H_{67}NO_{11}Na$, 784.4606). HR-MS for **4** $(M+Na)^+$: *m/z* 784.4634 (required for $C_{42}H_{67}NO_{11}Na$, 784.4606).

Results and Discussion

13-Demethoxy-13-methylascomycin (2)

A strain of *S. hygroscopicus* ATCC 14891 containing a mutated ascomycin PKS where the acyltransferase domain of module 8 was replaced by the methylmalonyl-CoA-specific acyltransferase domain from module 2 of the erythromycin PKS was found by LC-MS analysis to produce a new compound in place of ascomycin [3, 4]. The LC-APCI-mass spectrum of this compound (Fig. 1) revealed a series of fragment ions resulting from up to three dehydrations and one loss of MeOH from the parent $[M+H]^+$ together with a diagnostic fragment ion at *m/z* 549, each of which is observed at 16 amu less than the corresponding fragments observed in the APCI-mass spectrum of ascomycin. The diagnostic fragment ion is believed to arise from loss of the C25∼C34 segment of the molecule as indicated.

This compound was isolated, and HR-MS measurements indicated its molecular formula to be $C_{43}H_{69}NO_{11}$, one oxygen atom less than ascomycin.

As is typical for ascomycin analogues, the ^{1}H- and ^{13}C-NMR spectra of **2** in $CDCl_3$ displayed two sets of signals, in a ratio of approximately 2 : 1, corresponding to *cis-trans* rotameric forms about the amide bond. Preliminary inspection of the NMR spectra of **2** revealed signals for only two methoxyl groups and that it differed from **1** in the C-10 to C-16 region. The structure was determined by detailed analysis of NMR data, which in addition to ^{1}H and ^{13}C spectra, included DEPT, gHMBC, multiplicity-edited HSQC, gCOSY, and TOCSY experiments. In the discussion that follows, chemical shifts are given for the major rotamer. One of the methoxyl groups (δ_H 3.29, δ_C 57.0; 15-OMe) displayed an HMBC corrrelation to a methine signal at δ 76.7 (C-15). The proton attached to this carbon, resonating at δ 3.40 (H-15), was weakly coupled to a proton at δ 3.57 in the COSY and TOCSY spectra, which was assigned as H-14 based on it showing an HMBC correlation to C-10 (δ 97.6). HMBC crosspeaks were also observed from H-14 to a methine (δ 30.6), a methylene (δ 36.9), and a methyl group (δ 17.2). Two methyl doublets also displayed long-range heteronuclear couplings to the methylene resonance at δ 36.9, and an HMBC correlation from one of the methyl groups to C-10 established the substitution about the pyran ring. The remaining NMR shifts were in good agreement for those observed and reported [6] for **1** (Tables 1 and 2). The coupling pattern of H-14 appears as a doublet of doublet (J=10.0, 1.0 Hz), with the large coupling being to H-13. A coupling constant of this magnitude requires a diaxial orientation of H-13 and H-14 and suggests the stereochemistry at C-13 is as indicated [7].

The chemical shifts for C-2 and C-6 are diagnostic for the amide rotamer, as these are most highly affected by the orientation of the adjacent C-9 carbonyl group [8]. The corresponding chemical shift differences between the *trans* and *cis* rotamers of **1** and **2** for C-2 and C-6 are quite comparable, as are the absolute values of the chemical shifts (Table 2). Thus the major rotamer of **2** in $CDCl_3$ solution is expected to be *cis*, as is observed for **1**.

13-Demethoxyascomycin (3) and Its Oxepane (4)

A strain of *S. hygroscopicus* ATCC 14891 containing a mutated ascomycin PKS where the acyltransferase domain of module 8 was replaced by the malonyl-CoA-specific acyltransferase domain from module 3 of the rapamycin PKS was found to produce two new compounds [4]. Their LC-APCI mass spectra were essentially identical and consistent with a 13- or 15-demethoxylated ascomycin analogue (Fig. 1), as were HR-MS measurements. The ^{13}C-NMR spectrum of the mixture of compounds revealed the presence of four sets of resonances, apparently resulting from the *cis* and *trans* amide rotamers of two isomeric ascomycins. The two compounds could be separated by HPLC, but were found to interconvert upon standing at ambient temperature in aqueous acetonitrile within a few hours. Further examination revealed the two isomers to be quite stable in non-aqueous media, and so a separation protocol was developed to provide the two isomers of sufficient purity to allow for NMR characterization. The two isomers were separated using a water - acetonitrile gradient with a short (50 mm) preparative column at ambient temperature. The collected fractions were immediately dried on a rotary evaporator under high vacuum and low temperature, then dried under vacuum overnight. The ^{13}C-NMR spectrum of **3** revealed a mixture of two forms in a ratio of approximately 2 : 1, with

Table 1 ^{1}H NMR chemical shift assignments for **1** and major rotamers of **2**∼**4**

Position	**1** (major)[a]	**2**	**1** (minor)[a]	**3**	**4**
2	4.61	4.69 (d, 5.0)	5.00	5.12 (d, 5.0)	5.06 (d, 4.0)
3	2.09	2.08	2.33	2.27	2.20 (2H)
	1.99	1.91	1.80	1.58	
4	1.78	1.74	1.78	1.73	1.71
	1.43	1.39	1.43	1.23	1.43
5	1.78	1.73	1.78	1.54	1.61
	1.43	1.45	1.43	1.46	1.52
6	4.43	4.44 (d, 13.5)	3.73	3.53 (d, 13.0)	4.57 (d, 13.0)
	3.02	3.02	3.29	3.19	2.98
9-OH					5.36 (s)
10-OH	4.26	4.17 (s)		4.54 (s)	
11	2.19	2.10	2.31	2.23	3.12
11-Me	1.00	0.93	0.97	0.83 (d, 6.5)	1.17 (d, 6.0)
12	2.18	1.32	2.13	2.60	1.80
	1.48	1.65	1.54	1.52	1.23
13	3.40	1.94	3.45	1.58	1.72
				1.31	1.46
13-Me	3.39	0.88	3.38		
14	3.68	3.57 (dd, 10.0, 1.0)	3.88	3.92 (dd, 12.0, 7.0)	3.39
15	3.58	3.40	3.58	3.26	3.27
15-OMe	3.31	3.29 (s)	3.34	3.29 (s)	3.24
16	1.59	1.58	1.59	1.50 (2H)	1.48
	1.06	1.06	1.35		0.98
17	1.70	1.72	1.70	1.80	1.84
17-Me	0.94	0.90	0.84	0.89 (d, 7.0)	0.94 (d. 6.0)
18	2.18	2.17	2.18	2.08	2.06
	1.82	1.81	1.95	1.87	1.87
19-Me	1.60	1.60	1.63	1.69	1.63
20	5.02	5.02 (d, 9.5)	5.02	4.85 (d, 10.0)	4.81 (d, 10.0)
21	3.21	3.23	3.18	3.12	3.17
23	2.79	2.77 (dd, 16.0, 3.0)	2.73	2.74 (dd, 18.0, 2.0)	2.89
	2.09	2.10	2.33	2.45 (dd, 18.0, 7.0)	2.23
24	3.92	3.89	3.96	3.82	3.80
25	1.91	1.87	1.91	1.78	1.83
25-Me	0.88	0.89	0.92	0.89 (d, 7.0)	0.85 (d, 8.0)
26	5.33	5.31 (d, 2.1)	5.21	5.19 (d, 7.0)	5.15
27-Me	1.63	1.63	1.67		1.55
28	5.10	5.09 (d, 9.0)	5.06	5.22 (d, 9.0)	5.15
29	2.29	2.28	2.29	2.23	2.19
30	2.06	2.03	2.06	2.03	1.96
	0.97	0.95	0.97	0.93	0.91
31	3.02	3.00	3.02	2.93	2.95
31-OMe	3.41	3.40 (s)	3.41	3.35 (s)	3.31
32	3.40	3.40	3.40	3.35	3.34
33	2.01	2.00	2.01	1.95	1.91
	1.37	1.36	1.37	1.30	1.31
34	1.63	1.62	1.63	1.55	1.52
	1.06	1.03	1.06	1.06	1.05
35	1.78	1.74	1.75	1.71	1.69
	1.48	1.40	1.49	1.36	1.30
36	0.87	0.87	0.87	0.79 (t, 7.5)	0.76 (t, 7.0)

[a] Reported values [6].

Table 2 ^{13}C chemical shift assignments for **1** and major rotamers of **2**∼**4**

Position	**1** (major)[a]	**2**	**1** (minor)[a]	**3**	**4**
1	169.0	169.2	168.7	169.3	169.8
2	56.6	56.6	52.7	52.2	51.6
3	27.6	27.9	26.2	26.6	25.5
4	21.1	21.1	20.8	21.3	20.9
5	24.2	24.5	24.5	24.9	25.2
6	39.2	39.3	43.9	44.7	43.7
8	164.7	164.9	165.8	165.9	167.4
9	196.1	196.7	192.7	196.3	98.2
10	97.0	97.6	98.7	98.9	210.1
11	34.6	34.9	33.6	34.8	43.3
11-Me	16.2	16.3	16.0	15.8	16.8
12	32.7	36.9	32.5	34.8	35.5
13	73.7	30.6	73.7	27.0	27.4
13-Me	56.3	17.2	56.0		
14	72.9	75.5	72.3	73.8	77.0
15	75.2	76.7	76.6	82.1	83.0
15-OMe	57.0	57.0	57.5	57.9	56.5
16	33.0	32.9	35.5	34.4	32.9
17	26.3	26.5	26.0	27.4	29.7
17-Me	20.4	20.3	19.5	21.8	21.8
18	48.7	48.8	48.5	47.0	48.2
19	138.8	138.5	139.6	139.3	138.8
19-Me	15.8	15.9	15.7	17.6	16.8
20	123.1	123.3	123.3	124.5	123.7
21	54.7	54.4	55.0	54.9	54.5
22	213.4	213.4	213.4	212.0	212.7
23	43.2	43.5	43.6	44.9	44.3
24	70.0	70.2	69.0	67.7	69.6
25	39.8	39.9	40.4	39.4	39.1
25-Me	9.6	9.6	9.9	9.9	9.6
26	77.2	77.2	77.9	81.0	77.8
27	132.3	132.4	131.8	131.8	131.4
27-Me	14.1	14.1	14.2	12.9	13.6
28	129.7	129.8	129.6	133.0	130.4
29	34.9	34.9	34.9	34.9	34.9
30	34.9	34.9	34.8	34.4	34.6
31	84.2	84.2	84.2	84.1	84.2
31-OMe	56.6	56.6	56.6	56.5	56.5
32	73.5	73.6	73.5	73.4	73.5
33	31.2	31.3	31.2	31.2	31.2
34	30.6	30.6	30.6	30.5	30.5
35	24.5	24.6	24.5	23.6	23.5
36	11.6	11.7	11.7	11.7	11.7

[a] Reported values [6].

chemical shift patterns again suggestive of the *cis* and *trans* amide rotamers typical of ascomycin-like compounds. The assignments for the ^{1}H and ^{13}C resonances are listed in Tables 1 and 2. The structure was determined to be 13-demethoxyascomycin (**3**) as follows (chemical shifts are given for the major rotamer). The two methoxyl groups were assigned as being at positions 31 and 15 based on HMBC, HSQC, and COSY correlations and the APCI-MS fragementation pattern. For the 15-OMe, an HMBC correlation connected the proton resonance at δ 3.92 with the hemiacetal carbon signal at δ 98.9, establishing δ 3.92 as being due to H-14. H-14 in turn showed COSY correlations to a proton at δ 3.26, as well as an HMBC correlation to the attached carbon at δ 82.1. As the δ 3.26 proton showed a strong HMBC correlation to a methoxyl carbon at δ 57.9, this established the resonances of the adjacent methoxylated carbon. This was established as being at C-15 based on HMBC correlations between δ 57.9 and a geminal proton pair at δ 1.50, assigned as the H-16 pair based upon a further HMBC correlation to a methine carbon at δ 27.4, assigned as C-17. If the methoxyl were at C-13, this methine would be C-11, but C-11 can be assigned to δ 34.8 based on the observed HMBC correlation from the C-10-hemiacetal proton at δ 4.54 to it. The resonance for C-13 was identified most easily through a combination of HSQC and COSY spectra. The resonance for H-14 showed a strong COSY correlation with a proton at δ 1.31; HSQC revealed this to be one of a geminal pair of protons attached to carbon resonating at δ 27.0, which was thus identified as C-13. The NMR data thus supports the assignment of **3** as 13-demethoxyascomycin.

A comparison of the chemical shifts for the major rotamer of **3** and ascomycin reveal very close similarity, with two exceptions. The first is the expected deviation around C-13 due to the absence of the 13-methoxyl group in **3**. The second, unexpected deviation is around C-2 and C-6 of the pipecolic acid moiety. The difference in δ_C chemical shifts between the major and minor rotamers for C-2 (major−minor $\Delta\delta$ −4.16) and C-6 (major−minor $\Delta\delta$ +5.44) are comparable in magnitude to those observed for **1** and **2**, but are opposite in sign. This suggests that the *trans*-rotamer is the major form for **3**, unlike for **1** and **2** where the *cis*-rotamer predominates.

NMR analysis of the later-eluting peak revealed it to contain the 9,14-hemiacetal form of 13-demethoxy-ascomycin (**4**), predominantly as a single rotamer; the previously reported 9,14-hemiacetal form of ascomycin (**5**) exists as a 3 : 1 mixture of rotamers [9]. The assignments for the ^{1}H and ^{13}C resonances are listed in Tables 1 and 2. Several resonances are diagnostic for the 9,14-hemiacetal structure. First, there are no ^{13}C resonances for conjugated ketone carbonyls. Resonances for two ketones observed at δ 210.1 and 212.7, and resonances for a lactone and a

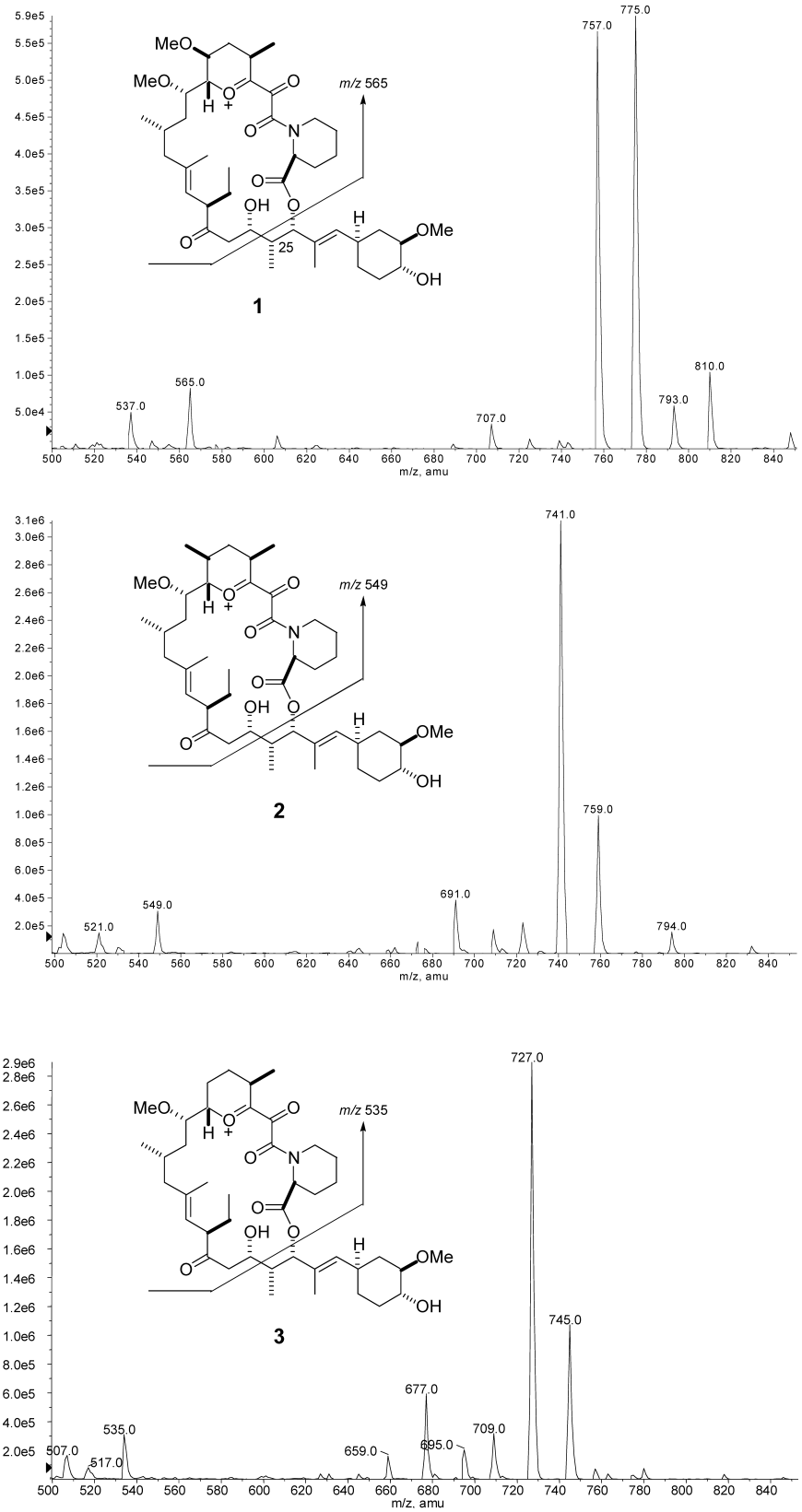

Fig. 1 HPLC-APCI mass spectra of ascomycin (**1**), 13-demethoxy-13-methylascomycin (**2**), and 13-demethoxyascomycin (**3**).

lactam at δ 169.8 and 167.4, respectively, are the sole carbonyl signals. Second, the hemiacetal hydroxyl proton at δ 5.36 shows HMBC correlations to the ketone at δ 210.1, the amide carbonyl function at δ 167.4, the hemiacetal carbon at δ 98.2, and a methine carbon at δ 43.3. This is consistent only with the proposed 9,14-hemiacetal structure. Based on the chemical shifts of the pipecolic acid residue carbons as discussed above, the isolated form of **4** again appears to be predominantly the *trans* amide rotamer. Several resonances for the other rotamer could be determined by difference from the spectra of the isomeric mixture and those of the separated forms, and are consistent with the minor form being the *cis*-amide rotamer (major−minor $\Delta\delta$ −3.94 for C-2; $\Delta\delta$ +4.34 for C-6).

The reasons for the change in preferred rotamer between the 13-substituted (*cis*-preferred) and 13-unsubstituted (*trans*-preferred) ascomycins are unclear. The apparent difference in equilibrium constant between the 10,14-hemiacetal and 9,14-hemiacetal forms of the ascomycin macrocycle is also curious. For the 13-substituted ascomycins, the 10,14-hemiacetal isomer is strongly preferred (95 : 5 for ascomycin [9]), whereas for 13-demethoxyascomycin the two forms exist in essentially equal amounts. This can be rationalized as resulting from a 1,3-*syn*-pentane-like interaction between the 13- and 15-substituents which become increasingly important in the 10,14-hemiacetal isomer. This interaction is relieved upon removal of the 13-substituent.

In these regards it is of interest that rapamycin, which is a naturally occurring 13-unsubstituted immunophilin, shares the *trans*-rotamer preference with **3** and is also readily isomerized into the 10,14-hemiacetal form [10]. The 13-substituent thus appears to play a role in stabilization of the biologically active forms of the ascomycins and selection of the preferred rotamer.

In summary, genetic engineering has provided novel analogues of ascomycin. The rationally designed alteration of the 13-substituent via domain substitution within the polyketide synthase provided the expected products, although with some interesting conformational consequences. It is thus likely that genetic engineering of polyketide synthases can be useful not only in altering the substitution pattern of polyketides, but also in controlling the conformational and tautomeric properties of a natural product leading to multiple means of improving biological activity.

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