Antimycins, Inhibitors of ATP-Citrate Lyase, from a *Streptomyces* sp.

COLIN J. BARROW,*,† JOSEPH J. OLEYNEK, VINCENT MARINELLI, HAO H. SUN, PAUL KAPLITA, DAVID M. SEDLOCK, AMANDA M. GILLUM, CHRISTOPHER C. CHADWICK and RAYMOND COOPER

Sterling Winthrop Pharmaceuticals Research Division, 25 Great Valley Parkway, Malvern, PA 19355, U.S.A.

(Received for publication March 31, 1997)

A related group of compounds belonging to the antimycin class of antibiotics was found in culture broth produced by a *Streptomyces* species. The group includes known antimycins A_1, A_2, A_3 and A_4, and new antimycins A_7 and A_8. These compounds inhibit ATP-citrate lyase with *Ki* values of 4 to 60 μM against the substrate magnesium citrate. The structures of the new antimycins were determined by spectroscopic analyses.

The ATP-dependent formation of acetyl-CoA from citrate is catalysed by a cytoplasmic enzyme called ATP-citrate lyase. This reaction provides the major source of acetyl-CoA for the biosynthesis of fatty acids and cholesterol.[1] Therefore, an ATP-citrate lyase inhibitor is expected to block *in vivo* lipogenesis and cholesterogenesis, leading to beneficial lowering of serum triglyceride and LDL-cholesterol levels.

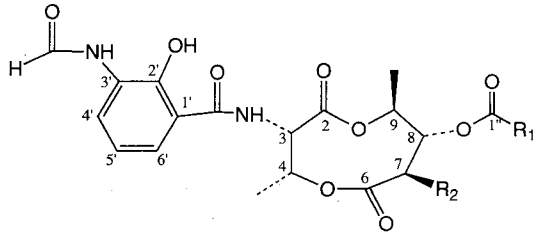
We recently reported the isolation of a potent and competitive inhibitor of the substrate magnesium citrate (Mg. citrate).[2] In the course of screening for other ATP-citrate lyase inhibitors we isolated a series of antimycins which also inhibit the substrate Mg. citrate. The antimycins were first isolated from a *Streptomyces* sp. in 1949.[3] Structure determination of antimycin A_1 was completed in 1961,[4,5] and the absolute configuration established by KINOSHITA *et al.*[6] More recently the isolation of related antimycins A_2 to A_6 was reported, and each shown to be a mixture of two closely related isomers.[7] The class is characterised by the presence of a carboxy phenol amido unit, a nine-membered cyclic bis-lactone, and two alkyl side chains of varying carbon length. Although first identified as antibiotics, more recently antimycins have been reported to inhibit the electron flow in the mitochondrial respiratory chain between cytochromes b and c_1. They have been used extensively to investigate the energy metabolism in eukaryotic organisms, and have been used commercially as fungicides.[7] We now report that antimycins are ATP-citrate lyase inhibitors. Two of these inhibitors, antimycin A_7 and antimycin A_8, are new compounds (Figure 1). In this study we wish to report the fermentation, isolation and structure determination leading to the identification of antimycin A_7 and A_8. We also report the ATP-citrate lyase inhibitory activity of the antimycins.

Materials and Methods

Microorganism

An actinomycete culture, deposited in Sterling Winthrop's Culture Collection, Collegeville, PA, USA, and

Fig. 1. Structures for antimycins A_1, A_2, A_3, A_4, A_7, and A_8.



Antimycin A_{1a} $R_1 = CH(CH_3)CH_2CH_3$, $R_2 = (CH_2)_5CH_3$
Antimycin A_{1b} $R_1 = CH_2CH(CH_3)_2$, $R_2 = (CH_2)_5CH_3$
Antimycin A_{2a} $R_1 = CH(CH_3)_2$, $R_2 = (CH_2)_5CH_3$
Antimycin A_{2b} $R_1 = CH_2CH_2CH_3$, $R_2 = (CH_2)_5CH_3$
Antimycin A_{3a} $R_1 = CH(CH_3)CH_2CH_3$, $R_2 = (CH_2)_3CH_3$
Antimycin A_{3b} $R_1 = CH_2CH(CH_3)_2$, $R_2 = (CH_2)_3CH_3$
Antimycin A_{4a} $R_1 = CH(CH_3)_2$, $R_2 = (CH_2)_3CH_3$
Antimycin A_{4b} $R_1 = CH_2CH_2CH_3$, $R_2 = (CH_2)_3CH_3$
Antimycin A_{7a} $R_1 = CH(CH_3)_2$, $R_2 = (CH_2)_2CH(CH_3)_2$
Antimycin A_{7b} $R_1 = CH_2CH_2CH_3$, $R_2 = (CH_2)_2CH(CH_3)_2$
Antimycin A_{8a} $R_1 = CH(CH_3)CH_2CH_3$, $R_2 = (CH_2)_2CH(CH_3)_2$
Antimycin A_{8b} $R_1 = CH_2CH(CH_3)_2$, $R_2 = (CH_2)_2CH(CH_3)_2$

† Present address: School of Chemistry, University of Melbourne, Parkville, Victoria 3052, Australia.

designated as SC2221, was isolated from a soil collected at Mountain Dandah, Nantu County, Taiwan. The culture was examined by standard fatty acid methyl ester analysis to determine the similarity grouping.[8] Results from this analysis classified this organism as a *Streptomyces* sp.

Fermentation

Seed culture for the inoculation of production media was typically prepared as follows: 7~10 day old agar slants were washed with sterile water and the culture wash was used as inoculum into seed medium. Alternatively, frozen culture was used directly as inoculum. In both cases approximately one ml was transferred from stock to the 30 ml of seed medium contained in a 250 ml shake flask. The seed medium consisted of (% w/v): glucose 2.0, Pharmamedia (Traders Protein) 1.5, (NH_4)-SO_4 0.3, $ZnSO_4 \cdot 7H_2O$ 0.003, $CaCO_3$ 0.4, yeast extract 0.5 (pH adjusted to 7.0 prior to sterilisation). The flask was incubated under humidity control (75%) at 27°C and aerated at 220 rpm in a New Brunswick Innova 4900 environmental shaker for 24~48 hours. One ml of seed culture was transferred to 30 ml production medium in 250 ml shake flasks containing (% w/v): glycerol 2.0, dextrin 2.0, soytone 1.0, yeast extract 0.3, $(NH_4)_2SO_4$ 0.2, soytone 1.0, $CaCO_3$ 0.2 (pH adjusted to 7.0 prior to sterilisation). The culture was harvested after 4 days incubation in the production medium. Production of antimycins was monitored both by bioassay and by reverse phased HPLC.

ATP-Citrate Lyase Binding Assay

Rat liver ATP-citrate lyase was purified as previously described[2] and incubated for 15~60 minutes at 37°C in 0.40 ml of 200 mM Tris-Cl (pH 8.4), 200 mM hydroxylamine, 20 mM tripotassium citrate, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 5 mM ATP and 0.1 mM CoA. The reaction was quenched by mixing with 0.48 ml of 20% trichloroacetic acid. Following mixing with 0.12 ml of 2 M $FeCl_3$, acetyl hydroxamate was determined by measurement of absorbance at 520 nm.[9] A standard curve was generated using succinyl hydroxamate as described.[10]

Isolation of Antimycins A_1, A_2, A_3, A_4, A_7, A_8

The whole culture (1 litre) was extracted with ethyl acetate (1 litre, 2×). The dried ethyl acetate extract (560 mg) was then extracted with hexane. The hexane insoluble material (108 mg) was further separated using preparative HPLC on an ODS C-18 reverse-phased silica gel column (YMC-Pack, 15×250 mm; YMC Co., Ltd.) using a gradient, from 75 : 25 acetonitrile - water to 90 : 10 acetonitrile - water over 30 minutes at a flow rate of 3 ml/minute. Antimycins eluted in the order A_4, A_7, A_3, A_8, A_2, A_1 (Figure 2).

Physical and Spectroscopic Data for Antimycins A_7 and A_8

Spectroscopic data for antimycins $A_1 \sim A_4$ were in agreement with literature data.[11~14]

Antimycin A_7: white solid; $[\alpha]_D^{25}$ +72° (*c* 0.5, chloroform); HRFAB-MS MH^+ 521.2499 (calculated for C_{26}-

Fig. 2. Preparative HPLC separation of antimycins, using an ODS column with a gradient from 75:25 acetonitrile - water to 90:10 acetonitrile - water over 30 minutes.

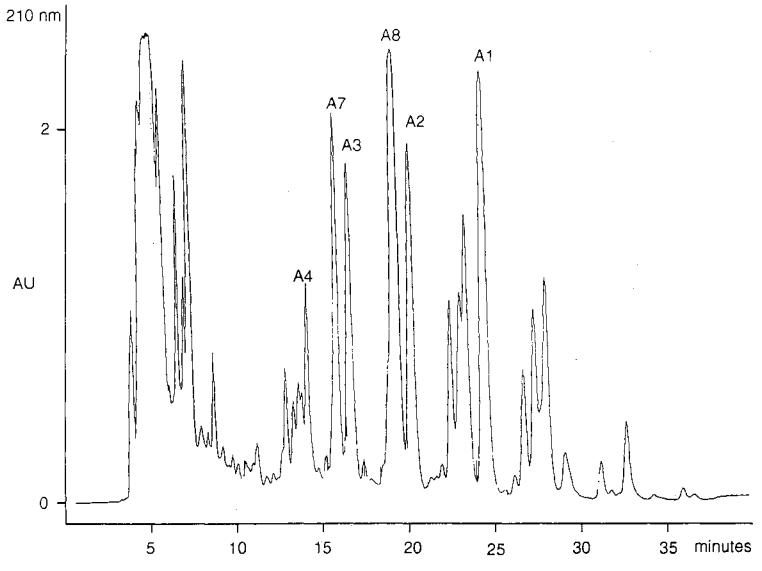


Table 1. ^{1}H NMR spectral data (in $CDCl_3$) for antimycins A_1, A_2, A_3, A_4, A_7, and A_8.

Atom	A_1	A_2	A_3	A_4	A_7	A_8
3	5.32 t (7.7)	5.31 t (7.7)	5.32 t (7.7)	5.29 t (7.7)	5.27 t (7.7)	5.28 t (7.6)
4	5.75 p (7.0)	5.75 p (7.2)	5.74 p (6.8)	5.73 p (6.9)	5.73 p (6.8)	5.73 p (7.0)
7	2.52 m	2.51 m	2.52 m	2.53 m	2.50 m	2.49 m
8	5.11 t (9.9)	5.09 t (10.0)	5.11 t (9.9)	5.08 m	5.09 t (9.8)	5.09 t (9.9)
9	4.97 m	4.98 m	4.99 m	4.98 m	5.00 m	4.99 m
4-Me	1.30 d (6.7)	1.31 d (6.7)	1.30 d (6.7)	1.30 d (6.7)	1.31 d (6.7)	1.31 d (6.6)
9-Me	1.28 d (6.3)	1.27 d (6.2)	1.28 d (6.3)	1.28 d (6.1)	1.29 d (6.2)	1.29 d (6.0)
4′	8.54 d (8.1)	8.54 d (7.9)	8.53 d (8.0)	8.54 d (8.1)	8.54 d (8.0)	8.54 d (8.1)
5′	6.89 t (8.1)	6.90 t (8.1)	6.90 t (8.1)	6.91 t (8.1)	6.91 t (8.0)	6.92 t (8.1)
6′	7.23 d (8.3)	7.23 d (8.2)	7.23 d (8.2)	7.23 d (8.2)	7.23 d (8.0)	7.23 d (8.3)
α	1.68 m, 1.23 m	1.72 m, 1.23 m	1.68 m, 1.24 m	1.72 m, 1.25 m	1.68 m, 1.36 m	1.67 m, 1.36 m
β	1.23 m	1.23 m	1.24 m	1.25 m	1.17 m	1.25 m
γ	1.23 m	1.23 m	1.24 m	1.25 m	1.50 m	1.47 m
δ	1.23 m	1.23 m	0.85 t (7.1)	0.86 t (7.2)	0.84 d (6.6)	0.84 d (6.6)
ε	1.23 m	1.23 m			0.84 d (6.6)	0.84 d (6.6)
ζ	0.85 t (7.1)	0.85 t (7.0)				
*H*CO	8.50 d (1.7)	8.50 d (1.6)	8.50 d (1.7)	8.49 d (1.5)	8.49 d (1.3)	8.50 s
HCON*H*	8.03 bs	8.02 bs	8.02 bs	7.92 bs	7.88 bs	7.91 bs
1′-CON*H*	7.09 d (7.7)	7.08 d (7.7)	7.08 d (7.7)	7.05 d (7.7)	7.04 d (7.7)	7.05 d (7.6)
2′-OH	12.58 s	12.59 s	12.59 s	12.59 s	12.60 s	12.61 s
component a						
2″	2.41 m	2.35 t (7.4)	2.41 m	2.34 t (7.3)	2.35 t (7.4)	2.42 m
3″	1.75 m, 1.50 m	1.66 m	1.75 m, 1.48 m	1.68 m	1.66 m	1.71 m, 1.49 m
4″	0.94 t (7.4)	0.97 t (7.4)	0.94 t (7.5)	0.98 t (7.4)	0.98 t (7.3)	0.94 t (7.4)
5″	1.18 d (7.0)		1.17 d (7.0)			1.19 d (7.1)
component b						
2″	2.24 d (6.8)	2.58 m	2.24 d (6.6)	2.60 m	2.61 hep (7.0)	2.24 d (6.7)
3″	2.13 m	1.20 d (7.0)	2.13 m	1.21 d (6.9)	1.21 d (7.0)	2.14 m
4″	0.98 d (6.6)	1.21 d (7.0)	0.98 d (6.6)	1.20 d (6.9)	1.20 d (7.0)	0.99 d (6.6)
5″	0.98 d (6.6)		0.98 d (6.6)			0.99 d (6.6)

$H_{37}N_2O_9$ is 521.2500); UV (methanol) λ_{max} 227 (ε 38000), 319 (6200); IR ν_{max} 3373, 2958, 1749, 1642, 1532, 1367, 1180, 1147, 748 cm^{-1}; MP 165~168°C; ^{1}H and ^{13}C NMR (Tables 1 and 2).

Antimycin A_8: white solid; $[\alpha]_D^{25}$ +70° (*c* 0.5, chloroform); HRFAB-MS MH^+ 535.2648 (calculated for $C_{27}H_{39}N_2O_9$ is 535.2657); UV (methanol) λ_{max} 227 (ε 37800), 319 (6000); IR ν_{max} 3372, 2958, 1748, 1642, 1533, 1367, 1181, 1147, 737 cm^{-1}; MP 160~164°C; ^{1}H and ^{13}C NMR, Tables 1 and 2.

Results and Discussion

Fermentation

Biosynthesis of the antimycins was reproducible using the fermentation conditions described above. Attempts to increase the titre of antimycins or to alter the ratios of the different homologues by modifying the threonine, soybean oil, olive oil, or Pharmamedia content of the medium were unsuccessful. Fermentation time studies revealed the optimum incubation time to be 96 hours, at which time the pH of the production medium was 8.0. Culture broth harvested at 96 hours yielded concentrations of antimycins, as follows: A_1, 23.2 μg/ml; A_2, 12.7 μg/ml, A_3, 9.3 μg/ml, A_4, 3.5 μg/ml, A_7, 8.5 μg/ml, A_8, 14 μg/ml. These concentrations were determined using quantitative HPLC comparison with commercial standards of A_1 to A_4.

Isolation and Structure Determination of Antimycins

The antimycins were isolated using a bioassay directed isolation, monitoring the inhibitory activity of ATP-citrate lyase. The structures of antimycins A_1, A_2, A_3, and A_4 were identified by comparison with reference compounds obtained from Sigma Chemical Co. The ^{1}H NMR spectrum for antimycin A_7 indicated that A_7 differed from antimycins A_2 and A_4 only at the 7-alkyl sidechain and that antimycin A_7 is a mixture of two closely related compounds, A_{7a} and A_{7b}, which could not be preparatively separated. Antimycins A_1~A_4 are each a mixture of two closely related compounds which have been separated analytically but not preparatively.[11] For antimycin A_7, high resolution mass spectrometry

Table 2. ^{13}C NMR spectral data (in $CDCl_3$) for antimycins A_1, A_2, A_3, A_4, A_7, and A_8.

Atom	A_1	A_2	A_3	A_4	A_7	A_8
2	170.10	170.10	170.10	170.11	170.10	170.09
3	53.69	53.65	53.66	53.70	53.82	53.71
4	70.89	70.90	70.91	70.91	70.94	70.96
6	172.92	172.90	172.93	172.89	172.93	172.93
7	50.15	50.13	50.11	50.14	50.35	50.38
8	75.40	75.35	75.31	75.36	75.39	75.51
9	74.91	74.90	74.92	74.93	74.92	74.92
4-Me	14.96	14.96	14.96	14.97	14.97	14.98
9-Me	17.82	17.80	17.81	17.80	17.82	17.89
1′	112.62	112.63	112.64	112.63	112.63	112.61
2′	150.65	150.64	150.64	150.66	150.67	150.67
3′	127.52	127.51	127.52	127.51	127.51	127.45
4′	124.82	124.81	124.82	124.85	124.84	124.86
5′	118.94	118.95	118.95	119.00	119.00	119.01
6′	120.08	120.07	120.08	120.06	120.08	120.09
HC=O	159.07	159.05	159.06	158.92	158.98	158.96
1′-CONH	169.38	169.38	169.38	169.43	169.41	169.42
α	28.45	28.20	28.14	28.04	26.25	26.36
β	22.43	22.40	22.40	22.38	36.11	36.10
γ	26.96	27.00	29.16	29.21	27.83	27.87
δ	31.44	31.47	13.69	13.72	22.17	22.13
ε	28.92	28.90			22.45	22.18
ζ	13.93	13.97				
component a						
1″	175.21	171.90	175.21	171.63	171.80	175.50
2″	41.27	36.08	41.29	36.10	36.11	41.33
3″	26.46	18.34	26.48	18.37	18.35	26.51
4″	11.66	13.94	11.66	13.68	13.66	11.73
5″	16.72		16.71			16.79
component b						
1″	171.66	175.50	171.66	175.53	175.52	171.65
2″	43.22	34.14	43.23	34.15	34.17	43.26
3″	25.47	18.91	25.47	18.93	18.93	25.50
4″	22.40	18.92	22.38	18.94	18.99	22.43
5″	22.40		22.38			22.43

gave a molecular formula of $C_{26}H_{36}N_2O_9$. This requires that the alkyl side chain has the formula C_5H_{11}. COSY connectivities from a methine at 2.50 ppm (7-H) to methylene hydrogens at 1.68 and 1.36 ppm (H_α), from H_α to a methylene at 1.17 ppm (H_β), from H_β to a methine at 1.47 ppm (H_γ), and from H_γ to a six hydrogen methyl doublet at 0.84 ppm (H_δ and H_ε), indicated that the alkyl side chain is isopentyl (3-methylbutyl). HMBC connectivities from H_δ and H_ε to C_γ and C_β support this side chain structure.

The structure of antimycin A_8 was determined similarly to that of A_7, and differed from antimycins A_1 and A_3 only at the 7-alkyl side chain. The structures of antimycins $A_1 \sim A_4$ and $A_7 \sim A_8$ are shown in Figure 1 and the NMR data in Tables 1 and 2. NMR assignments for all compounds were obtained with the aid of COSY, DEPT, HMQC and HMBC spectra.

Biological Activity of the Antimycins

The antimycins are inhibitors of ATP-citrate lyase against the substrate Mg. citrate with the following *Ki* values: Antimycin A_1, 29.5 μM; antimycin A_2, 4.2 μM; antimycin A_3, 60.1 μM; antimycin A_4, 64.8 μM; antimycin A_7, 55.0 μM; antimycin A_8, 4.0 μM.

Acknowledgments

We thank Panlabs Inc. (Bothell, WA, USA) for initial isolation of the producing culture.

References

1) LOWENSTEIN, J. M.: Citrate and the Conversion of Carbohydrate into Fat. In the Metabolic Roles of Citrate. Biochem. Soc. Symposia, Vol. 27, pp. 61～68, 1968
2) OLEYNEK, J. J.; C. J. BARROW, M. P. BURNS, D. M. SEDLOCK, D. J. MURPHY, P. V. KAPLITA, H. H. SUN, R. COOPER, A. M. GILLUM & C. C. CHADWICK: Anthrones, naturally occurring competitive inhibitors of ATP-citrate lyase. Drug v. Res. 36: 35～42, 1995

3) DUSHEE, B. R.; C. LEBEN, C. W. KEITT & F. M. STRONG: The isolation and properties of antimycin A. J. Am. Chem. Soc. 71: 2436~2437, 1949
4) VAN TAMELEN, E. E.; J. D. DICKIE, M. E. LOOMANS, R. S. DEWEY & F. M. STRONG: The chemistry of antimycin A. X. Structure of the antimycins. J. Am. Chem. Soc. 83: 1639~1646, 1961
5) DIRCH, A. J.; D. W. CAMERON, Y. HARADA & R. W. RICKARDS: The structure of the antimycin-A complex. J. Chem. Soc. 889~895, 1961
6) KINOSHITA, M.; S. ABURAKI & S. UMEZAWA: Absolute configurations of antimycin lactones and antimycin A. J. Antibiotics 25: 373~375, 1972
7) Dictionary of Antibiotics and Related Substances; *Ed.*, BYCROFT, D. W; Chapman and Hall: London, 1988
8) LIPMANN, F. & L. C. TUTTLE: A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. 159: 21~28, 1945
9) TAKEDA, Y.; F. SUZUKI & H. INOUE: ATP Citrate Lyase (Citrate Cleavage Enzyme). Methods Enzymol. 13: 153~160, 1969
10) LIPMANN, F. & L. C. TUTTLE: A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. 159: 21~28, 1945
11) ABIDI, S. L.: High-performance liquid chromatoghraphic separation of subcomponents of antimycin A. J. Chromatoghraphy 447: 65~79, 1988
12) ABIDI, S. L. & B. R. ADAMS: ^{1}H and ^{13}C Resonance designation of antimycin A_1 by two-dimentional NMR spectroscopy. Mag. Res. in Chemistry 25: 1078~1080, 1987
13) SCHILLING, G.; D. BERTI & D. KLUEPFEL: Antimycin A components II. J. Antibiotics 23: 81~90, 1970
14) The Merck Index; *Ed.*, BUDAVARI, S., Merck & Co. Inc., Rathway, 1989