of fast decomposition. TLC analysis of the product on a silica gel plate (pretreated with hexane–ethyl acetate–triethylamine (9:1:0.2) and eluted with ethyl acetate) indicated the product to be 99% pure: UV (MeOH) 270, 280 (40 000), 290 nm. The specific activity (determination of mass by UV) was 39.6 Ci/mmol.

(5*S*,6*R*,7*E*,9*E*,11*Z*,14*Z*)-6-(*S*-L-Cysteinylglycinyl)-5-hydroxy-7,9,11,14-eicosatetraenoic Acid Dipotassium Salt (Leukotriene D_4 (4b) Dipotassium Salt). Cysteinylglycine[40] (1 g, ca. 4.5 mmol; this material contained 1 equiv of lithium chloride) was dissolved in 10 mL of 6:1 methanol–water containing 1.5 mL of triethylamine. To the resulting solution was added 1 g (3 mmol) of LTA_4 methyl ester (**5**). After being stirred at room temperature for 18 h, the solution was diluted with 50 mL of water and most of the methanol was removed in vacuo, at 45 °C. The aqueous residue was freeze-dried. The residue was dissolved in water (30 mL), containing hydroquinone (10 mg), at 10 °C, and the solution was treated with aqueous KOH solution (600 mg in 10 mL). After 5 min at room temperature, water (50 mL) was added, the pH was adjusted to 9 with 0.5 N phosphoric acid, and the mixture was freeze-dried. The residue was dissolved in water and applied to a C_{18}-silica gel reverse-phase chromatography column (30 × 2 cm) packed in water. Elution with water removed the potassium phosphate while 7:3 methanol–water eluted the desired material (monitored by UV). Removal of methanol from the appropriate fractions in vacuo and freeze-drying of the aqueous residue gave 700 mg (40%) of LTD_4 dipotassium salt as a solid: UV (CH_3OH) 268 (ε 39 400), 280 (48 700), 292 nm (36 800). RPLC analysis (30 cm × 4 mm i.d. Waters C_{18}-silica gel; 65% CH_3OH, 35% pH 5.6 NH_4OAc; 280 nm) of this material revealed a purity of at least 94%. This salt was stored at −80 °C under argon, in the dark.

(5*S*,6*R*,7*E*,9*E*,11*Z*,14*Z*)-6-(*S*-Glutathionyl)-5-hydroxy-7,9,11,14-eicosatetraenoic Acid Dipotassium Salt (Leukotriene C_4 (4a) Dipotassium Salt). Glutathione (2 g, 6.5 mmol) was dissolved in 30 mL of 9:1 methanol–water, and the pH of the solution was adjusted to 8 by the addition of triethylamine. LTA_4 methyl ester (**5**, 1.5 g, 4.5 mmol) was added and the resulting mixture was stirred for 2 h at room temperature, then concentrated in vacuo. Flash chromatography[37] of the residue on silica gel (150 mL; CH_3OH–CH_2Cl_2–concd. NH_4OH 10:10:1) yielded 1.02 g of the adduct after concentration in vacuo and freeze-drying of the appropriate fractions (monitored by UV). This material was dissolved in 30 mL of water; the solution was treated with aqueous KOH solution (1 g in 10 mL) and allowed to stand at room temperature for 15 min. The pH of the solution was brought to 9 by the addition of 0.5 N phosphoric acid. The mixture was freeze-dried and the residue was digested with methanol. The insoluble solids were removed by filtration and the filtrate was evaporated in vacuo. The residue was desalted as described in the preceding experiment (30 × 2 cm C_{18}-silica gel RP column) giving 700 mg (21.2%) of LTC_4 dipotassium salt as a colorless solid: UV (CH_3OH) 268 (ε 39 000), 280 (49 400), 291 nm (40 000). Analysis of this material by RPLC (30-cm Waters C_{18}-silica gel; 65% CH_3OH, 35% pH 5.6 NH_4OAc; 280 nm) revealed a purity of at least 95%. This salt was stored at −80 °C, in the dark, under argon.

Anal. Calcd for $C_{30}H_{44}K_2N_3O_6S\cdot 4H_2O$: C, 46.60; H, 6.78; N, 5.43; K, 10.09. Found: C, 46.82; H, 6.51; N, 5.67; K, 10.14.

(5*S*,6*R*,7*E*,9*E*,11*Z*,14*Z*)-6-(*S*-L-Cysteinyl)-5-hydroxy-7,9,11,14-eicosatetraenoic Acid Monopotassium Salt (Leukotriene E_4 (4c) Monopotassium Salt). L-Cysteine methyl ester hydrochloride (2 g, 11.6 mmol) was dissolved in 20 mL of 6:1 methanol–water, and the solution was treated with triethylamine until pH 9 was reached. To this solution was added 2 g (6.0 mmol) of LTA_4 methyl ester (**5**), and after 1 h at room temperature the methanol was evaporated in vacuo. The residue was worked up with ethyl acetate in the usual manner, giving crude LTE_4 dimethyl ester. This material was purified by HPLC (deactivated silica gel,[3e] one column, 1:1 hexane–ethyl acetate containing 3% CH_3OH), giving 2.65 g (94%) of pure diester. The spectral characteristics of this material were identical with those of material prepared by an alternative route.[3e] A solution of this diester (5.57 mmol) in 20 mL of methanol was cooled to 5 °C, treated with KOH solution (2.5 g in 15 mL of H_2O), and stirred at room temperature for 15 min. Water (50 mL) was added and the solution was adjusted to pH 9 by the addition of 0.5 N phosphoric acid. *n*-Butyl alcohol (20 mL) was added and the organic solvents were evaporated in vacuo to yield a clear aqueous solution which was freeze-dried. The residue was digested with methanol (3 × 20 mL) and the methanol extracts were combined and concentrated in vacuo. The residue was dissolved in water and applied to a 30 × 2 cm RP column of C_{18}-silica gel. Desalting was carried out as described above. Elution with 1:4 CH_3OH–H_2O and 4:1 CH_3OH–H_2O gave two fractions (0.4 g and 1.8 g, respectively) after freeze-drying, both of which exhibited the characteristic leukotriene UV chromophore. The major fraction (less polar material—monopotassium salt) was at least 96% pure as determined by RPLC (same conditions as for LTC_4 and LTD_4): UV (CH_3OH) 270 (ε 40 000), 280 (49 400), 291 nm (40 000). In several runs, microanalytical data indicated that the more polar material was the dipotassium salt and the less polar material was the monopotassium salt bis-hydrate. The 1.8 g obtained corresponds to a 62% yield based on a molecular weight of 513 for the latter species. This salt was stored at −80 °C, in the dark, under argon.

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Structure of the Sodium and Potassium Ion Activated Adenosinetriphosphatase Inhibitor L-681,110

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Abstract: Structure proposals for the major component A_1 of the Na^+,K^+-ATPase (EC 3.6.1.3; from porcine cerebral cortex or dog kidney) inhibitor L-681,110 and two minor components A_2 and B_1 (see Figure 4) are presented on the basis of spectroscopic evidence. They represent a new class of 16-membered macrocyclic lactones and are of considerable biological interest in that component A_1 also has comparable activity to the potent anthelmintic avermectins in stimulating GABA release from rat brain synaptosomes. Stereochemical assignments around the tetrahydropyran ring were deduced on the basis of spin–spin coupling constants.

The Na^+,K^+-ATPase [EC 3.6.1.3] inhibitor L-681,110 is produced by *Streptomyces* species MA-5038 and was isolated by solvent extraction of the mycelia.[1] Besides inhibiting ATPase from dog kidney or porcine cerebral cortex, the major component

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Table I. ^1H NMR Assignments of L-681,110 Components A_1, A_2, and B_1 at 25 °C[a]

assignment	A_1 (CD_3OD)	A_2 (CD_3OD)	B_1 ($CDCl_3$)
C2-OCH_3	3.63 s	3.66 s	3.64 s
H3	6.72 s	6.70 s	6.68 s
C4-CH_3	1.98 br s	1.98 br s	1.98 br s
H5	5.90 br d (9)	5.89 br d (9.5)	5.77 br d (~8.5)
H6	2.54 ddq (1.5, 7, 9)	2.53 ddq (2, 7, 9)	2.54 ddq (1.5, 7, 8.5)
C6-CH_3	1.05 d (7)	1.05 d (7)	1.05 d (7)
H7	3.30 dd (1.5, ~8)	3.28 dd (2, 7)	~3.29 obsc
H8	~1.90 m obsc	~1.89 m obsc	~1.87 m obsc
C8-CH_3	0.82 d (7)	0.92 d (7)	0.75 d (7)
C9 H	~2.00 obsc	~2.00 obsc	~1.95 obsc
C10-CH_3	1.90 br s	1.89 br s	1.93 br s
H11	5.78 br d (11)	5.79 br d (11)	5.82 br d (~10.5)
H12	6.65 dd (11, 15.5)	6.61 dd (11, 15)	6.52 dd (10.5, 15)
H13	5.13 dd (9, 15.5)	5.15 dd (~9.5, 15)	5.17 dd (9, 15)
H14	4.00 dd (7.5, 8.5)	3.97 dd (7.5, 8.5)	3.89 t (9.5)
C14-OCH_3	3.26 s	3.25 s	3.26 s
H15	5.05 dd (1.5, 7.5)	5.14 dd (1.5, 7.5)	4.97 dd (~1.5, 8.5)
H16	~2.08 m	~2.03 m	~2.11 m
C16-CH_3	0.87 d (7)	1.06 d (7)	0.82 d (7)
H17	4.16 dd (1.5, 10.5)	3.56 dd (~1.5, 10)	4.15 ddd (1.5, 4, 10.5)
C17-OH			4.64 d (4)
H18	1.82 dq (1.5, 7)	2.13 dq (~1.5, 7)	1.77 dq (1.5, 7)
C18-CH_3	0.99 d (7)	0.98 d (7)	1.04 d (7)
C19-OH			5.51 d (2)
C19-OCH_3		3.10 s	
H20α	1.33 t (12)	1.61 dd (11, 12.5)	~1.00 obsc
H20β	2.32 dd (5, 12)	2.30 dd (5, 12.5)	2.45 dd (5, 12)
H21	5.05 dt (5, ~11, ~11)	5.01 dt (5, 11, 11)	3.22 dt (5, 10.5, 10.5)
C21-OCH_3			3.38 s
H2'	6.65 br d (16)	6.75 br d (16)	
H3'	6.91 v br d (16)	6.85 v br d (16)	
H22	1.68 tq (6.5, 11, 11)	~1.61 m obsc	1.58 m obsc
C22-CH_3	0.85 d (7)	0.83 d (7)	0.90 d (7)
H23	3.60 dd (2, 10)	3.23 dd (~2, 10)	3.50 dd (2, 10)
H24	~1.90 m obsc	~2.03 m obsc	~1.90 m obsc
C24-CH_3	0.92 d (7)	0.92 d (7)	0.89 d (7)
C24-CH_3	0.97 d (7)	0.92 d (7)	0.93 d (7)

[a] Chemical shifts are in ppm downfield of internal Me_4Si. Coupling constants in hertz (±0.5) are given in parentheses. Abbreviations: s = singlet, d = doublet; t = triplet; q = quartet; m = multiplet; br = broad; obsc = obscured (overlapping signals); v = very.

A_1 displays anthelmintic activity and is of considerable interest in being comparably active in stimulating GABA release from rat brain synaptosomes as the potent anthelmintic avermectins which were recently discovered in these laboratories.[2] L-681,110 belongs to a new class of 16-membered macrocyclic lactones with a C14 side chain which incorporates a tetrahydropyran ring having both a hemiketal hydroxy group and fumarate half-ester group as substituents. In this paper we present spectroscopic evidence for the proposed structures of the major component A_1 of L-681,110 and minor components A_2 and B_1.

Structure Determination

The component mixture with ATPase activity, isolated by acetone extraction of the mycelia, was separated by silica gel TLC into two slow running components A_1 and A_2 and a faster running component B_1. Components A_1 and A_2 behave chromatographically as carboxylic acids as their mobilities on TLC are retarded when ammonia is present in the solvent system. A_1 is the major component and is readily converted to A_2 on heating in methanol, suggesting the latter to be an artifact, as the preparative TLC was carried out by using methanol–methylene chloride mixtures. This was subsequently confirmed[3] as extraction and chromatography in the absence of methanol produced no A_2 component.

High-resolution electron impact mass spectral measurements suggested isomeric structures for components A_1 and A_2 as both gave an apparent molecular ion of m/z 586.3861 corresponding to the molecular formula $C_{35}H_{54}O_7$ (calculated 586.3855) even though the ^1H NMR spectrum of A_2 indicated three compared to two methoxy groups in A_1. The ^{13}C NMR spectra indicated 39 and 40 carbons for A_1 and A_2, respectively, confirming the extra methoxy group in A_2 and suggesting loss of a C_4 fragment during volatilization in the mass spectrometer. The nature of this small fragment became apparent from ^1H NMR studies of both components under various conditions. The unusual broadening of two coupled doublets at δ 6.65 and 6.91 (J = 16 Hz) in the spectrum of A_1 in CD_3OD at 25 °C (see Table I) varied with solvent and temperature, and the signals in fact coalesced at δ 6.85 in $CDCl_3$ at 45 °C. The coupling constant of 16 Hz suggested a trans-substituted double bond flanked by two carbonyl groups, which is corroborated by the appearance of four broadened resonances at 130.5, 140.0, 166.7, and 171.1 ppm in the ^{13}C NMR spectrum of A_1 in CD_3OD–$CDCl_3$ (1:4) (see Table II). As noted above, A_1 and A_2 behave chromatographically as carboxylic acids, suggesting, therefore, the presence of a fumarate

(1) L. Huang, R. L. Monaghan, K. Jakubas, C. C. Wang, S. S. Pong, and R. W. Burg, Discovery, Production, and Purification of a New Type of Na^+,K^+-Activated ATPase Inhibitor L-681,110, from the Fermentation Broth of Streptomyces sp., Abstracts, 20th Interscience Conference on Anti-Microbial Agents and Chemotherapy, 1980.

(2) (a) Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, GA, Oct 1–4, 1978. Avermectins. A new family of potent anthelmintic agents. Abstract No. 462: Producing Organism and Fermentation, R. W. Burg, B. M. Miller, E. E. Baker, J. Birnbaum, S. A. Currie, R. Hartman, Y. L. Kong, R. L. Monaghan, G. Olson, I. Putter, J. B. Tunac, H. Wallick, E. O. Stapley, R. Oiwa, and S. Omura. Abstract No. 463: Isolation and Chromatographic Properties, T. W. Miller, L. Chaiet, D. J. Cole, L. J. Cole, J. E. Flor, R. T. Goegelman, V. P. Gullo, A. J. Kempf, W. R. Krellitz, R. L. Monaghan, R. E. Ormond, K. E. Wilson, G. Albers-Schönberg, and I. Putter. Abstract No. 464: Structure Determination, G. Albers-Schonberg, B. H. Arison, J. C. Chabala, A. W. Douglas, P. Eskola, M. H. Fisher, J. M. Hirshfield, K. Hoogsteen, A. Lusi, H. Mrozik, J. L. Smith, J. P. Springer, and R. L. Tolman. Abstract No. 465: Efficacy of the B_{1a} Component, J. R. Egerton, D. A. Ostlind, L. S. Blair, C. H. Eary, D. Suhayda, R. F. Riek, and W. C. Campbell. Full papers covering Abstracts No. 462, 463, and 465 have appeared in: *Antimicrob. Agents Chemother. 15*, 361, 368, 372 (1979). (b) D. A. Ostlind, S. Cifelli, and R. Lang, *Vet. Rec., 105*, 168 (1979).

(3) L. Cole, personal communication.

Table II. ^{13}C NMR Assignments of L-681,110 Components A_1 and A_2 [a]

assignment	A_1 [b]	A_1 [c]	A_2 [c]	assignment	A_1 [b]	A_1 [c]	A_2 [c]
C4-CH_3 [d]	7.1 q (127)	7.0 q	7.1 q	C21	76.1 d (~146)	76.1 d	76.7 d
C10-CH_3 [d]	9.9 q (127)	9.9 q	10.5 q	C15	77.1 d (145)	76.9 d	77.0 d
C24-CH_3 [e]	12.3 q (126)	12.2 q	12.0 q	C7 [g]	81.3 d (141)	80.6 d	80.3 d
C6-CH_3 [e]	14.0 q	14.0 q	13.6 q	C17 [g]	82.5 d (143)	82.9 d	83.3 d
C22-CH_3	14.4 q (126)	14.3 q	13.9 q	C19	99.2 s	99.4 s	103.0 s
C8-CH_3 [e]	17.3 q (126)	17.4 q	17.1 q	C12 [h]	125.6 d (~146)	124.8 d	124.6 d
C16-CH_3 [e]	20.2 q	20.3 q	19.9 q	C13 [h]	127.4 d (~151)	126.3 d	126.1 d
C24-CH_3	21.2 q (126)	21.2 q	20.4 q	C4	133.3 s	132.9 s*	132.7 s*
C18-CH_3 [e]	21.8 q (126)	21.8 q	21.7 q	C11 [i]	133.5 d (149)	134.3 d	133.6 d
C24	28.0 d (124)	28.1 d	28.1 d	C3 [i]	134.0 d (151)	135.2 d	134.4 d
C6 [f]	36.9 d (124)	36.9 d*	36.7 d*	C2'	~136.4 v br	140.0 d	138.2 d
C8 [f]	37.3 d	37.5 d*	37.3 d*	C3'	~130.5 v br	130.5 d	131.4 d
C16 [f]	38.2 d	38.2 d*	38.2 d*	C2	~141.1 s	141.1 s	140.9 s
C22	40.2 d	40.7 d*	38.9 d*	C5	143.4 d (153)	145.2 d	144.5 d
C20	40.0 t	39.8 t	35.1 t	C10	143.6 s	144.8 s*	144.3 s*
C9	41.4 t	41.4 t	41.1 t	C1'	~165.4 v br	166.7 s	166.2 s
C18	42.1 d	42.1 d*	40.2 d*	C1	167.8 s	168.2 s	167.3 s
C19-OCH_3			46.4 q	C4'	~170.4 v br	171.1 br s	169.9 br s
C14-OCH_3	55.7 q (142)	55.8 q	55.5 q				
C2-OCH_3	60.1 q (145)	60.3 q	60.3 q				
C14	70.9 d (146)	70.9 d	69.7 d				
C23	75.4 d (~144)	75.1 d	74.7 d				

[a] In ppm downfield of Me_4Si at 25 °C. [b] In $CDCl_3$; $^1J_{CH}$ values in hertz ± 1.0 Hz are given in parentheses; where not given, ^1J values could not be determined because of overlap of signals. [c] In CD_3OD-$CDCl_3$ (1:4); [2-^{13}C]propionate-enriched carbons are marked with an asterisk (*). [d-i] Interchangeable assignments.

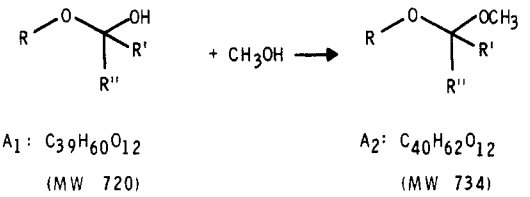


Figure 1. Structural relationship between components A_1 and A_2.

half-ester moiety. This accords well with the ^{13}C NMR resonances observed at 134.1, 134.3, 165.7, and 167.3 ppm for ethyl fumarate in the same solvent as well as with the chemical shift of δ 6.90 reported[4] for the olefinic protons in $CDCl_3$. The differential exchange broadening of the two proton and four carbon signals is attributed to slow dissociation of the acid under these conditions.

^1H NMR evidence on the chromatographically faster running B_1 component indicated the presence of two secondary and one tertiary alcohol groups, the latter being associated with the hemiketal functionality (see below). ^1H and ^{13}C NMR data on A_1 and A_2 implicated the presence of two ester, one carboxylate, one hemiketal, two secondary alcohol, and two methoxy groups. This gives a total of twelve oxygens and suggests the presence of one more molecule of water than allowed by the MS data, giving a new formula for the major component A_1.

$$C_{35}H_{54}O_7 + C_4H_4O_4 + H_2O \rightarrow C_{39}H_{60}O_{12}$$

Indirect confirmation of this formula comes from the mass spectrum of the minor component A_2 which although appearing isomeric with A_1 shows a very weak but reproducible peak at m/z 734, $C_{40}H_{62}O_{12}$. Conversion of A_1 to A_2 was originally witnessed in the NMR tube at elevated temperatures and could be reproduced quantitatively by refluxing briefly in methanol. The new methoxy peak at δ 3.10 readily exchanges in CD_3OD at 60 °C, suggesting methyl ketal formation, which is supported by singlets at 99.4 and 103.0 ppm in "gated" decoupled ^{13}C NMR spectra of A_1 and A_2, respectively, and by the presence of a third methoxy quartet at 46.4 ppm in the spectrum of A_2 (see Table II). The relationship between A_1 and A_2 is thus simply depicted as in Figure 1 in agreement with M^+ 734 observed for A_2. Losses in the mass spectrum of A_2 of methanol and fumarate and in A_1 of water and fumarate would account for the same apparent molecular ion b, m/z 586.

Analysis of the ^1H and ^{13}C NMR spectra of components A_1 and A_2 is fully consistent with the proton and carbon counts as illustrated for A_1 in Table III. To account for the twelve oxygens in the molecule, several combinations of functionalities are possible but only two remain for consideration (Table IV) after most of the ^1H and ^{13}C NMR evidence is taken into account. The proposed structure has to account for two

Table III. Proton and Carbon Counts for Component A_1 from ^1H and ^{13}C NMR Data

^{13}C NMR	^1H NMR	number of carbons	number of protons
9 × CH_3	1 × $(CH_3)_2CH$	9	6
	5 × CH_3CH		15
	2 × CH_3C=		6
6 × CH	~6 × CH	6	6
2 × CH_2	~2 × CH_2	2	4
2 × CH_3O	2 × CH_3O	2	6
6 × CHOR	2 × CHOH	2	4
	2 × CHOR	2	2
	2 × CHOCOR	2	2
1 × C(=O)O	1 × C(OH)OR	1	1
7 × CH=	7 × CH=	7	7
3 × –C=		3	
3 × CO_2R	1 × CO_2H	3	1
	total	39	60

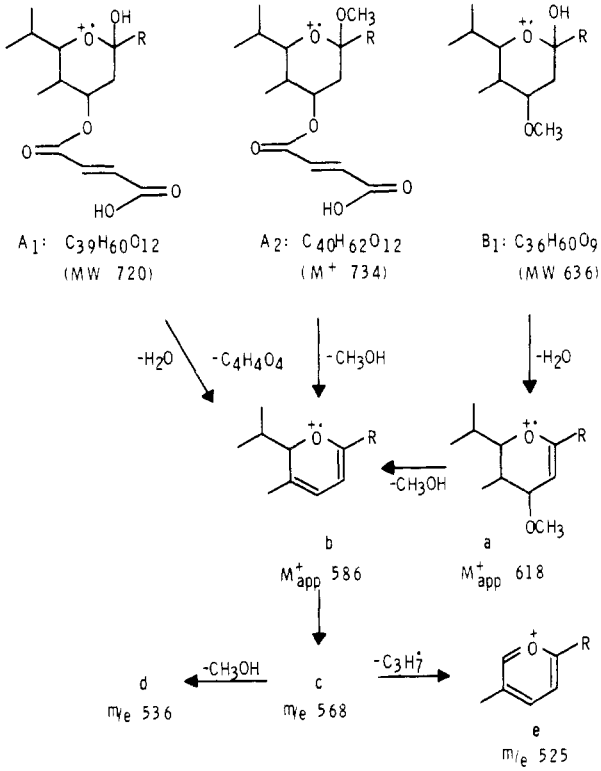
Table IV. Possible Combinations of Oxygen Functionalities for Component A_1 from ^1H and ^{13}C NMR Data

alternative A	number of O	number of *CH*OR	number of *O*H	alternative B	number of O	number of *CH*OR	number of *O*H
2 × CHOH	2	2	2	2 × CHOH	2	2	2
2 × CO_2CH	4	2		2 × CO_2CH	4	2	
1 × CO_2H	2			1 × CO_2H	2		
1 × CH_3OCH	1	1		2 × CH_3OCH	2	2	
1 × CH_3OC=	1						
1 × C(OH)OCH	2	1	1	1 × C(OH)OC=	2		1
total	12	6	3	total	12	6	3

methoxy groups, six methine carbons attached to oxygen, one carboxyl and two carboxyl ester groups, one hemiketal functionality, and two secondary alcohol groups. Of the two possibilities in Table IV, alternative A is favored and satisfactorily accounts for the different environments

(4) "The Aldrich Library of NMR Spectra", C. J. Pouchert and J. R. Campbell, Eds., Aldrich, Milwaukee, WI, 1974, Vol. 3, p 69.

Scheme I



of the two methoxy groups at δ 3.26 and 3.63. This is also reflected in their $^1J_{^{13}C-H}$ values of 142 and 145 Hz, respectively, the value of 142 Hz being typical for an alkyl methyl ether whereas the value of 145 Hz is intermediate to that of the latter and a methyl ester (~148 Hz).[5] The correctness of possibility A is borne out from detailed ^1H NMR decoupling studies on all three components under a variety of conditions. In particular, the nature of the hemiketal functionality became clear from a ^1H NMR study of component B_1, which, in contrast to A_1 and A_2, gives sharp spectra in $CDCl_3$ and benzene-d_6, allowing hydroxyl protons to be readily detected. Derivation of the various partial structures will therefore be mainly illustrated with reference to component B_1 although most of the features were similarly confirmed for components A_1 and A_2. The presence of five double bonds and three carboxyl functions argues for the presence of two rings. From a consideration of the number of chain terminations [C–CH_3 (9) + O–CH_3 (2) + OH (3) + CO_2H (1) = 15] and branch points [>C= (3) + O_2 (2) + CHOR (6) + CH (6) = 17], the number of rings [$^1/_2$(branch points − terminations) + 1 = 2] can be independently found[6] and provides assurance that the correct ^{13}C NMR assignments have been made.

Partial Structure 1

Besides the ability to detect OH signals in the ^1H NMR spectra of B_1 in $CDCl_3$ or benzene-d_6, advantage was taken of the induced solvent shifts, particularly of the overlapping proton signals in the high-field region between δ 1.6 and δ 2.2. Comparison of the ^1H NMR spectrum of B_1 in $CDCl_3$ with those of A_1 and A_2 in CD_3OD (see Table I) indicates great similarity but immediately reveals the absence of the fumarate protons and the presence of an additional methoxy group at δ 3.26. The absence of the fumarate chromophore in B_1 is also apparent from the ultraviolet spectrum of B_1 which lacks the maximum near 209 nm of both A_1 and A_2. That the methoxy group does not belong to a methyl ketal functionality was unambiguously demonstrated by conversion of B_1 to its methyl ketal derivative by refluxing in methanol under acid catalysis. This strongly suggested substitution of the fumarate moiety of A_1 and A_2 by a methoxy group in B_1, and this was further supported by the observed upfield shift of the doublet of triplets at δ 5.05 in the spectrum of A_1 to δ 3.22 for B_1. An OH group at this position could be immediately ruled out because of the lack of further coupling to an active proton. Corroboration

(5) O. D. Hensens, unpublished observations.
(6) A. W. Douglas, personal communication.

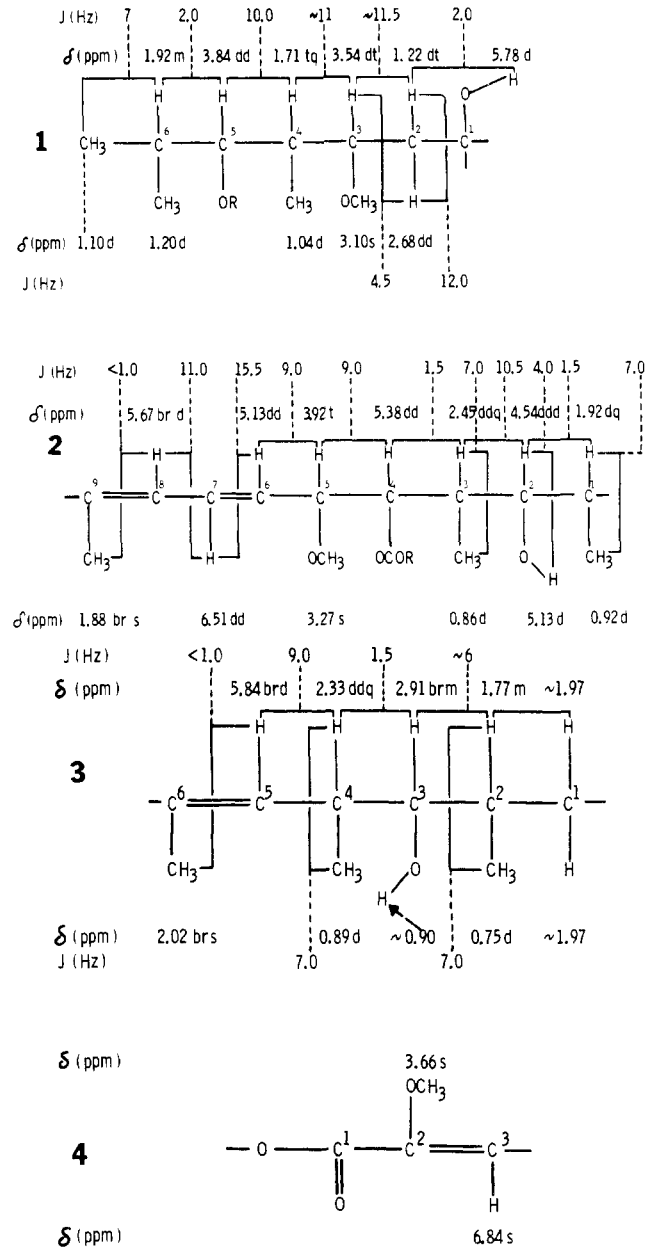


Figure 2. ^1H NMR parameters of partial structures derived from ^1H NMR decoupling studies of component B_1 in benzene-d_6 at 40 °C.

was obtained from the mass spectrum (see Scheme I) which displays prominent peaks at m/z 618, 586, 568, and 525, the last three ions also being common to the spectra of A_1 and A_2. Replacement of the fumarate by a methoxy group gives $C_{36}H_{60}O_9$ for the molecular formula of B_1. The compound, as in the case of A_1, readily loses the hemiketal hydroxyl as water to give the apparent molecular ion at m/z 618. Further loss of methanol, instead of fumarate as in the case of A_1 and A_2, gives m/z 586, the apparent molecular ion for both A_1 and A_2.

The resonance in the spectrum of B_1 at δ 3.22 in $CDCl_3$ and δ 3.54 in benzene-d_6 at 40 °C was shown by decoupling experiments to be part of sequence **1** as shown in Figure 2. Irradiation of the active proton at δ 5.78 (d, J = 2 Hz) resulted in collapse of the doublet of triplets at δ 1.22 to a clean triplet ($J \simeq 11.5$ Hz), strongly suggesting long-range coupling involving a tertiary rather than a secondary alcohol proton. The coupling appears to be stereospecific as no coupling to the other methylene proton at δ 2.68 is observed. With the exception of H3 no further coupling to the methylene protons is evident, establishing the quaternary nature of C1 which can therefore be assigned to the hemiketal carbon as no other quaternary carbon-carrying oxygen is indicated by the ^{13}C NMR data. The J_{gem} of 12 Hz is consistent with this

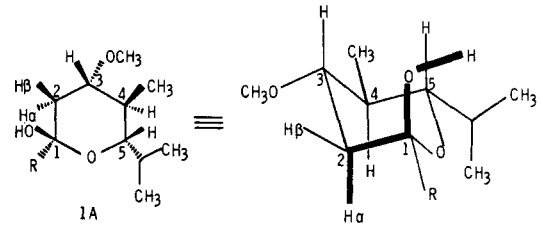


Figure 3. Partial structure **1a** of component B_1.

proposal and rules out juxtaposition to a carbonyl or olefinic carbon.[7] The large coupling constants between vicinal protons along the chain are compatible with a chair conformation of a six-membered ring carrying equatorial substituents, and it is therefore concluded that the second oxygen at C1 bridges C1 and C5. The appreciably shielded position of the axial methylene proton at δ 1.22 in benzene-d_6 and near δ 1.0 in $CDCl_3$ can be cited as a further example of the shielding associated with an oxygen lone pair in a rigid tetrahydropyran ring via a syn–axial relationship between the lone pair and the affected proton, as previously noted for the avermectins.[8] Preference for the axial orientation of the OH group at the anomeric carbon C1 is evidenced by the large long-range coupling of 2.0 Hz between H2α and the OH proton involving only σ bonds. This is expected to be optimal for a planar W arrangement of the four bonds H–O–C1–C2–H,[7] which, because of the axial nature of the upfield methylene proton at δ 1.22, can be readily accommodated by an axial OH group as depicted in Figure 3.

The ^{1}H NMR spectra of all components indicate seven methyl doublets whereas the ^{13}C NMR spectra of A_1 and A_2 give evidence for only six methine carbons in the upfield region of the spectra, inferring the presence of at least one isopropyl group. Three of the methylmethine groups can be unequivocally assigned as their methine protons are well separated, leaving four to be assigned. We therefore relied on solvent shifts to completely define the terminal moieties of the three sequences **1**, **2**, and **3** (Figure 2). In $CDCl_3$ spiked with CD_3OD at 40 °C, H1 of sequence **2** appears as a doublet of quartets (J = 1.5, 7 Hz) at δ 1.75 which collapses to a sharp quartet (J = 7 Hz) on irradiation of the doublet of doublets at δ 4.12 (H2, J = 1.5, 10.5 Hz). C1 of sequence **2** must therefore be adjacent to a quaternary carbon, and this demonstration of a fourth methylmethine group limits the number of isopropyl groups to one. H1 of sequence **2** and H6 of **1** overlap at δ 1.92 in C_6D_6 at 40 °C (see Figure 2), and irradiation at this frequency collapses the three methyl doublets at δ 0.92, 1.10, and 1.20 to singlets, which therefore locates the isopropyl group at the end of sequence **1**. Furthermore, irradiation at δ 1.77 collapses the remaining methyl doublet at δ 0.75 to a singlet and sharpens the overlapping multiplet near δ 1.97 which places the second hitherto unaccounted for methylene group, as required by ^{13}C NMR analysis, at the end of sequence **3**.

Confirmation of partial structure **1a** was readily obtained from mass spectral evidence as depicted in Scheme I. Facile loss of water and methanol in addition to fumaric acid from the molecular ion of A_1 and A_2 gives fragment b (m/z 586) corresponding to the apparent molecular ion, which, after loss of water from another portion of the molecule, readily aromatizes through loss of the isopropyl side chain to give the abundant fragment c (m/z 525). Loss of the side chain in an analogous situation has very recently been observed for the avermectins.[9] Loss of water from the molecular ion of component B_1 to give M^+_{app} 618 is followed by loss of methanol to give ion b which undergoes further fragmentation as noted for A_1 and A_2.

(7) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2nd ed., Pergamon Press, Oxford, 1969.
(8) J. P. Springer, B. H. Arison, J. M. Hirshfield, and K. Hoogsteen, *J. Am. Chem. Soc.*, *103*, 4221 (1981).
(9) G. Albers-Schönberg, B. H. Arison, J. C. Chabala, A. W. Douglas, P. Eskola, M. H. Fisher, A. Lusi, H. Mrozik, J. L. Smith, and R. L. Tolman, *ibid.*, 4216.

Partial Structure 2

It has already been concluded above that C1 of sequence **2** (Figure 2) is adjacent to a quaternary carbon. The position of the hydroxyl group at C2 was unambiguously determined by addition of CD_3OD to the benzene-d_6 solution which resulted in disappearance of the doublet at δ 5.13 (J = 4 Hz) and collapse of the H2 multiplet (doublet of doublet of doublets, J = 1.5, 4, 10.5 Hz) at δ 4.54 to a doublet of doublets (J = 1.5, 10.5 Hz). This allowed the second ester functionality to be placed at C4 because of the downfield ^{1}H NMR chemical shift at δ 5.38 for H4 relative to H5. Also, the methoxy and not a hydroxy group could be placed at C5 because of the lack of further coupling of H5 to an active proton. The transoid diene follows readily from the coupled resonances at δ 5.13, 6.51, and 5.67 with the large coupling constant of 15.5 Hz between H6 and H7 and the upfield ^{13}C NMR chemical shift of the C9 methyl group[10] at 7.1 or 9.9 ppm assigned on the basis of SFORD experiments. The planarity of the diene chromophore is evident from the large coupling between H7 and H8 (J = 11 Hz) as well as the intense absorption at 246 nm (ϵ 38 200) in the ultraviolet spectrum similar to that observed for the transoid diene in the milbemycins[11] and avermectins.[9]

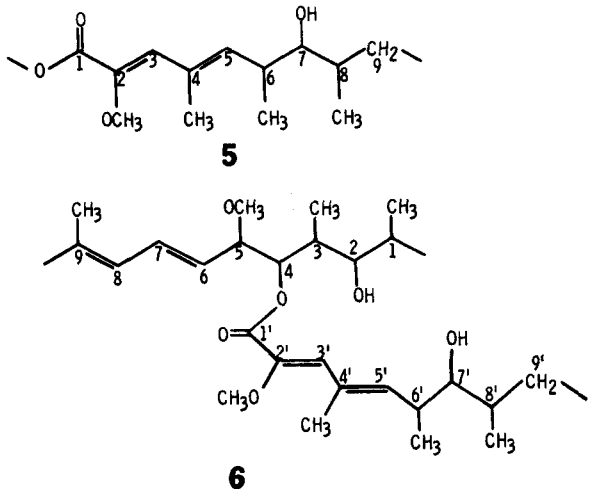
Partial Structure 3

The remaining hydroxyl group was unambiguously placed at C3 of sequence **3** (Figure 2) from a ^{2}H-exchange experiment with CD_3OD which resulted in sharpening of the broad multiplet at δ 2.91 to a clean doublet of doublets (J = 1.5, 6 Hz). The OH resonance appears to be obscured under the high-field envelope near δ 0.90 as irradiation at this frequency sharpens H3 from a broad multiplet to a broad doublet ($J \simeq 6$ Hz). The resonance at δ 1.77 (H2) to which H3 is coupled was shown above to be coupled to both the methyl doublet at δ 0.75 and protons of the second CH_2 group near δ 1.97. Similar to the situation in sequence **2**, the olefinic proton at δ 5.84 is coupled to the allylic methyl at δ 2.02 which places the remaining vinylic methyl unambiquously at the end of this sequence. Trans subsitution of the double bond follows from the upfield ^{13}C NMR chemical shift of the methyl group at 9.9 or 7.1 ppm as in sequence **2**.[10] The three partial structures **1**, **2**, and **3** readily account for all the high-field resonances required by ^{1}H and ^{13}C NMR analysis.

Partial Structure 4

The only two resonances that remain to be assigned are the sharp one-proton singlet at δ 6.84 and methoxy singlet at δ 3.66. The previously made argument favoring an enol methyl ether (alternative B in Table IV) when considered with the ^{13}C NMR evidence for the presence of a third unsaturated carboxylic function suggested partial structure **4** (Figure 2) where C3 is adjacent to a quaternary center. In order to account for the remaining ultraviolet absorption maximum near 284 nm, the sequence must be combined with the double bond of sequence **3** to give the α-methoxydienoic acid chromophore **5**. This accords well with the value of 281 nm, calculated from the absorption maxima reported for the γ-methylsorbic acid derivatives streptolic[12] and tirandamycic acid[13] by using an increment of +20 for an α-methoxy substituent.[14] Whereas α-alkoxysorbic acid derivatives do not appear to be reported in the literature, absorption maxima of 265 and 270 nm were found for the β-methoxy analogues[15] which is surprising as a β-alkoxy substituent would be expected to shift the maximum of a conjugated ester by more than an

(10) N. K. Wilson and J. B. Stothers in "Topics in Stereochemistry", E. L. Eliel and N. L. Allinger, Eds., Wiley, New York, 1974, Vol. 8.
(11) H. Mishima, M. Kurabayashi, C. Tamura, S. Sato, H. Kuwano, and A. Saito, *Tetrahedron Lett.*, 711 (1975).
(12) K. L. Rinehart, Jr., J. R. Beck, W. W. Epstein, and L. D. Spicer, *J. Am. Chem. Soc.*, *85*, 4035 (1963).
(13) F. A. MacKellar, M. F. Grostic, E. C. Olson, R. J. Wnuk, A. R. Braufman, and K. L. Rinehart, Jr., *ibid.*, *93*, 4993 (1971).
(14) A. I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products", Pergamon Press, Oxford, 1964, p 242.
(15) H. B. Henbest and E. R. H. Jones, *J. Chem. Soc.*, 3628 (1950).



5

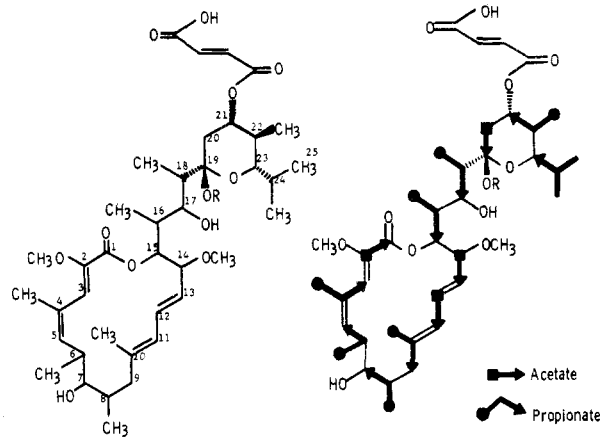
6

α-alkoxy group.[14,16] The α-methoxy substitution is, however, corroborated by ^1H[17] and ^{13}C NMR[18] predictions based on model compounds.

Total Structure

It now remains to link partial structures **1a**, **2**, and **5** into the correct gross structure. As there is only one ester linkage in component B_1, sequence **5** must be attached to sequence **2** through C4 to form sequence **6**. Detailed ^1H NMR analyses of decoupling experiments on all components under a variety of conditions have shown above that C1 of sequence **6** is adjacent to a quaternary carbon, either C9 of **6** or C1 of sequence **1**. The first possibility can be ruled out on the basis of ring size, mass spectral evidence, and biogenetic considerations. Incorporation of a planar transoid diene chromophore, as required by ^1H NMR and ultraviolet data, into a nine-membered ring is impossible from a consideration of Dreiding models. Furthermore, the expected facile mass spectral loss of this ring residue via a simple pyrolysis involving the ester group to form a fragment $C_{23}H_{40}O_7$ (and smaller fragments resulting from elimination of water, methanol, carbon dioxide, and/or the isopropyl side chain) is not observed. Biosynthetic feeding experiments with [2-^{13}C]propionate show enrichment of the two olefinic singlets at 132.5 and 144.4 ppm and five high-field methine carbons in the ^{13}C NMR spectra of components A_1 and A_2, demonstrating that all methyl groups with the exception of the isopropyl methyls are derived from propionate. A simple biosynthetic scheme for the nine-membered ring containing three propionate-derived methyl groups is thus precluded.

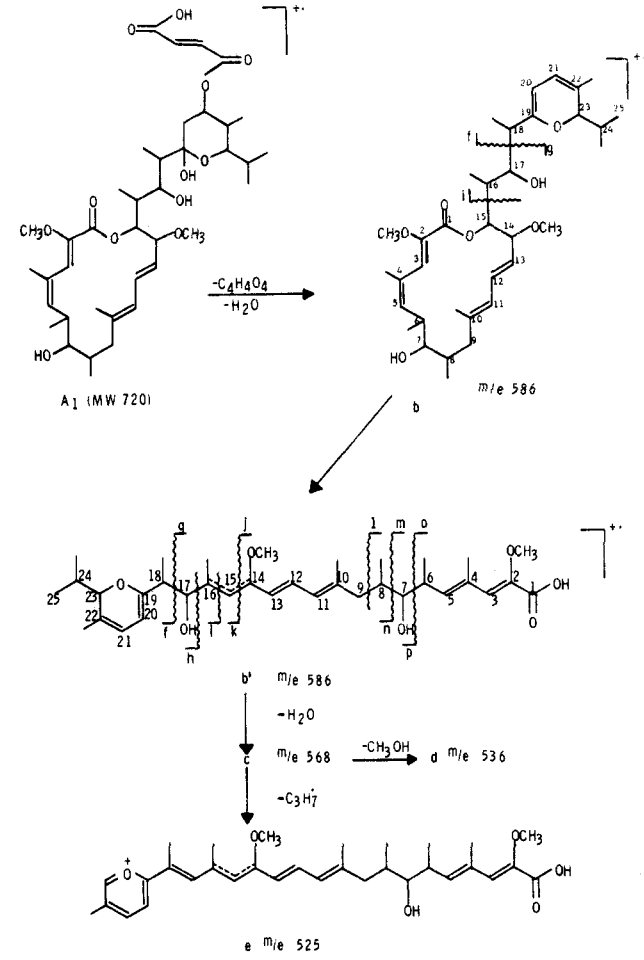
The other possibility involving attachment of C1 of **6** to C1 of **1a** and C9 to C9′ of sequence **6**, is proposed as the structure for component B_1. Components A_1 and A_2 both have a fumarate moiety at C21 instead of the methoxy group as shown in Figure 4. A detailed mass spectral analysis of component A_1 provides ample confirmation for the proposed structure as shown in Scheme II. The various bond cleavages as indicated in fragments b and b′ were confirmed by high-resolution measurements as summarized in Table V. Reference has already been made to the dominant and highly conjugated fragment e, m/z 525 (Scheme I), resulting from loss of fumarate, two molecules of water, and the isopropyl side chain at C23. Other fragments showing the characteristic loss of the side chain are f, h, i, and n. In addition, fragments h, i, k, and n show further loss of water and fragment n loss of methanol, providing therefore strong evidence for the sequence C8–C25. Thermal elimination of the ester function at C15 may



Component A_1, R = H
A_2, R = CH_3
B_1, R = H and substitution of OCH_3 for fumarate moiety

Figure 4. Proposed structures of L-681,110 components and biosynthetic scheme.

Scheme II



(16) This apparent inconsistency was pointed out by a referee and suggests the need for care in predicting UV maxima of substituted sorbic acid derivatives.

(17) The α-proton in streptolic acid[12] absorbs at δ 5.85, well upfield of that found in sequence **5** in various solvents. Substitution by a methoxy group at the β-position would be expected to lead to even more increased shielding of the α-proton.[7,19]

(18) The ^{13}C NMR assignments of sorbic acid in Me_2SO-e_6 at 25 °C were unequivocally determined by SFORD experiments [CO_2H 168.4 s; Cα 120.4 d (161.5 Hz); Cβ 145.0 d (155 Hz); Cγ 130.2 d (156 Hz); Cδ 139.3 d (155.5 Hz); CH_3 18.4 q (127.5 Hz)]. The introduction of a β-methoxy substituent in *cis*-methylcrotonate leads to shielding of the α-carbon and deshielding of the β-carbon to approximately the same extent (~30 ppm).[19] Whereas the magnitude of both shifts in a series of substituted vinyl ethers appears to depend on the steric environment of the double bond,[20] the above trends nevertheless confirm α over β-substitution in **5**.

(19) H. Brouwer and J. B. Stothers, *Can. J. Chem.*, *50*, 601 (1972).

(20) A. C. Rojas and J. K. Crandall, *J. Org. Chem.*, *40*, 2225 (1975).

Table V. High-Resolution Mass Spectral Data for Component A_1

fragment ion[a]	elemental composition	calcd mass	found mass
b or b′	$C_{35}H_{54}O_7$	586.3855	586.3861
c (b′ − H_2O)	$C_{35}H_{52}O_6$	568.3750	568.3809
d (b′ − H_2O − CH_3OH)	$C_{34}H_{48}O_5$	536.3489	536.3464
e (b′ − H_2O − C_3H_7)	$C_{32}H_{45}O_6$	525.3204	525.3176
g − H	$C_{24}H_{36}O_6$	420.2512	420.2597
p − H_2O	$C_{26}H_{39}O_3$	399.2899	399.2897
n − H_2O − H	$C_{25}H_{36}O_2$	368.2715	368.2654
n − H_2O − CH_3OH	$C_{24}H_{33}O$	337.2531	337.2526
n − H_2O − C_3H_7 − H	$C_{22}H_{29}O_2$	325.2167	325.2188
j − H_2O + H	$C_{20}H_{30}O_4$	334.2144	334.2142
k	$C_{15}H_{23}O_2$	235.1698	235.1697
k − H_2O	$C_{15}H_{21}O$	217.1592	217.1582
l − H_2O	$C_{12}H_{17}O_3$	209.1178	209.1176
l − CO_2 − CH_3OH	$C_{10}H_{15}O$	151.1123	151.1127
i − C_3H_7 + H	$C_{11}H_{17}O_2$	181.1228	181.1232
i − C_3H_7 − H_2O + H	$C_{11}H_{15}O$	163.1123	163.1132
m − H_2O	$C_{10}H_{13}O_3$	181.0865	181.0876
m − H_2O − CH_3OH	$C_9H_9O_2$	149.0602	149.0592
m − H_2O − CO_2	$C_9H_{13}O$	137.0966	137.0944
o	$C_9H_{13}O_3$	169.0865	169.0883
o − CH_3OH	$C_8H_9O_2$	137.0603	137.0584
o − CO_2	$C_8H_{13}O$	125.0966	125.0936
o − CO_2 − CH_3OH	C_7H_9	93.0703	93.0705
o − C_3H_7	$C_9H_{12}O_2$	152.0837	152.0841
h − C_3H_7 − H_2O + H	$C_9H_{11}O$	135.0809	135.0795
f − C_3H_7 + H	$C_8H_{11}O$	123.0809	123.0794

[a] See Scheme II.

involve the proton either at C14 or at C16, and in spite of this, cleavage occurs on both sides of C15 as characterized by the fragment ions i, j, and k.

Confirmation of the α-methoxy-γ-methylhexadienoic acid portion of the molecule is readily obtained from fragments l, m, and o which show characteristic losses of methanol and/or carbon dioxide. In addition the ions m and l both lose water involving the hydroxyl group at C7. The fragment j − H_2O (m/z 334) is the most significant ion above m/e 200 in the spectrum of component B_1 and represents cleavage near the point of attachment of the lactone carboxyl group which, together with fragments l, m, and o, provides strong support for the sequence C1–C14. The main mass spectral ions as summarized in Table V can therefore be readily accounted for in terms of the proposed structure.

Biosynthesis and ^{13}C NMR Assignments

The presence of a large number of methyl groups in L-681,110 led us to explore its biosynthesis with [2-^{13}C]propionate. Incorporation of ^{13}C label resulted in significantly increased (~4×) signal strengths for the five high-field methine and two olefinic quaternary carbons which are marked with an asterisk in Table II. Incorporation of seven propionate units is readily allowed by the proposed structures in addition to a postulated isobutyrate (probably derived from valine[9]) and four acetate residues as shown in Figure 4. Oxygenation at the unexpected C2 and C14 positions may suggest the incorporation of glycollate or glycerate rather than acetate at these positions.[21] The remaining high-field methine carbon at 28.1 ppm is therefore readily assigned to C24 of the isopropyl group. The two labeled methines at 40.7 and 42.1 ppm in A_1 which are displaced to higher field by ca. 2 ppm in the methyl ketal A_2 must implicate C18 and C22 and the remaining methines are collectively assigned to C6, C8, and C16. On the same basis the two methylene carbons at 39.8 and 41.4 ppm are assigned to C20 and C9, respectively. Of the three olefinic quaternary carbons, the one at 141.1 ppm is not enriched by [2-^{13}C]propionate and can therefore be unequivocally assigned to C2. Most other resonances were tentatively assigned on the basis of single-bond ^{13}C–^1H coupling constants obtained for A1 in $CDCl_3$ (see Table II), the effect of methyl ketal formation, and comparison with model compounds.

(21) These alternatives were suggested by a referee.

Stereochemistry

The relative stereochemistry of the tetrahydropyran ring as shown in Figure 3 follows unequivocally from an analysis of the coupling constant data. The large vicinal couplings between H2, H3, H4, and H5 are compelling evidence that the substituents at C3, C4, and C5 are equatorial. Moreover, the appreciable long-range coupling of 2.0 Hz between the hemiketal OH proton and H2 can be satisfactorily explained by invoking the axial configuration of the OH group at C1 (see discussion under partial structure **1**) and consequently the preferred equatorial configuration of the side chain. The ease of methyl ketal formation from A_1 in the absence of an external acid catalyst is of interest as only one product (A_2) is obtained. This is in contrast to component B_1 where the addition of acetic acid is required and where both anomers are produced in a ratio of ca. 3:1. Anchimeric assistance by the fumarate carboxyl group is invoked to account for the ease and stereospecificity of methyl ketal formation from A_1. The conversion involves several appreciable ^1H NMR shifts including C16–CH_3 (−0.19 ppm), H17 (+0.60 ppm), H18 (−0.31 ppm), H20 (−0.28 ppm), and H23 (+0.37 ppm) and is believed to be due, in large part, to conformational changes involving disruption of a tightly hydrogen bonded network involving the hydroxyl groups at C17 and C19 and the ester oxygen at C15. Because of these changes, consideration of ring substituent effects could not be used to unequivocally rule in favor of retention or inversion of configuration at C19.[22] Presumably both occur in the case of component B_1.

Of the four possible *Z,E* geometrical isomers of the α-methoxy-γ-methylsorbate[1] chromophoric group, only the αβ double bond can be rigorously assigned as having the *E* configuration on the basis of chemical shift comparison of the β-proton with those in model compounds. The β-proton in *Z,E*-methylsorbate absorbs at δ 6.47, about 0.7 ppm upfield of that in the *E,E* isomer,[23] whereas the effect of methyl substitution at the γ-position in the latter leads to deshielding of ca. 0.4 ppm by comparison with tirandamycic acid.[13] The shielding of this proton upon introduction of a methoxy group at the α-position is expected to be close to 1.0 ppm,[17,24] therefore predicting values of ca. δ 6.60 and 5.90 for the *E,E* and *Z,E* isomers, respectively. The β-proton in component B_1 occurs at δ 6.68 (H3), thereby strongly favoring the *E* configuration of the αβ-unsaturated double bond.

The geometry of the diene chromophore is fully trans from ^1H NMR data and the upfield ^{13}C NMR chemical shift of the methyl group (see above). The latter argument may not be valid, however, in assigning the trans geometry of the αβ double bond of the sorbic acid chromophore.

Experimental Section

Ultraviolet spectra were recorded on a Cary 15 instrument. ^1H NMR and ^{13}C NMR spectra were obtained at 300 and 75 MHz, respectively, by using a Varian SC-300 NMR spectrometer in the FT mode. Chemical shifts in both types of spectra are quoted in ppm downfield of internal Me_4Si. Gated decoupled ^{13}C NMR spectra were obtained by using an acquisition time of 0.4 s, pulse delay of 0.2 s, sweep width of 15 000 Hz, and a pulse flipping angle of ca. 50°. Low-resolution mass spectra were obtained on a LKB-9000 and high-resolution spectra on a Varian MAT-731 spectrometer.

Separation of L-681,110 Components. Crude L-681,110 (122 mg) was put on 10 prewashed silica gel HF_{254} plates (20 × 20 × 0.05 cm) and developed continuously for 20 h in methylene chloride–methanol (9:1) containing 1% ammonium hydroxide. Less polar components including B_1 migrated to the front. The major component A_1, having the lowest R_f, and the very similar minor component A_2, having a slightly higher R_f, completely separated under these conditions. The respective zones were collected from all plates and eluted with chloroform–methanol (9:1). The eluates were evaporated, redissolved in methylene chloride, filtered through a tight cotton plug, diluted with glass-distilled, VPC-quality benzene, and lyophilized. Component A_1 (76.9 mg): UV (CH_3OH) 210

(22) H. Booth in "Progress in Nuclear Magnetic Resonance Spectroscopy", J. W. Emsley, J. Feeney, and L. H. sutcliffe, Eds., Pergamon Press, Oxford, 1969, Vol. 5, p 149.

(23) J. A. Elvidge and P. D. Ralph, *J. Chem. Soc. B*, 243 (1966).

(24) W. Brugel, "Handbook and NMR Spectral Parameters", Heyden, London, 1979, Vol. 1, pp 143–144.

nm (ε 26 300), 246 (ε 35 590), 284.5 (ε 15 880); mass spectrum m/z 586, 568, 525 (see Table V): ^{1}H NMR and ^{13}C NMR (see Table I and II). Component A_2 (7.7 mg): UV (CH_3OH) 209 nm (ε 24 170), 246 (ε 32 870), 283 nm (ε 14 120); mass spectrum m/z 734 (M^+), 586, 568, 525; ^{1}H NMR and ^{13}C NMR (see Tables I and II).

In another experiment crude L-681,110 (270 mg) was put on nine silica gel HF_{254} plates (20 × 20 × 0.025 cm) and developed 3 times with chloroform–methanol (4:1) containing 2% ammonium hydroxide. Under these conditions the components running faster than A_1 and A_2 separated into several bands which were individually recovered. The major band with the second highest R_f gave 41 mg of lypophilized component B_1: UV (CH_3OH) 246 nm (ε 38 200), 284.5 (ε 16 950); mass spectrum m/z 618 ($M^+ - H_2O$), 600, 586, 568, 525; ^{1}H NMR (see Table I and figure 2).

Conversion of A_1 to A_2. Component A_1 (50 mg) was dissolved in methanol (25 mL) and refluxed for 3.5 h. The solvent was removed under reduced pressure giving a glassy white solid (45 mg) indistinguishable by ^{1}H NMR in CD_3OD from component A_2.

Methanolysis of B_1. Component B_1 (2.5 mg) was recovered unchanged when refluxed in methanol (10 mL) for 3 h. Under the same conditions but in the presence of 5 drops of glacial acetic acid, an oil was obtained on evaporation of the solvent which by ^{1}H NMR showed predominantly two methyl ketal isomers in the proportion of ca. 3:1. Well-resolved resonances of the major component are as follows: δ ($CDCl_3$) 1.41 (dd, 1, J = 11, 12 Hz, H20α), 1.92 (br s, 3, C10–CH_3), 1.99 (br s, 3, C4-CH_3), 2.41 (m, 1, H20β), 2.54 (m, 1, H6), 3.04 (s, 3, C19-OH_3), 3.24 (s, 3, C14-OCH_3), 3.35 (s, 3, C21-OCH_3), 3.71 (s, 3, C2-OCH_3), 3.88 (t, 1, J = 9.5 Hz, H14), 5.12 (br d, 1, J = 7.5 Hz, H15), 5.22 (dd, 1, J = 9, 15 Hz, H13), 5.78 (br d, 1, $J \simeq$ 10.5 Hz, H11), 5.83 (br d, 1, J = 9 Hz, H5), 6.52 (dd, 1, J = 10.5, 15 Hz, H12), 6.68 (s, 1, H3).

Production of Labeled Material. ^{13}C-labeled product was produced by growing a *Streptomyces* species MA 5038 (Merck Culture Collection) in an inoculum medium which contains dextrose (1.0 g/L), soluble starch (10 g/L), beef extract (3.0 g/L), yeast autolysate (5.0 g/L), peptone (5.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.05 g/L), KH_2PO_4 (0.182 g/L), Na_2HPO_4 (0.190 g/L), and $CaCO_3$ (0.5 g/L) and was adjusted to pH 7.0–7.2 with NaOH. After 1 day of incubation at 28 °C with agitation, a portion of the culture broth (5% final concentration) is transferred into a production medium of the following composition: lactose (20.0 g/L), distillers solubles (10.0 g/L), and yeast autolysate (5.0 g/L) adjusted to pH 7.0 with NaOH. Following incubation at 28 °C for 3 days with agitation, a portion of this growth (5% final concentration) is transferred into flasks of fresh production media. After 18 h of incubation with agitation at 28 °C, a sterile solution of sodium [*methylene*-^{13}C]propionate (90 atom % ^{13}C, MSD Isotopes) is added to the flasks to a final concentration of 100 μg/mL. Flasks are then incubated as before for another 54 h before product is isolated.

Registry No. L-681,110 component A_1, 82621-00-9; L-681,110 component A_2, 82623-58-3; L-681,110 component B_1, 82620-99-3; ATPase, 9000-83-3; GABA, 56-12-2.

pH Dependency of the Zinc and Cobalt Carboxypeptidase Catalyzed Enolization of (*R*)-2-Benzyl-3-(*p*-methoxybenzoyl)propionic Acid

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Abstract: The pH dependency of the kinetics of the CPA-catalyzed enolization of the ketonic substrate (*R*)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid ((−)-**1**) has been determined. The study of this relatively simple reaction allows us to examine the catalytic properties and ionization behavior of the enzyme-bound active-site bases and acids without the complications that would be encountered for peptides and esters where the formation and breakdown of a multiplicity of intermediates along the reaction pathway must be considered. The pK_a values of 6.03 ± 0.35 and 6.04 ± 0.31 measured from the pH dependency of k_{cat} for the Zn(II) and Co(II) CPA catalyzed enolization of (−)-**1**, respectively, correspond to the pK_a for the ionization of the γ-carboxyl of Glu-270. The binding of (−)-**1** to CPA was investigated by examining the inhibition by this compound of the enzyme-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate. The K_I–pH dependencies for the inhibitory activity of (−)-**1** show that in alkaline solution one ionization of an enzyme-bound group occurs with pK_a = 7.56 ± 0.15 for the Zn(II) enzyme and pK_a = 8.29 ± 0.32 for Co(II) CPA while the other occurs above pH 9. The pK_a values in the vicinity of 8 represent the ionization of the phenolic hydroxyl of Tyr-248, in good agreement with earlier assignments of this pK. Finally, a reasonable interpretation of the pH dependency on a group with pK_a <9 is that it corresponds to the ionization of the active-site metal ion bound water.

The action of many acyl transfer enzymes is known to involve the participation of highly reactive nucleophiles present in their active sites. Although kinetic and chemical modification studies on the hydrolytic processes provide powerful techniques, a multiplicity of reaction intermediates, the possibility of changes in rate-determining step with changes in pH, and other factors often conspire to make the precise nature of the participation of the enzyme-bound nucleophile obscure. For example, in the instance of the well-known enzyme pepsin, although many studies have been made on the chemistry of the active site and the X-ray structures of related acid proteases are known, the interpretation of the wealth of kinetic and chemical modification data available in terms of a detailed reaction mechanism remains highly controversial.[1] With these problems in mind, in our laboratory an investigation was undertaken on the zinc-containing enzyme carboxypeptidase A (CPA) to establish whether we could find a simple reaction where the problem of the existence of multiple intermediates would not occur and that could be used to probe the catalytic activity of the presumed nucleophile in the active site of CPA, the γ-carboxylate group of the essential Glu-270 residue.[2] Indeed, we have demonstrated that the active site of the hydrolytic enzyme CPA is capable of catalyzing stereospecifically an enolization reaction.[3] In particular, the enzyme catalyzes stereospecifically[3,4] the exchange of hydrogens present in an activated methylene group of a ketonic substrate. More recently, it has been shown that CPA catalyzes the α,β-elimination

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