AB023, NOVEL POLYENE ANTIBIOTICS

II. ISOLATION AND STRUCTURE DETERMINATION

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AB023, a complex of new polyene antibiotics, was isolated from a soil *Streptomyces* strain. The two main components, pentaene antibiotics AB023a and AB023b, were separated and purified by preparative chromatographic methods and their structures were determined by extensive NMR and mass spectrometric studies.

In the course of our screening program for active substances of microbial origin, a new antifungal antibiotic was isolated from the mycelium of a *Streptomyces* sp. SD581 and designated AB023.

AB023 is a complex of polyenic antibiotics: The major components are the pentaenes AB023a and AB023b, whose structures differ only by a $-CH_2-$ moiety (Fig. 1).

In the companion paper[1], taxonomy of the producing organism, fermentation and biological activity were presented. The present paper describes the isolation and the chemical properties of both AB023a and AB023b and their structure determination.

Inside this class of antibiotics, all the structurally identified compounds are methylpentaenes as fungichromin[2,3], elizabethin[4], chainin[5,6] and the filipin complex[7,8]: AB023a and AB023b are sugar free, 28-membered macrocyclic pentaenes with no substituent on the conjugated double-bond chain.

Experimental

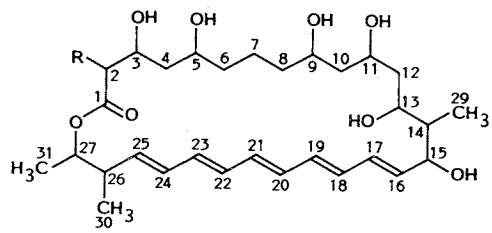
HPLC assays were performed on a LiChrosorb RP-18 cartridge column (250 × 4 mm, Merck), equipped with a guard-cartridge μBondapak RP-18 (Millipore/Waters, Milford, MA) maintained at 35°C. The chromatograms were analyzed with a photodiode array UV detector. Instrumentation included two M510 pumps, a U6K injector, a 990S photodiode array detector, a RCM-100 column chamber (all from Millipore/Waters, Milford, MA) and a computer APC IV (NEC Corporation, Tokyo).

All mass spectra were obtained on a Finnigan MAT 8400 double focusing reverse geometry mass spectrometer, equipped with a SuperIncos data system for data acquisition and elaboration.

All NMR spectra were recorded on a Bruker AM 300 NMR spectrometer. ^1H NMR spectra were recorded in DMSO-d_6 and in MeOD-d_4: Chemical shifts are given in ppm, referenced to the methyl group of the solvent, at 2.56 ppm in DMSO-d_6 and at 3.30 ppm in MeOD-d_4.

^{13}C NMR spectra were recorded in MeOD-d_4 and in DMSO-d_6, at 75 MHz, using broad-band proton decoupling; chemical shifts are given in ppm, referenced to the methyl group of the solvent, at

Fig. 1. Structures of AB023a and AB023b.



AB023a R = CH_3 (28-C)
AB023b R = CH_2CH_3 (28-C, 32-C)

49.0 ppm in MeOD-d_4, and at 39.5 ppm in DMSO-d_6.

^{1}H-^{1}H correlation spectra (COSY) were recorded in phase-sensitive, double-quantum mode, using the standard pulse sequence in MeOD-d_4. A 2K × 1K data matrix was accumulated in 512 increments with 80 scans for each increment. The delay time between scans was 1 second. ^{1}H-^{13}C correlation spectrum (HETCOR) was recorded in MeOD-d_4, using the standard pulse sequence. A 2K × 512W data matrix was accumulated in 128 increments with 1,024 scans for each increment (2.5 days). The experiment was optimized for a $^1J_{CH}=135$ Hz. The delay time between scans was 2 seconds and the total acquisition time was 2.5 days.

Results and Discussion

Isolation of the Antibiotics

Fifty liters of the culture broth of *Streptomyces* SD581[1)] were separated from the mycelium by centrifugation and the broth discarded, the mycelium cake was suspended in a solution of ethanol - water (1 : 1) and then filtered to remove cells. The filtrate was concentrated under vacuum until total removal of ethanol and the residue was applied to a column of macroreticular neutral resin XAD-4 (Rohm and Haas Co., Philadelphia, USA). The column was washed with two bed volumes of water and then eluted with a linear gradient from 20% to 80% of a solution of acetonitrile - methanol (1 : 1) in water.

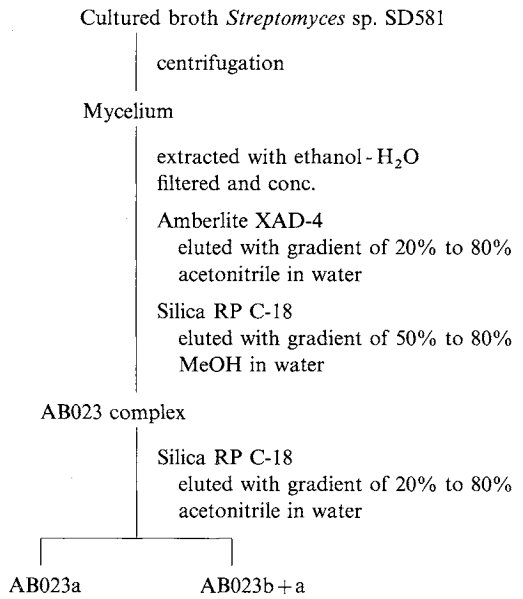
The active fractions were pooled, loaded on a column of reverse phase silica C-18 (Amicon Europe, Lausanne, CH) and eluted with a linear gradient from 50% to 80% of methanol in water. AB023 precipitated from the fraction containing it as a homogenous peak by HPLC in methanol - water (86 : 14) condition and as a double peak in acetonitrile - water (46 : 54) on a C-18 reverse phase column in agreement with DCI-MS experiments that demonstrated two distinct ions.

The partial separation of the two components, AB023a and AB023b, was achieved by a preparative chromatography on reverse phase silica C-18, using as eluent a linear gradient from 20% to 80% of acetonitrile in water.

After concentration and drying, 50 mg of pure AB023a and about 100 mg of the complex AB023 (enriched in AB023b) were obtained.

The steps leading to the isolation are outlined in Fig. 2.

Fig. 2. Isolation procedure of the antibiotics.



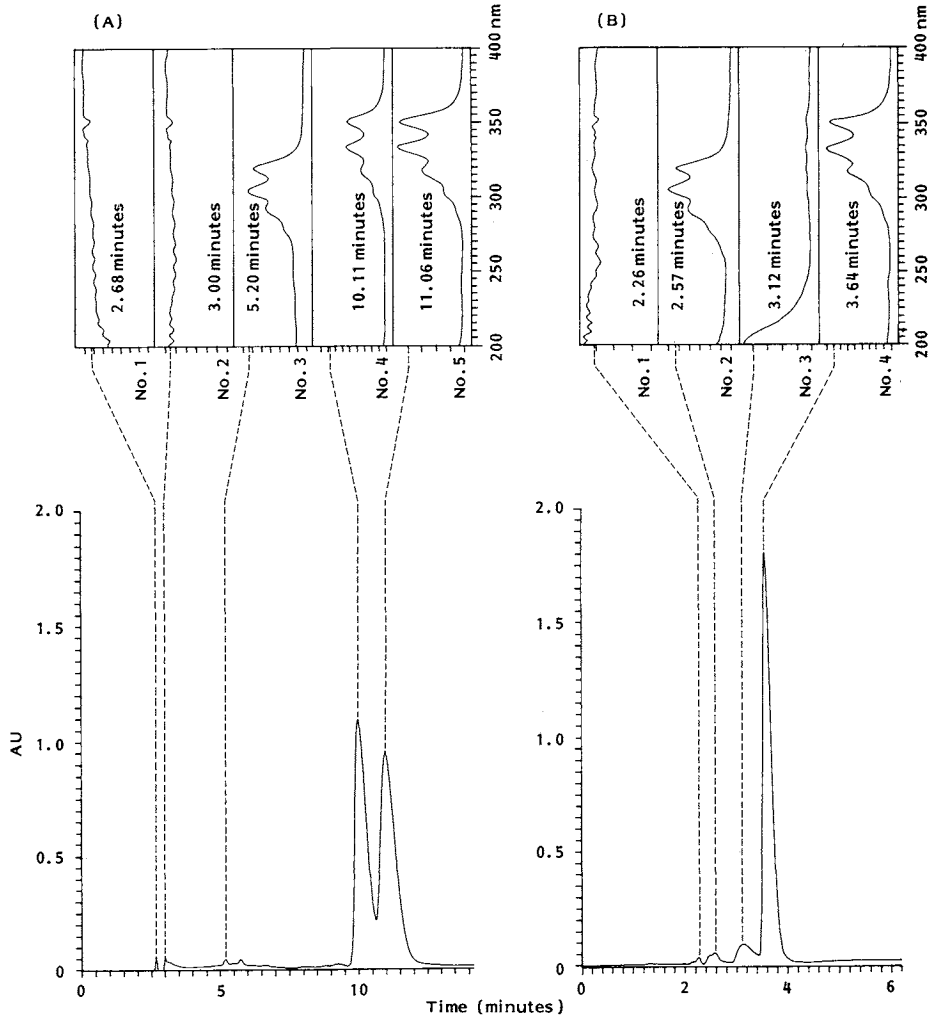
Physico-chemical Properties

AB023 is a complex of polyenic antibiotics, whose major components are the pentaenes AB023a and AB023b in about a 1 : 1 relative ratio. The purification step was rather difficult and only AB023a was isolated as a pure compound while AB023b was isolated in an enriched form (60% component b and 40% component a).

The complete separation of the complex could be achieved only by HPLC in acetonitrile - water on reverse phase C-18 column, while only a single peak was observed in methanol - water condition (Fig. 3).

Fig. 3. HPLC profile of the complex AB023.

The upper part of the figure shows the UV spectra of every peak in the chromatogram.



A) Column = RP-18 250 × 4 mm, Merck. Eluents = acetonitrile - water 46 : 54, flow = 1 ml/minute, T = 40°C.

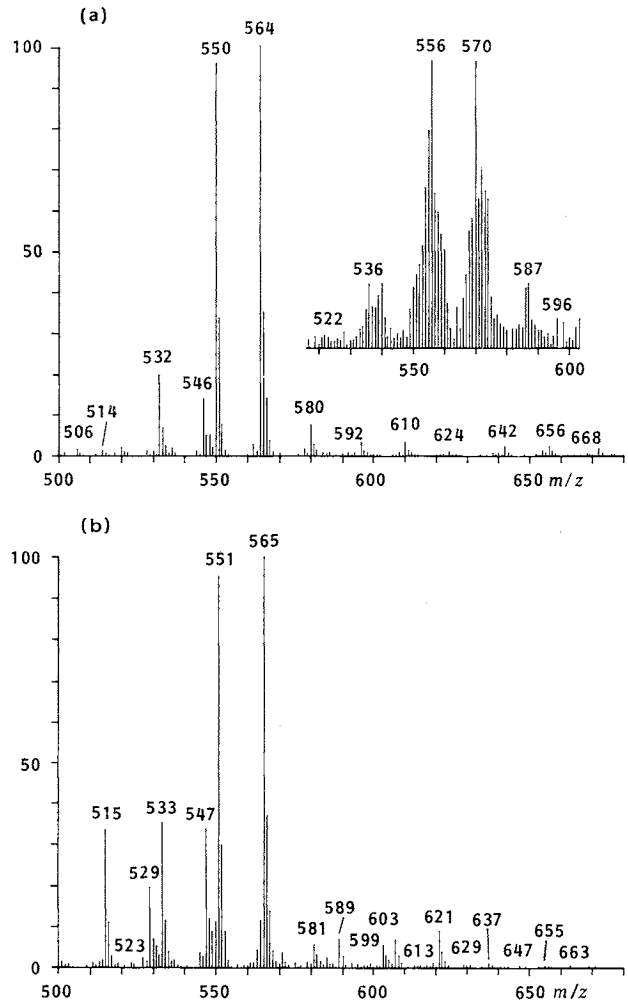
B) Column = RP-18 250 × 4 mm, Merck. Eluents = methanol - water 86 : 14, flow = 1 ml/minute, T = 40°C.

The antibiotic components were analyzed by desorption chemical ionization mass spectrometry (DCI-MS) that proved to be a suitable ionization technique for the characterization of non-polar biomolecules[9].

AB023a showed a molecular ion at *m/z* 551 $(M+H)^+$ in the positive and an ion at *m/z* 550 $(M)^-$ in the negative ion mode while AB023b showed ions at *m/z* 565 $(M+H)^+$ and *m/z* 564 $(M)^-$, respectively (Fig. 4). The 14 m.u. difference found between the molecular ions clearly indicated the presence of one more $-CH_2-$ group in AB023b which was identified at 22.1 ppm on the ^{13}C NMR spectrum (Fig. 11).

The molecular formula of $C_{31}H_{50}O_8$ was established for AB023a by high resolution mass spectrometry (HRMS) in the electron impact ionization mode. The accurate mass values, the ions due to consecutive

Fig. 4. DCI mass spectra of AB023 (a+b).



a) Negative ion mode. b) Positive ion mode.
The insert on part (a) shows a DCI-MS experiment using ND_3 as reagent gas.

water losses (*m/z* 532, 514 and 496) and their ^{13}C isotopic ions are reported in Table 1. All data are the average values of six or more measurements.

The elemental analysis confirmed that the nitrogen is not a constituent of AB023a and AB023b.

The ^1H NMR fully-decoupled ^{13}C NMR spectrum in MeOD-d_4 and the DEPT experiments at 45°, 90° and 135°, confirmed the presence of 31 carbons for AB023a including one –COOR, ten CH=, seven –CH–OH, three –CH–, six $-CH_2-$, four $-CH_3$ (Fig. 5) and 32 carbons for AB023b including one –COOR, ten CH=, seven –CH–OH, three –CH–, seven $-CH_2-$ and four $-CH_3$.

Consequently a molecular formula of $C_{32}H_{52}O_8$ could be assigned to AB023b.

The carboxylic group and the five double bonds accounted for all but one the structure insaturations in both AB023a and AB023b hence only a ring was confirmed in their structures.

In the ^1H NMR spectrum of AB023a in DMSO-d_6 (Fig. 6), six signals, in the range 5.25~4.25 ppm, were observed, lacking in the MeOD-d_4 spectrum. They were assigned to –OH groups because of the

Table 1. HREI-MS experiments.

Molecular formula	Ions	Calculated	Found	(ppm)
$C_{31}H_{50}O_8$	M^+	550.3505	550.3474±0.004	6
$C_{31}H_{48}O_7$	$(M-H_2O)^+$	532.3400	532.3407±0.002	1
$C_{30}[^{13}C]H_{48}O_7$	$[(M+H)-H_2O]^+$	533.3433	533.3458±0.003	5
$C_{31}H_{46}O_6$	$(M-2H_2O)^+$	514.3294	514.3309±0.0004	3
$C_{30}[^{13}C]H_{46}O_6$	$[(M+H)-2H_2O]^+$	515.3327	515.3332±0.001	1
$C_{31}H_{44}O_5$	$(M-3H_2O)^+$	496.3188	496.3194±0.001	1
$C_{30}[^{13}C]H_{44}O_5$	$[(M+H)-3H_2O]^+$	497.3221	497.3218±0.002	1

Fig. 5. ^1H broad-band decoupled ^{13}C NMR spectrum of AB023a in MeOD-d_4.

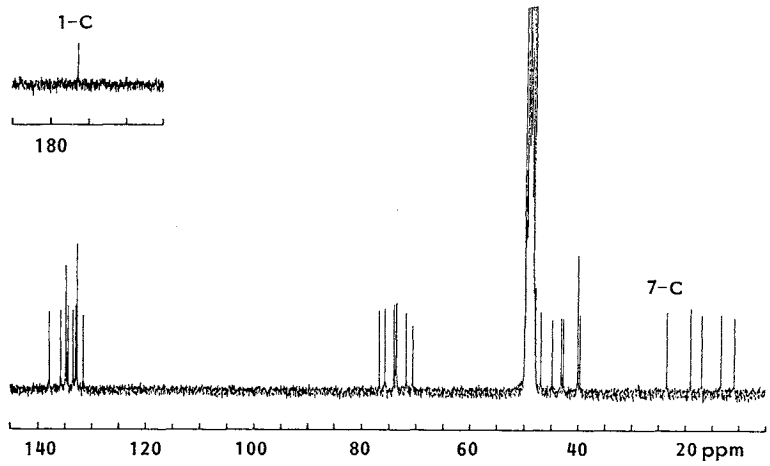
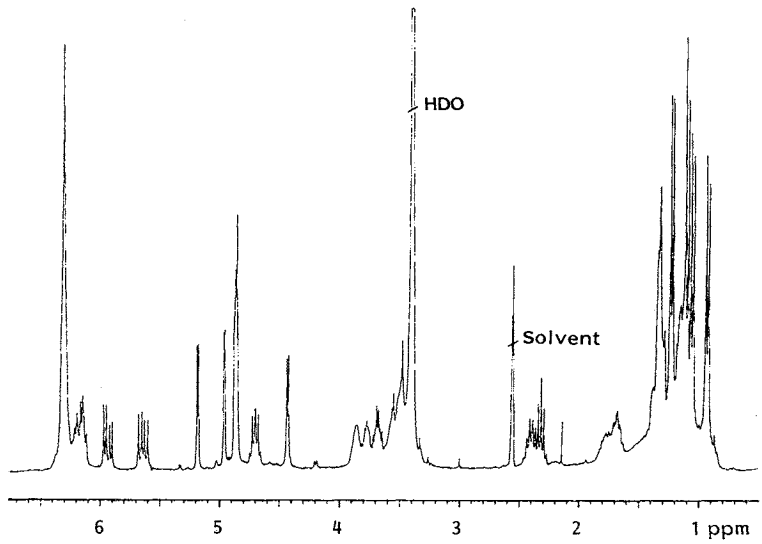


Fig. 6. ^1H NMR spectrum of AB023a in DMSO-d_6.



absence of nitrogen atom in the molecular formula. The presence of six exchangeable hydrogens was further demonstrated in both the biomolecules by DCI-MS experiments with deuterated-ammonia as the reagent gas[10] (see insert in Fig. 4a).

The other signals were grouped as follow: δ 6.30~5.30 (10H, m, -CH=), 4.74 (1H, m, -CH-O-CO-), 4.00~3.50 (6H, m, -CH-O-), 2.35 (1H, m, -CH-), 2.34 (1H, m, -CH-), 1.91~1.61 (2H, m, -CH-), 1.45~1.10 (11H, m, $-CH_2-$), 1.24 (3H, d, $-CH_3$), 1.15 (3H, d, $-CH_3$), 1.05 (3H, d, $-CH_3$), 1.02 (3H, d, $-CH_3$).

The UV spectra of AB023a and AB023b were characteristic of the polyene antibiotics system (Fig. 7) and the structure of the conjugated double bonds was easily ascertained to be a pentaenes on the precise wavelength of the absorption bands[11] at 350, 332 and 317 nm.

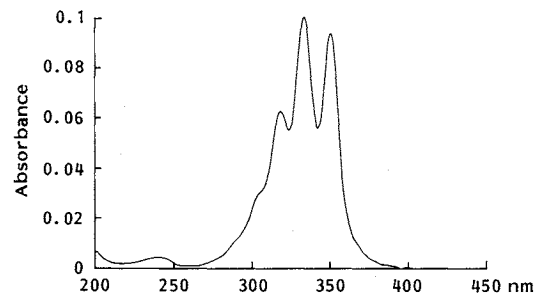
In the ^1H NMR spectrum no olefinic hydrogen shifts to low fields (7~8 ppm) were observed, indicating that the polyene system was not conjugated to the lactonic carbonyl; on the other hand, oxopentaenes should show strong modifications in the UV spectrum[11].

Moreover, in AB023a and AB023b any substitution on the pentaene system was excluded because of the presence of ten distinguishable olefinic use hydrogens in the ^1H NMR spectra.

The UV spectra also, confirmed this hypothesis, since the substitution of the hydrogens on the chromophoric double bond system would lead to a 4 or 6 nm red shift of the absorption bands[12].

The IR spectrum of AB023a showed the expected stretching absorption of hydroxy groups (3400 cm^{-1}), lactone carbonyl group (1725 cm^{-1}) and C=C double bonds (1636 cm^{-1}) (Fig. 8).

Fig. 7. UV spectrum of AB023a in MeOH-H_2O (50:50).



Structure Determination of AB023a

By NMR analysis the complete structure of AB023a was determined. The structure of AB023b was then elucidated in comparison with the NMR

Fig. 8. IR spectrum of AB023a.

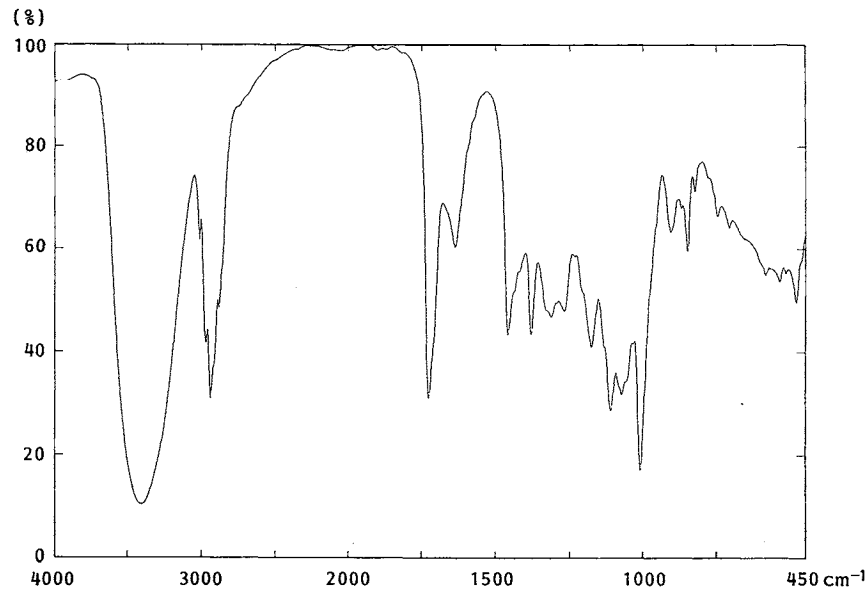
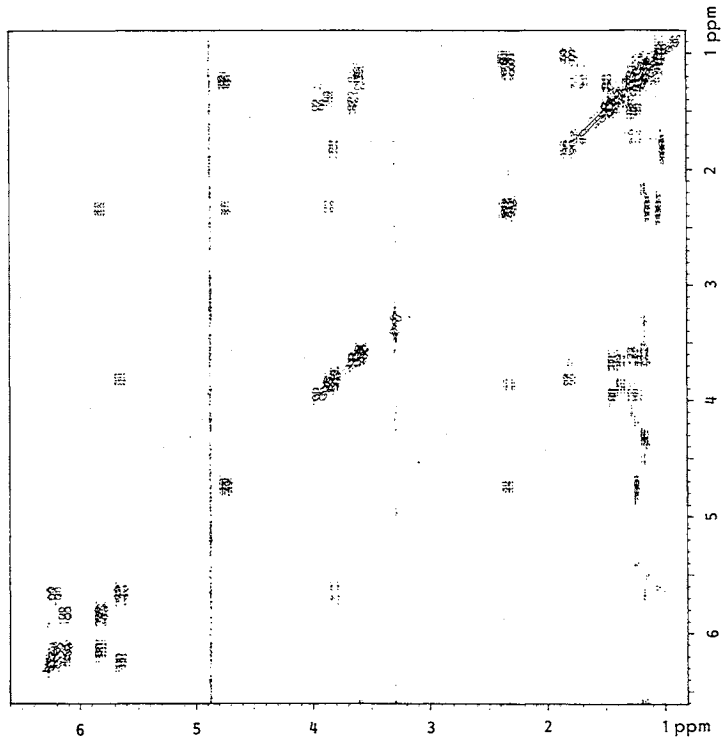


Fig. 9. Double-quantum phase-sensitive COSY spectrum of AB023a in MeOD-d_4.



signals of the two compounds (Fig. 1).

The assignment of the ^{1}H NMR spectrum of AB023a was based on the homonuclear double-quantum, phase-sensitive COSY experiments in MeOD-d_4 and in DMSO-d_6, while the correlation between proton and carbon chemical shifts was performed by means of the ^{1}H-^{13}C COSY (HETCOR) experiment. On the COSY spectrum (Fig. 9), the resonances of the protons in the central part of the pentaenic system were all overlapped in one multiplet at 6.24 ppm. Only the four terminal olefinic protons were distinguishable; at δ 6.15 (1H, m, 24-H), 5.82 (1H, dd, 25-H), 6.22 (1H, m, 17-H), 5.65 (1H, dd, 16-H). The protons 26-H and 15-H, directly coupled to the olefinic ones, could be assigned without ambiguities, as well. The proton 26-H, at δ 2.35 was clearly coupled to the methyl group at δ 1.05 and to the methine signal that, on the basis of the chemical shift at δ 4.74 and the lack of other couplings, was assigned to 27-H adjacent to the carboxylic group. On the other side of the pentaenic system, the proton 15-H (δ 3.82, CH–O–) showed a coupling with 14-H (δ 1.82, –CH–) which was coupled to 29-H (δ 1.02, CH_3) and to 13-H (δ 3.66, –CH–O–). The assignment of the remaining part of the structure was much less straightforward because of the signal overlapping in two narrow regions (–OH and $-CH_2-$ regions) due to the lacking of some expected cross-peaks, probably because of a too weak coupling between unfavorably oriented hydrogens. The ^{1}H-^{13}C heteronuclear COSY experiment (Fig. 10) was fundamental to complete the structure of AB023a, because it enabled us to determine the proton chemical shifts of the six methylene groups overlapped in the ^{1}H-^{1}H COSY spectrum: δ 1.73/1.02, 1.45, 1.45/1.26, 1.44/1.31, 1.40, 1.23.

On this basis, these other connections could be observed on the ^{1}H-^{1}H COSY map: The proton 13-H was connected to the methylene group at δ 1.45 (12-H); 12-H was clearly connected to the

Fig. 10. Heteronuclear COSY of AB023a in MeOD-d_4.

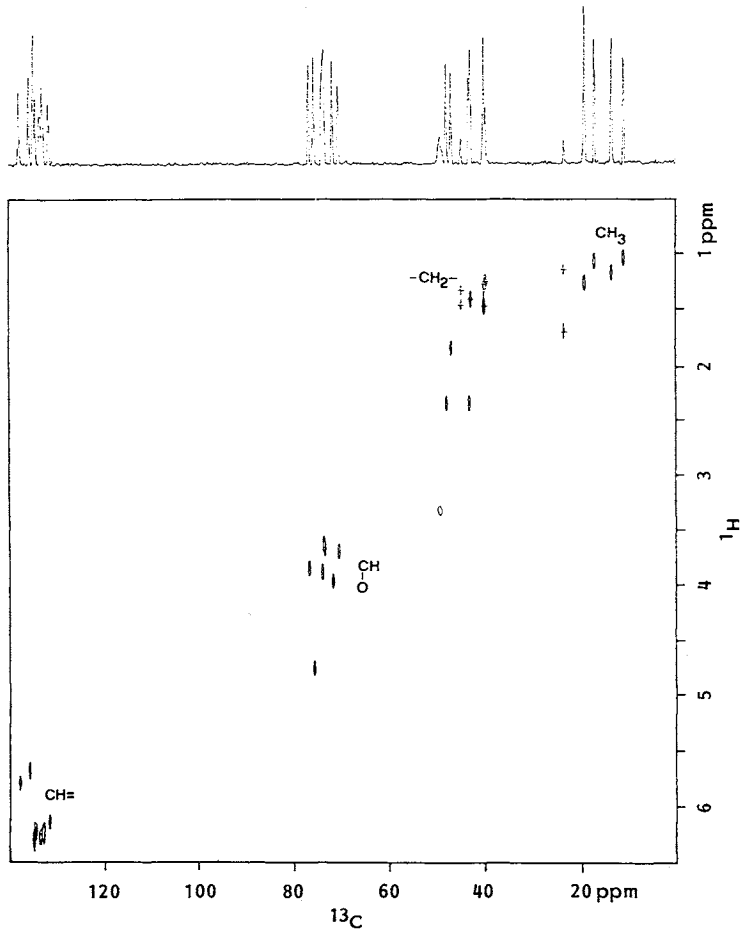
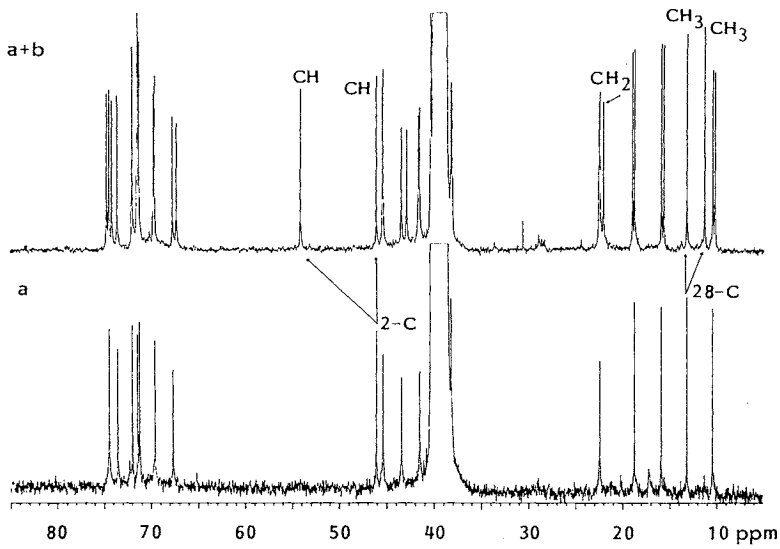


Table 2. ^{1}H and ^{13}C NMR data for AB023a in MeOD-d_4.

Position	^{1}H (ppm)	^{13}C (ppm)	Position	^{1}H (ppm)	^{13}C (ppm)
1		176.4 s	17	6.22 m	132.7 d
2	2.34 m	47.8 t	18	6.24 m	134.7～132.7 d
3	3.86 m	74.0 d	19	6.24 m	134.7～132.7 d
4	1.44/1.31 m	44.8 t	20	6.24 m	134.7～132.7 d
5	3.65 m	73.5 d	21	6.24 m	134.7～132.7 d
6	1.23 m	39.6 t	22	6.24 m	134.7～132.7 d
7	1.73/1.02 m	23.5 t	23	6.24 m	134.7～132.7 d
8	1.40 m	42.7 t	24	6.15 dd	131.5 d
9	3.59 m	73.7 d	25	5.82 dd	137.8 d
10	1.26/1.45 m	40.0 t	26	2.35 m	43.1 t
11	3.95 m	71.8 d	27	4.74 m	75.7 d
12	1.45 m	40.0 t	Me-28	1.15 d	13.4 q
13	3.66 m	70.5 d	Me-29	1.02 d	10.9 q
14	1.82 m	46.9 t	Me-30	1.05 d	17.0 q
15	3.82 m	76.7 d	Me-31	1.24 d	19.0 q
16	5.65 dd	135.7 d			

Fig. 11. ^{1}H broad-band decoupled ^{13}C NMR spectrum of the complex AB023 (a+b) and the pure AB023a in DMSO-d_6.



–CH–O– at δ 3.95 (11-H); 11-H was coupled with $-CH_2-$ at δ 1.45/1.26 (10-H); 10-H seemed to be coupled to the –CH–O– at δ 3.59 (9-H) which was connected to another methylene group at δ 1.40 (8-H).

On the other side of the carbonyl group: The methine hydrogen at δ 2.34 (2-H) was coupled, in addition to the methyl group at δ 1.15 ppm, to the –CH–O– (δ 3.86, 3-H) which was connected to the methylene group (δ 1.44/1.31, 4-H); 5-H (δ 3.65, –CH–OH) was connected to 4-H and to the methylene group 6-H at δ 1.23. Furthermore the latter (6-H) exhibited a connectivity with another $-CH_2-$ in the upper part of the homonuclear COSY spectrum at δ 1.02/1.73. Its corresponding ^{13}C chemical shifts, δ 23.5, is at the highest field among all methylene groups, thereof a position between two methylene groups could be supposed for it.

Table 3. ^{13}C NMR data for AB023b in DMSO-d_6.

Position	^{13}C (ppm)	Position	^{13}C (ppm)
1	173.1 s	17	130.6 d
2	54.1 t	18	133.1~131.1 d
3	71.9 d	19	133.1~131.1 d
4	42.9 t	20	133.1~131.1 d
5	71.4 d	21	133.1~131.1 d
6	38.1 t	22	133.1~131.1 d
7	22.5 t	23	133.1~131.1 d
8	41.7 t	24	129.2 d
9	71.5 d	25	136.5 d
10	38.9 t	26	39.5 t
11	69.7 d	27	74.1 d
12	38.7 t	CH_2-28	22.1 t
13	67.3 d	Me-28	11.3 q
14	45.5 d	Me-29	10.2 q
15	74.7 d	Me-30	15.6 q
16	136.5 d	Me-31	19.0 q

The remaining methylene group at δ 1.40 was assigned 8-H but there were no direct evidences for the connection with the methylene 7-H.

All the protons and carbon assignments of AB023a are reported in Table 2.

Structure Determination of AB023b

The characteristic carbon chemical shifts of AB023b were based on a comparison with the ^{13}C NMR spectra of AB023 (a+b) and AB023a (Fig. 11): AB023b owned a $-CH_2-$ group at δ 22.1 more than AB023a; the methyl group (28-C) on 2-C in AB023a (at 13.2 ppm) was shifted to high fields (δ 11.3 ppm)

in AB023b and the 2-C signal, as well, was positioned at lower-field in AB023b (δ 54.1 ppm) than in AB023a (δ 46.1). From these observations it is clear that the 2-C methyl group in AB023a is replaced by an ethyl group in AB023b. In fact the observed shift of 2-C is in agreement with the expected downfield shit of 9 ppm for the substitution of a β-hydrogen by a methyl group (β-effect)[13].

A support for this hypothesis is that in the ^{1}H NMR spectrum of AB023 (a+b) a triplet pattern appears at δ 0.88 due to a different methyl group and the 2-H multiplet is shifted upfield (0.2 ppm).

All the carbons observed in the spectra of AB023 (a+b) and assigned to AB023b, are reported in Table 3.

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