ARIZONINS, A NEW COMPLEX OF ANTIBIOTICS RELATED TO KALAFUNGIN

II. ISOLATION AND CHARACTERIZATION

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A new complex of anti-Gram-positive antibiotics was produced by the fermentation of *Actinoplanes arizonaensis* sp. nov. The antibiotics were recovered from the fermentation broth with Amberlite XAD-7 resin and from the mycelium by acetone lysis. UV, IR, MS and NMR spectral studies characterized these compounds as kalafungin-type antibiotics. They differ from other known members by an unusual oxidation pattern on the aromatic ring. They vary from one another by the degree and position of *O*-methylation on the aromatic ring and in the aliphatic portion of the molecules. The structure of one component was confirmed by X-ray diffraction analysis.

In the course of screening microorganisms for the production of novel antibiotics, a new Actinoplanete species was discovered. This species produced 6 novel anti-Gram-positive antibiotics (**1**~**6**) related to kalafungin. A later paper will describe the identification and characterization of this microorganism. The biological activity of the antibiotics has previously been presented.[1] This paper will describe the isolation of the antibiotic components, their physico-chemical characteristics, and the elucidation of their structures.

A butanol extract of the broth from a 20-liter fermentation of *Actinoplanes arizonaensis* sp. nov. exhibited antibiotic activity against a number of Gram-positive bacteria. Analytical thin-layer chromatography and further isolation work indicated that this activity was produced in such small quantities by the wild-type strain, that a 5,000-liter fermentation was required to isolate sufficient material for full characterization.

Whole broth (5,000 liters) was adjusted to pH 5.0 with H_2SO_4 and Amberlite XAD-7 was added (350 liters) with overnight stirring. The XAD-7 and mycelium were removed by centrifugation, washed with water and eluted three times with acetone (3×350 liters). The eluate was concentrated to an aqueous phase (50 liters) and extracted seven times with methylisobutylketone (MIBK) (7×10 liters). The MIBK extract was evaporated under reduced pressure to a residue which was partitioned between hexane - MeOH (1.5 liters of each). The MeOH layer was partitioned between $CHCl_3$ - MeOH - H_2O (900 ml of each) and this partitioning repeated three times, the activity remaining each time in the lower layer. The combined lower layers were concentrated to an oil which was dissolved in EtOAc (2 liters). The EtOAc solution was extracted three times with distilled water (3×1.5 liters) and the aqueous layers were discarded. The EtOAc layer was concentrated under reduced pressure to an oil (209 g) which was then subjected to chromatography in two batches on a Sephadex LH-20 column (7.5×100 cm bed volume) eluted with MeOH - $CHCl_3$ (3:2). Active fractions from both LH-20 columns were combined and concentrated under reduced pressure to leave an oily solid. This

Fig. 1. Structures of the arizonins.

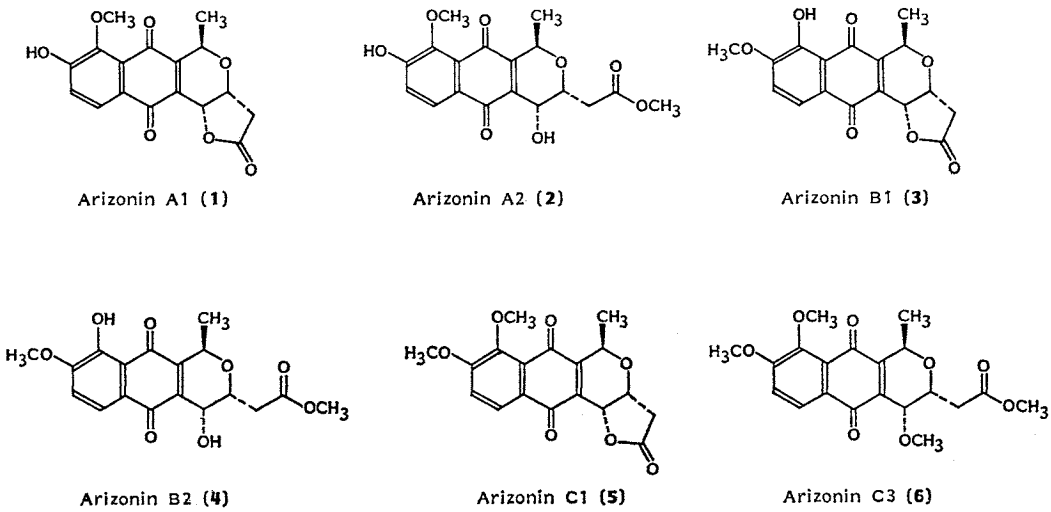
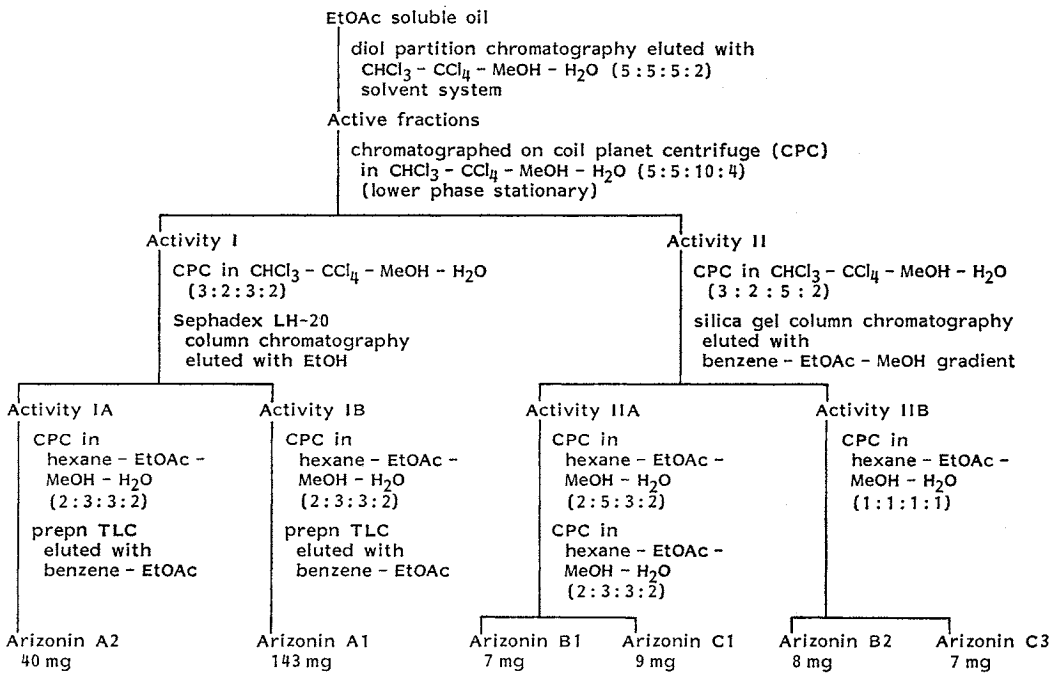


Fig. 2. Isolation of the arizonins.



was subjected to partition chromatography on a diol column[2] (2.5×90 cm bed volume) eluted with the lower phase of $CHCl_3$ - CCl_4 - MeOH - H_2O (5:5:5:2). Active fractions were combined and concentrated under reduced pressure to leave a solid residue (1.68 g). This residue was subjected to a separation scheme (see Fig. 2) comprised of multiple counter-current chromatographies on an Ito multi-layer coil planet centrifuge (CPC), silica gel columns and preparative thin-layer chromatographies (see Fig. 2).

Structural Elucidation of the Arizonins

A high resolution mass spectrum of arizonin B1 in the electron impact mode yielded a parent peak of *m/z* 330.0731 establishing a molecular formula of $C_{17}H_{14}O_7$ (calcd 330.0735). The ^{13}C NMR (Table 1) and ^1H NMR (Fig. 3) spectra of arizonin B1 revealed both aliphatic and aromatic portions within the structure. Thirteen protons were attached to carbon as indicated by the ^{13}C NMR multiplicities and hence arizonin B1 contains one exchangeable proton.

An UV spectrum of arizonin B1 in MeOH (Fig. 4) contains absorption maxima at 268 and 457 nm shifting to 270, 308 and 550 nm in basic MeOH. UV, together with an AB system (J=8.4 Hz) of aromatic proton signals at δ 7.84 and 7.23 and carbon signals at δ 188.6, 180.4, 154.4, 152.6, 149.5, 135.8, 123.4, 121.4, 115.8 and 114.8 suggested the presence of an hydroxynaphthoquinone moiety (**7**).

Comparison of ^1H NMR data between the aliphatic portions of arizonin B1 (**3**) and kalafungin (**8**) (Table 2) and single frequency proton decoupling experiments revealed the close structural similarities between these antibiotics. Nuclear Overhauser effect (NOE) experiments on arizonin B1

Table 1. ^{13}C NMR chemical shifts of arizonins in $CDCl_3$.

Arizonin A1	Arizonin A2	Arizonin B1	Arizonin C1
182.7 (Q)	183.3 (Q)	188.6 (Q)	182.4 (Q)
181.4 (Q)	182.0 (Q)	180.4 (Q)	181.3 (Q)
175.0 (Q)	171.6 (Q)	173.9 (Q)	174.2 (Q)
157.3 (Q)	156.8 (Q)	154.4 (Q)	159.2 (Q)
150.0 (Q)	146.8 (Q)	152.6 (Q)	150.6 (Q)
147.4 (Q)	146.6 (Q)	149.5 (Q)	149.7 (Q)
134.7 (Q)	138.4 (Q)	135.8 (Q)	133.1 (Q)
132.9 (Q)	124.4 (Q)	123.4 (Q)	128.4 (Q)
125.1 (CH)	124.3 (CH)	121.4 (CH)	125.3 (Q)
124.6 (Q)	124.1 (Q)	115.8 (CH)	124.9 (CH)
121.2 (CH)	120.3 (CH)	114.8 (Q)	116.2 (CH)
69.2 (CH)	67.3 (CH)	68.8 (CH)	69.1 (CH)
66.7 (CH)	66.8 (CH)	66.6 (CH)	66.9 (CH)
66.4 (CH)	60.2 (CH)	66.2 (CH)	66.3 (CH)
61.2 (CH_3)	58.9 (CH_3)	56.2 (CH_3)	61.2 (CH_3)
36.8 (CH_2)	50.9 (CH_3)	37.0 (CH_2)	56.4 (CH_3)
18.2 (CH_3)	35.1 (CH_2)	18.7 (CH_3)	37.0 (CH_2)
	16.8 (CH_3)		18.7 (CH_3)

Q: Quaternary.

Fig. 3. ^1H NMR spectrum of arizonin B1 in MeOH-d_4.

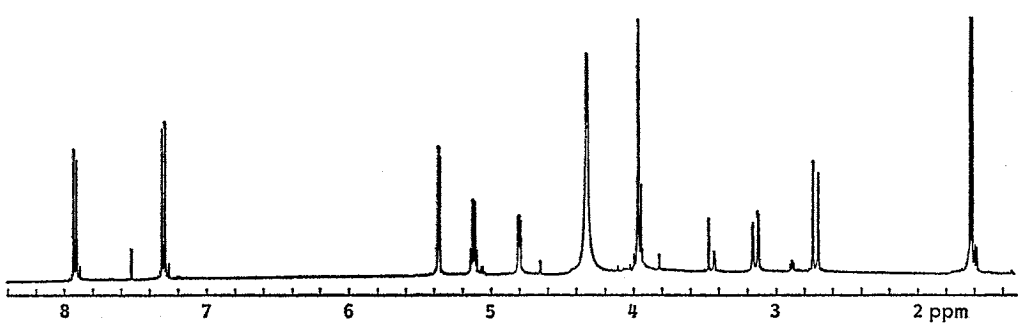


Fig. 4. UV spectrum of arizonin B1.

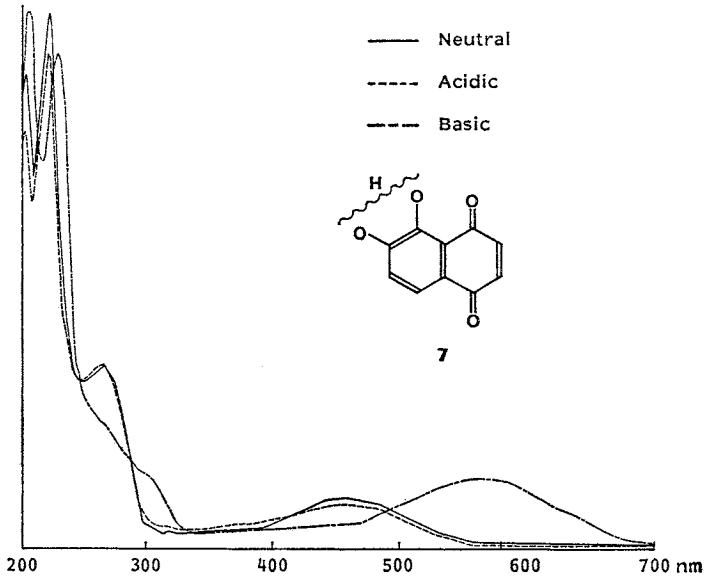


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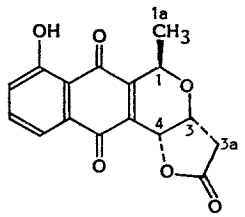
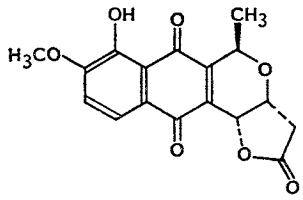
Table 2. ^{1}H NMR comparison of arizonin B1 and kalafungin.



Kalafungin (8)



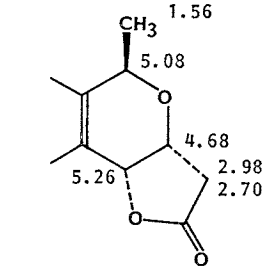
Arizonin B1 (3)

Position	Kalafungin (8)	Arizonin B1 (3)
1a	1.52 (d, J=7.0 Hz)	1.56 (d, J=7.4 Hz)
1	5.05 (q, J=7.0 Hz)	5.08 (q, J=7.4 Hz)
3	4.69 (dt, J=4.5 and 3.0 Hz)	4.68 (dt, J=5.0 and 2.7 Hz)
4	5.20 (d, J=3.0 Hz)	5.26 (d, J=2.7 Hz)
3a	3.02 (dd, J=18 and 4.5 Hz),	2.98 (dd, J=17.4 and 5.4 Hz),
	2.57 (d, J=18 Hz)	2.70 (d, J=17.4 Hz)

established the relative stereochemistry about the aliphatic portion (**9**). Protons whose signals are observed at δ 5.26 and 4.68 display a strong NOE to one another and in addition, each shows a similar but weaker NOE to the methyl signal at δ 1.56. The relative stereochemistry of **9** must therefore be identical to that of the kalafungins and other previously reported members of this antibiotic class.

The remaining proton signal, a methoxyl signal at δ 3.86, must be attached to the hydroxynaphthoquinone portion of the molecule. Nuclear Overhauser experiments position this methoxyl at C-8 based upon a strong NOE between the methoxyl protons and the aromatic signal at δ 7.23.

A hetero-nuclear COSY experiment determined the relative orientation of the hydroxynaphthoquinone to the aliphatic system as that in structure **3** as opposed to **10**. Carbonyl carbon signals for the hydroxynaphthoquinone appear at δ 188.6 and 180.4. The signal at δ 188.6 is assigned to the



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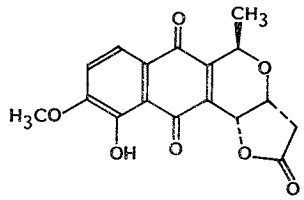

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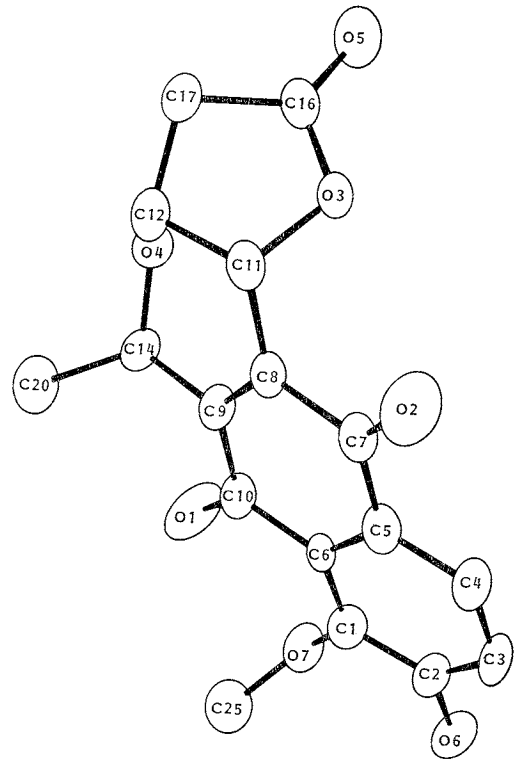
Fig. 5. X-Ray structure of arizonin A1.



hydrogen-bonded carbonyl due to its relative downfield chemical shift. The hydrogen-bonded carbonyl carbon signal exhibits a long range hetero-nuclear coupling to the proton at δ 5.08, whereas the non-hydrogen bonded carbonyl carbon signal exhibits a long range coupling to the proton at δ 5.26.

Absolute stereochemistry for arizonin B1 was determined by a comparison of the optical rotary dispersion curves between arizonin B1 and those of nanaomycin D and kalafungin. Based upon this comparison the absolute stereochemistry was determined to be that of kalafungin.

Arizonin A1 is isomeric with arizonin B1 ($C_{17}H_{14}O_7$) as evidenced by high resolution mass spectroscopy (observed m/z 330.0738, calcd 330.0735). ^{1}H NMR spectra of the two compounds were nearly superimposable. The ^{13}C NMR spectrum (Table 1) of arizonin A1 contains two quinone carbonyl signals at δ 182.7 and 181.4 indicating that neither carbonyl is hydrogen-bonded to a hydroxyl proton. Structure **1** was therefore assigned. This was confirmed and the substitution pattern of the aromatic ring for the family unequivocally established by a single crystal X-ray diffraction analysis of arizonin A1, the results of which are presented in Fig. 5.

The ^{1}H NMR spectrum of arizonin A2 contained two methoxyl signals at δ 3.77 and 3.91. UV spectra of arizonins A1 and A2 were nearly identical at all pH values and hence the methoxy-hydroxynaphthoquinone portions of these two structures must be identical. ^{13}C NMR spectra of arizonin A2 (Table 1) varied, only significantly in the portions assigned to aliphatic carbons and the ester lactone carbonyl, from the ^{13}C NMR spectra of arizonin B1. The additional methoxyl evident in the ^{1}H NMR spectrum is also observed as a peak at δ 50.9 in the ^{13}C NMR spectrum of A2. An IR spectrum of arizonin A2 contains a band at 1737 cm^{-1} indicative of a typical ester functionality rather than the γ-lactone carbonyl of arizonin A1. Interpretation of these data led to the assignment of the structure **2** to arizonin A2. The highest peak in the electron impact mass spectrum of arizonin

A2 occurred at *m/z* 344.0896 (calcd for $C_{18}H_{16}O_7$ *m/z* 344.0891). This arises from loss of water from the parent molecule $C_{18}H_{18}O_8$.

Arizonin C1 had a molecular formula of $C_{18}H_{16}O_7$ established by mass spectroscopy (observed *m/z* 344.0887, calcd 344.0891). Two aromatic methoxyl signals in the ^1H NMR spectrum were observed at δ 3.93 and 3.98 and in the ^{13}C NMR spectrum at δ 61.2 and 56.4. The UV spectrum of arizonin C1 in neutral or acidic solution was similar to that of arizonin A1, however, in contrast, the UV spectrum of C1 exhibited no bathochromic shift in base. An IR spectrum of arizonin C1 contained a γ-lactone type carbonyl band at 1780 cm^{-1}. These data were interpreted to indicate structure **5** for arizonin C1.

The ^1H NMR spectrum of arizonin C3 contained four methoxyl signals at δ 3.98, 3.85, 3.75 and 3.49 ppm. The IR spectrum of arizonin C3 contained a band at 1735 cm^{-1} indicative of a normal ester functionality. These data led to the assignment of structure **6** to arizonin C3.

Experimental

General Procedures

Optical rotations were measured in 1 dm tubes on a Perkin-Elmer model 241 polarimeter. Melting points were recorded on a Hoover Unimelt. UV spectra were recorded on a Perkin-Elmer Lambda 3B ultraviolet-visible spectrophotometer and IR spectra on a Perkin-Elmer 683 dual beam dispersive instrument. NMR spectra were acquired on either a General Electric QE 300 MHz or a Nicolet WM-360 MHz spectrometer. Hetero-nuclear COSY spectra were acquired by the method of BODENHAUSEN and FREEMAN[3] and carbon multiplicities were determined by the DEPT (distortionless enhancement of NMR signals by polarization transfer) of DODDRELL *et al.*[4] Nuclear Overhauser enhancement experiments were acquired by NOE difference spectroscopy.[5] Mass spectra were measured on a Kratos MS-50 spectrometer in the electron impact mode.

X-Ray Diffraction Analysis of Arizonin A1

Crystals of arizonin A1 suitable for single crystal X-ray diffraction analysis could be grown from $CHCl_3$ - MeOH solutions. A crystal of approximate dimensions $0.3\times0.4\times0.6$ mm was selected for the analysis. The crystal exhibited orthorhombic symmetry and accurate lattice constants of $a=14.112$, $b=23.185$, $c=4.510$ were determined from a least-squares fit of 15 diffractometer measured 2θ-values. Systematic extinctions and crystal density were uniquely accommodated by space group $P2_12_12_1$ with one molecule of composition $C_{17}H_{14}O_7$ forming the asymmetric unit. All diffraction maxima with $2\theta\leq135°$ were collected on a computer controlled four-circle diffractometer using graphite monochromated CuK_α radiation (1.54178 Å) and variable speed, 1° ω-scan. A total of 1,272 reflections were collected in this manner and after correction for Lorentz polarization and background effects, 1,149 (93.6%) were judged observed ($Fo\geq3\sigma(Fo)$) and used in subsequent calculations.*

A phasing model was found uneventfully using a multi-solution sign determining approach, and all non-hydrogen atoms were clearly visible in the first E-synthesis. Hydrogen atoms were located in a ΔF-synthesis following partial refinement of the non-hydrogen atoms. Least-square refinement with anisotropic non-hydrogen atoms and isotropic hydrogens converged to a conventional crystallographic residual of 0.059 for observed reflections.

Arizonin A1, $[\alpha]_D^{25}$ −92° (*c* 0.06, MeOH), was an orange crystalline solid, mp 233~235°C with decomposition. An electron impact mass spectrum established the molecular formula as $C_{17}H_{14}O_7$ with an exact mass of *m/z* 330.0738 (calcd 330.0735). An UV spectrum acquired in MeOH or MeOH - 0.1 N HCl contained bands at λ_{max} nm (ε) 215 (22,900), 262 (14,600), 406 (3,600) and in MeOH - 0.1 N NaOH at 225 (17,600), 258 (14,300), 303 (10,000) and 531 (4,400). An IR spectrum measured as a

* All crystallographic calculations were done on a Digital VAX 11/780. Principal programs employed were Multan 80 and Dirdif phase refinement supplied by Molecular Structure Corporation.

film contained bands at 3375, 2984, 2939, 1788, 1773, 1732, 1661, 1572, 1483, 1452, 1429, 1400, 1369, 1332, 1293, 1282, 1233, 1201, 1156, 1084, 1039, 991, 908 and 732 cm^{-1}.

Arizonin A2, $[\alpha]_D^{25}$ −149° (*c* 0.203, MeOH), was an orange crystalline solid, mp 170~174°C with decomposition. An electron impact mass spectrum gave a highest ion at *m/z* 344.0896 (calcd 344.0891) indicating a formula of $C_{18}H_{16}O_7$. This was interpreted as a loss of water from a parent formula of $C_{18}H_{18}O_8$. An UV spectrum acquired in MeOH or MeOH - 0.1 N HCl contained bands at λ_{max} nm (ε) 214 (22,500), 263 (14,000) and 405 (2,200) and in MeOH - 0.1 N NaOH at 226 (17,700), 258 (13,000), 303 (9,800), 372 (700) and 527 (3,700). An IR spectrum measured as thin film contained bands at 3400, 2985, 2940, 1737, 1660, 1572, 1483, 1374, 1282, 1228, 1200, 1158, 1121, 1081, 1042, 911, 765 and 732 cm^{-1}.

Arizonin B1, $[\alpha]_D^{25}$ −53.0° (*c* 0.112, MeOH), was an orange semi-solid. An electron impact mass spectrum established the molecular formula as $C_{17}H_{14}O_7$ with an exact mass of *m/z* 330.0731 (calcd 330.0735). An UV spectrum acquired in MeOH or MeOH - 0.1 N HCl contained bands at λ_{max} nm (ε) 223 (46,300), 268 (17,500) and 457 (7,300) and in MeOH - 0.1 N NaOH at 232 (45,500), 270 (9,000), 308 (5,000) and 550 (9,800). An IR spectrum measured as a thin film contained bands at 3440, 2956, 2925, 2854, 1788, 1648, 1620, 1601, 1581, 1460, 1366, 1270, 1200, 1154, 1227 and 1071 cm^{-1}.

Arizonin B2 was an orange semi-solid. An electron impact mass spectrum gave a parent peak at *m/z* 362 consistent with a molecular formula of $C_{18}H_{18}O_8$. An UV spectrum acquired in MeOH or MeOH - 0.1 N HCl contained bands at λ_{max} nm (ε) 223 (74,200), 264 (23,100) and 450 (4,800) and in MeOH - 0.1 N NaOH at 234 (68,100), 260 (sh), 300 (sh) and 560 (10,000).

Arizonin C1, $[\alpha]_D^{25}$ −84.3° (*c* 1.017, MeOH), was an orange crystalline solid, mp 110~135°C with decomposition. An electron impact mass spectrum established the molecular formula as $C_{18}H_{16}O_7$ with an exact mass match of *m/z* 344.0887 (calcd 344.0891). An UV spectrum acquired in MeOH, MeOH - 0.1 N HCl or MeOH - 0.1 N NaOH contained bands at λ_{max} nm (ε) 217 (8,200), 260 (4,500) and 402 (760). An IR spectrum measured as a thin film contained bands at 3360, 2979, 2941, 2848, 1780, 1694, 1663, 1575, 1487, 1451, 1403, 1377, 1337, 1278, 1232, 1208, 1164, 1126, 1090, 1071, 1054, 998, 916, 754 and 729 cm^{-1}.

Arizonin C3, $[\alpha]_D^{25}$ −104° (*c* 0.969, MeOH), was an orange crystalline solid, mp 195~208°C with decomposition. An UV spectrum acquired in MeOH, MeOH - 0.1 N HCl or MeOH - 0.1 N NaOH contained bands at λ_{max} nm (ε) 206 (29,100), 258 (6,300) and 390 (650). An IR spectrum measured as a thin film contained bands at 3469, 2938, 2918, 1735, 1698, 1662, 1578, 1486, 1452, 1439, 1416, 1374, 1333, 1273, 1199, 1166, 1127, 1055, 913 and 732 cm^{-1}.

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