# ISOLATION AND STRUCTURE DETERMINATION OF CRISAMICIN A, A NEW ANTIBIOTIC FROM *MICROMONOSPORA PURPUREOCHROMOGENES* SUBSP. *HALOTOLERANS*

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(Received for publication May 25, 1985)

A new antibiotic, crisamicin A, with *in vitro* activity against Gram-positive bacteria, B16 murine melanoma cells, and herpes simplex, vaccinia, and vesicular stomatitis viruses, has been isolated from *Micromonospora purpureochromogenes* subsp. *halotolerans*. On the basis of $^{1}H$ and $^{13}C$ NMR spectroscopic, high resolution field desorption mass spectrometric, and circular dichroism studies of the antibiotic and several of its derivatives, the structure of crisamicin A has been assigned.

A new antibiotic, crisamicin A, isolated from *Micromonospora purpureochromogenes* subsp. *halotolerans*[1~4], has been found active against Gram-positive bacteria, B16 murine melanoma cells, and herpes simplex, vaccinia, and vesicular stomatitis viruses (C. P. SCHAFFNER, personal communication). We assign here the structure of crisamicin A as **1** from chemical and physical data for the pure compound and several of its derivatives (see Scheme 1).††

## Isolation, Purification, and Spectroscopic Properties

Crude crisamicin A, obtained from a fermentation medium of *M. purpureochromogenes* subsp. *halotolerans*, was extracted repeatedly with acetonitrile and each extract was analyzed by HPLC. Fractions enriched in crisamicin A were collected, combined, and evaporated to dryness at reduced pressure to give a yellow powder, which was washed twice with a small amount of acetonitrile and crystallized from dioxane to afford orange needles. Attempts, using various solvents, to obtain a single crystal suitable for X-ray analysis were unsuccessful. The compound decomposed gradually and turned brown during heating so that its melting point could not be determined. It is moderately soluble in dioxane, tetrahydrofuran, acetone, chloroform, ethyl acetate, and acetonitrile, but is almost insoluble in methanol and water.

The $^{1}H$ and $^{13}C$ NMR, IR, and ORD spectra of crisamicin A are shown in Figs. 1~4. Crisamicin A was assigned the molecular formula $C_{32}H_{22}O_{12}$ on the basis of high resolution field desorption mass spectrometry (HRFD-MS) ($M^+$, $m/z$ 598.1124). The broad band decoupled $^{13}C$ NMR spectrum (Fig. 2), however, revealed only 16 signals and only eleven protons were observed in the $^{1}H$ NMR spectrum (Fig. 1). These observations argued the presence in crisamicin A of identical halves con-

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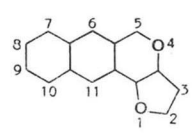
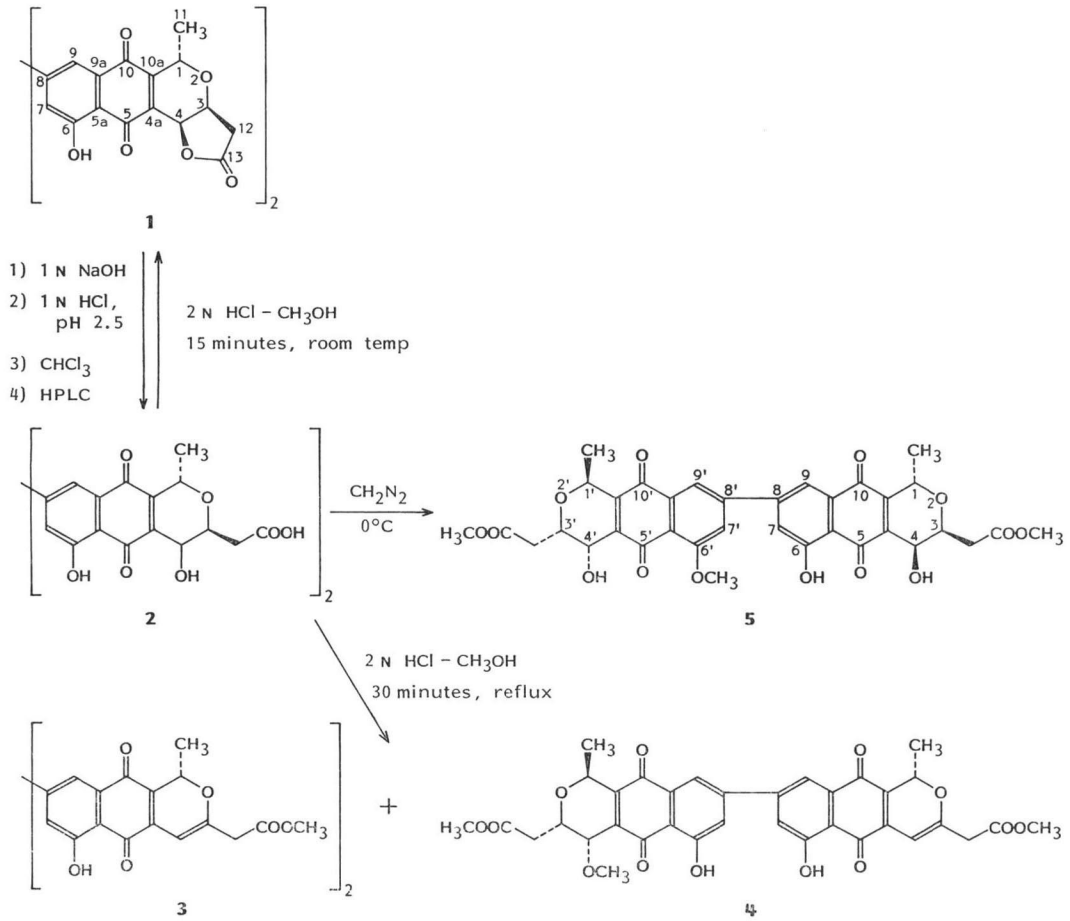
†† The official numbering of this ring system (a furonaphthopyran) should be that shown here (PATTERSON, A. M.; L. T. CAPELL & D. F. WALKER: The Ring Index. 2nd Ed., pp. 463, 620, American Chemical Society, Washington, D.C., 1960). However, to maintain agreement with the previous numbering system employed for kalafungin[10] and, sometimes, for the nanaomycins[11], we have numbered the system as a substituted naphthopyran.



Scheme 1.




nected by a symmetry element. Thus, the $C_{16}H_{11}O_8$ unit, with 11 elements of unsaturation, and the point(s) of connection of the halves were to be determined.

## Structure Assignment

The $^{13}C$ NMR signals for crisamicin A included two quinone carbonyl carbons ($\delta$ 187.6 and 182.3)[5] and eight aromatic (or olefinic) carbons, one of them oxygen substituted ($\delta$ 162.4) and two of them bearing hydrogens ($\delta$ 122.2 and 118.6). The FD mass spectrum is also in agreement with a quinone, showing peaks of variable intensity for the hydroquinone form at $m/z$ 600. These data, together with signals for a hydrogen-bonded phenol ($\delta$ 11.88, lost in $D_2O$) and two *meta*-substituted aryl hydrogens ($\delta$ 7.97 and 7.63, $J$=1.57 Hz) in the $^1H$ NMR spectrum, assign the juglone-type chromophore **a**. The UV spectrum of crisamicin A, with maxima at 215, 231, 267, and 435 nm, is also characteristic of a juglone-type chromophore[6], as are IR absorptions at 1653 and 1626 $cm^{-1}$ (quinone carbonyl groups, one of them hydrogen-bonded) and a phenolic hydroxyl group[7], giving a broad peak at *ca.* 3450 $cm^{-1}$. The NMR spectra of crisamicin A also afforded valuable information about the remaining structural details. The off-resonance $^{13}C$ NMR spectrum shows methyl ($CH_3$), methylene ($CH_2$), and three carbinyl (–$\underset{|}{C}$HO) units. These are juxtaposed into units **b** and **c** on the basis of the $^1H$ NMR spectrum and spin decoupling experiments.

Fig. 1. $^1H$ NMR spectrum of crisamicin A ($CD_2Cl_2$).

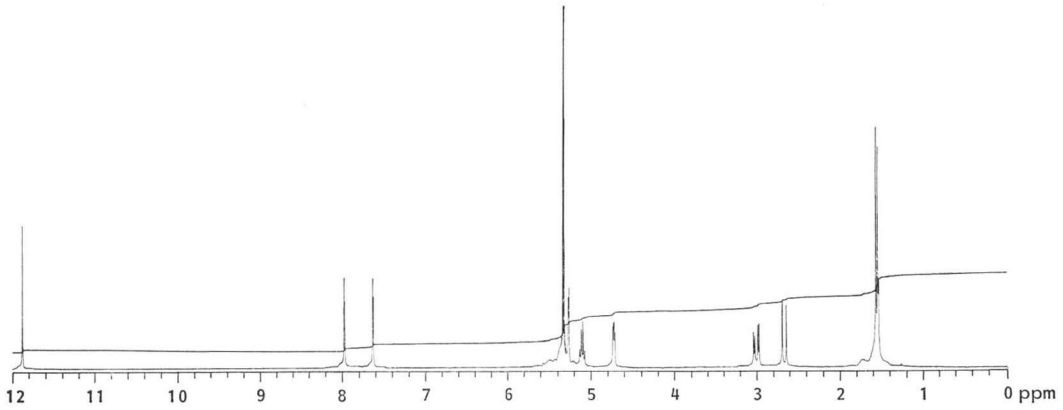
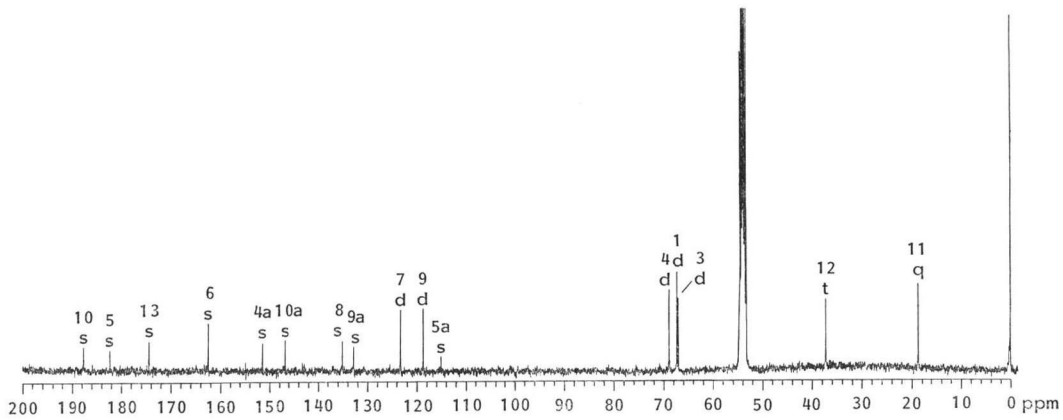



Fig. 2. Broad band $^1H$-decoupled $^{13}C$ NMR spectrum of crisamicin A ($CD_2Cl_2$).

Multiplicities: s=singlet, d=doublet, t=triplet and q=quartet in off-resonance proton-decoupled spectrum. The assignments of C-7 and C-9 are based on those of the corresponding carbons of juglone derivatives[5].




The remaining carbon of the half-molecule is a carboxyl carbon (COOR at 174.4 ppm). To satisfy the half molecular formula and its degree of unsaturation, this should be present in a lactone ring (in **c**), which would agree with the loss of two mol of carbon dioxide (from two lactone rings) in the FD mass spectrum to give ions at *m/z* 554 (together with 556) and 510. The IR band for the lactone (1796 $cm^{-1}$, KBr) identifies it as a strained $\gamma$-lactone (Y=CO) or a $\beta$-lactone (X or Y=CO). When the chemical shifts of the methylene protons alpha to the carbonyl group are compared with the corresponding data for propiolactone (3.56 ppm)[8] and butyrolactone [2.2~2.5 (m) ppm][9], however, the $\gamma$-lactone is far more likely (*i.e.*, X=Ar, Y=CO in **c**). Combination of partial structures **b** and **c** with the hydroxynaphthoquinone unit **a** then completes the structure of crisamicin A, since the remaining oxygen of **c** and that of **b** are the same, leading to unit **d** or **e**.

Structures **d** and **e** have a striking resemblance to kalafungin (**6**)[10] and nanaomycin D (**7**)[11]. In accord with that resemblance, the $^1H$ NMR signals for kalafungin[10] and crisamicin A are nearly identical (Table 1). The lactone absorption, however, was reported at 1760 $cm^{-1}$ (Nujol) in the IR spectrum

Fig. 3. IR spectra of crisamicin A (**1**) and its derivatives **2** and **5** in the carbonyl stretching region.

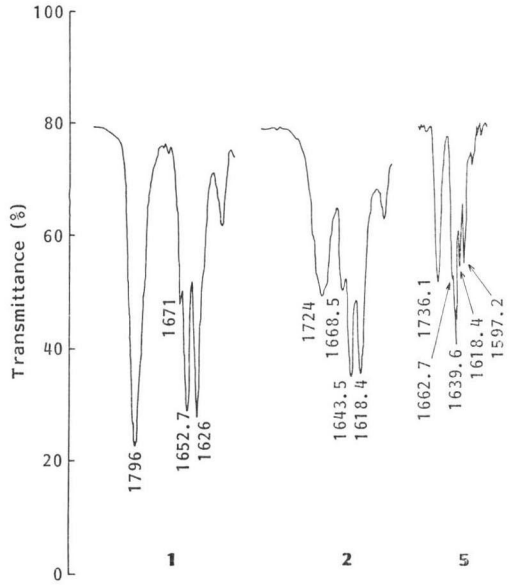
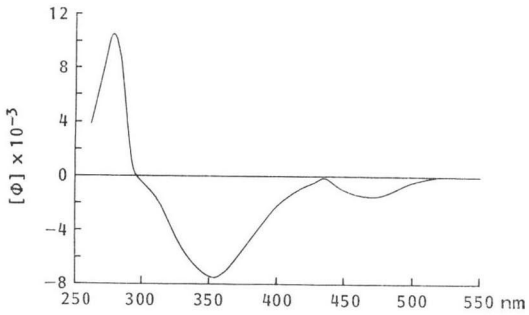
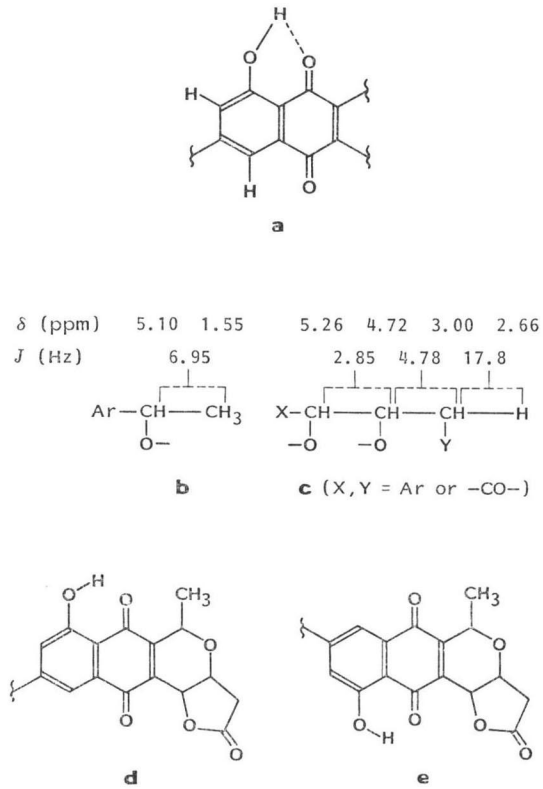



Fig. 4. Optical rotatory dispersion curve of crisamicin A.




of kalafungin vs. 1796 $cm^{-1}$ for crisamicin A. Although other related compounds show higher frequency IR carbonyl absorptions (nanaomycin D, 1775 $cm^{-1}$; $\gamma$-naphthocyclinone[12] (**8**), 1792 $cm^{-1}$; griseusin A[13] (**9**), 1788 $cm^{-1}$), we felt it necessary to confirm the $\gamma$-lactone assignment chemically (Scheme 2).

## Derivatization

In base, crisamicin A was transformed rapidly to **2**, an acid showing an IR band at 1724 $cm^{-1}$ rather than the lactone absorption at 1796 $cm^{-1}$. When **2** stood at room temperature for 15 minutes with 2 N methanolic hydrochloric acid, the lactone **1** re-formed, as confirmed by thin-layer chromatography (TLC) and IR spectroscopy. When **2** was treated with 2 N methanolic hydrochloric acid at reflux for 30 minutes, **3** and **4** were formed, while reaction of **2** with diazomethane yielded compound **5** (Scheme 1). $^1$H NMR data for **1**~**5** and for kalafungin (**6**) are given in Table 1. The chemical stability of crisamicin A (difficult to hydrolyze with acetic acid but easy with 1 N sodium hydroxide, reversion of **2** to the lactone) would be difficult to explain for a $\beta$-lactone.

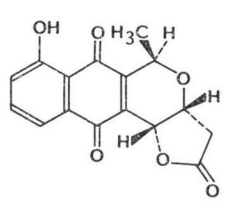
Compounds **3** and **4**, obtained by saponification of crisamicin A followed by methanolysis, both showed olefinic one-proton singlets in their $^1$H NMR spectra at 6.07 ppm. If crisamicin A contained a $\beta$-lactone ring, the product of saponification-methanolysis would have been compound **3A** and the

Table 1. $^1$H NMR data for crisamicin A and related compounds.

| Proton | 1 | 2 | 3 | 4 | | 5 | | 6[b] |
|---|---|---|---|---|---|---|---|---|
| | | | | Right moiety | Left moiety | Right moiety | Left moiety | |
| | $\delta$, m ($J$)[a] | | | | | | | |
| H-1 | 5.10, q (6.95) | 4.94, q (6.74) | 5.75, q (6.60) | 5.75, q (6.55) | 5.06, q (6.85) | 5.03, q (7.28) or | 5.05, q (7.08) | 5.05, q (7.0) |
| H-3 | 4.72, dd (4.78, 2.85) | 4.21, m | | | 4.30, t (6.09) | 4.37, m | 4.37, m | 4.69, dd (4.5, 3.0) |
| H-4 | 5.26, d (2.85) | 4.56, m | 6.07, s | 6.07, s | 4.33, s | 4.74, dd (6.79, 1.34) | 4.68, bs | 5.20, d (3.0) |
| 6-OH | 11.88, s | 12.01, s | 11.96, s | 11.97, s | 12.05, s | 11.94, s | | 7.4~8.0 (H-6, H-7, H-8) |
| H-7 | 7.63, d (1.57) | 7.77, d (1.22) | 7.50, d (1.31) | 7.50, d (1.49) | 7.56, d (1.48) | 7.49, d (0.95) or | 7.56, d (1.25) | |
| H-9 | 7.97, d (1.57) | 7.95, d (1.22) | 7.88, d (1.31) | 7.88, d (1.49) | 7.90, d (1.48) | 8.00, d (0.95) or | 7.89, d (1.25) | 11.80, s (9-OH) |
| $H_3$-11 | 1.55, d (6.95) | 1.47, d (6.74) | 1.45, d (6.60) | 1.46, d (6.55) | 1.53, d (6.85) | 1.57, d (7.28) or | 1.55, d (7.08) | 1.52, d (7.0) |
| H-12a | 2.66, d (17.77) | 2.66, dd (16.66, 5.02) | 3.35, s | 3.35, s | 2.86, d (6.09) | 2.86, d (6.26) | 2.86, d (6.26) | 2.57, d (18.0) |
| H-12b | 3.00, dd (17.77, 4.78) | 2.75, dd (16.66, 7.76) | | | | | | 3.02, dd (18.0, 4.5) |
| COOH | | 10.33, br | | | | | | |
| $COOCH_3$, 3H | | | 3.77, s | 3.77, s | 3.77, s | 3.76, s | or 3.77, s | |
| 4-$OCH_3$, 1H | | | | | 3.61, s | | | |
| 4-OH, 1H | | 2.55, d (6.53) | | | | 2.47, d (6.79) | 2.70, d (4.50) | |
| 6-$OCH_3$, 3H | | | | | | | 4.12, s | |

[a] In $CD_2Cl_2$; $\delta$ in ppm downfield from TMS as internal standard; multiplicity, where s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet; $J$ in Hz.
[b] Ref 10.

Scheme 2.



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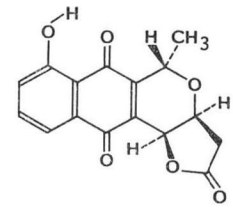

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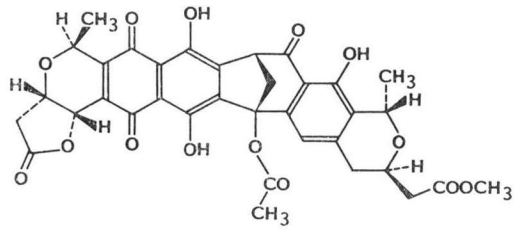

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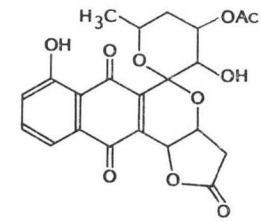

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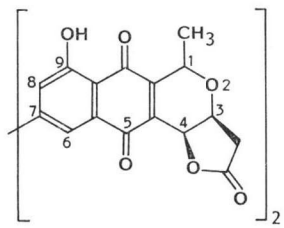
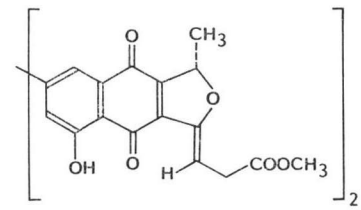
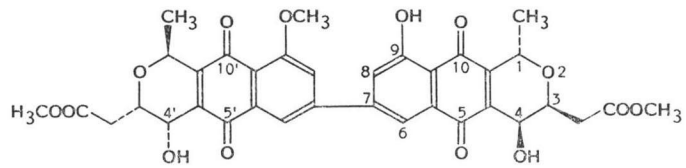


1A



3A



5A

$^1$H NMR signals for the olefinic proton would have been split, presumably to a triplet, by the vicinal protons of the methylene group.

The one remaining gross structural feature of crisamicin A is the location of the phenolic hydroxyl group. An intramolecular hydrogen bond with a quinone carbonyl group could form either with a C-6 hydroxyl as in structures **1** and **5** or with a C-9 hydroxyl as in structures **1A** and **5A**. The phenolic hydroxyl groups in kalafungin (**6**) and nanaomycin D (**7**) were located at C-9 by X-ray analysis[14] and biosynthetically incorporated $^{18}$C[15], respectively. The $^1$H NMR and IR spectra of the product of the reaction of **2** with diazomethane place the phenolic hydroxyl group at C-6, as shown in structure **5**, rather than at C-9 as in **5A**, according to the following reasoning. The chemical shifts of the C-4 and C-4′ hydroxyl protons, appearing at 2.47 and 2.70 ppm, respectively, suggest different chemical surroundings for those two hydroxyl groups. This is consistent with structure **5** but not with **5A**,

where the two hydroxyl groups appear in essentially identical surroundings.

Structure **5** is also in agreement with the IR spectrum (Fig. 3). The intensities of the two quinone carbonyl peaks are almost equal (due to two different types of carbonyl groups — non-hydrogen-bonded near 1653 $cm^{-1}$ and hydrogen-bonded near 1626 $cm^{-1}$) in the spectra of both the lactone **1** and the acid **2**. The IR spectrum of **5**, however, has three peaks due to quinone carbonyl groups, at 1640, 1618, and 1597 $cm^{-1}$, the first peak being almost twice as intense as the other two. These three peaks can be assigned to three types of quinone carbonyl groups in compound **5**. The C-10 and C-10′ quinone carbonyl groups are free from intramolecular hydrogen bonding and should be superimposed to give the double-intensity, highest frequency peak of the three. On the other hand, the C-5 carbonyl group is hydrogen-bonded to both the 4- and 6-hydroxyls and the C-5′ carbonyl group is hydrogen-bonded only to the 4′-hydroxyl. The absorbances of the C-5 and C-5′ carbonyl groups should be of approximately equal intensities, but that of the C-5 carbonyl group should be at lower frequency because of the two hydrogen bonds with the 4- and 6-hydroxyls. Thus, the IR spectrum agrees with the predictions based on structure **5**.

The alternative structure **5A** also contains four quinone carbonyl groups which can be divided into three types, but its absorption pattern would differ from that described above. For **5A**, the absorptions of the C-5 and C-5′ carbonyl groups would overlap and be most intense, but would appear at a frequency lower than that of the 10′-carbonyl group, which is not hydrogen-bonded. The absorption of the hydrogen-bonded 10-carbonyl group might be distinct (at lower frequency than the 10′-carbonyl) or it might overlap those of C-5 and C-5′. In view of these IR data, structures **5A** and **1A** can be eliminated.

The aromatic ring pattern is unusual in that the hydroxyl group is in a different position from that in kalafungin or nanaomycin D. It is also unusual in that the coupling is at a position *meta* to the hydroxyl rather than at the usual *ortho* or *para* position found for phenolic coupling. (The latter would also be the case for pattern **1A**). A reasonable explanation would be that coupling took place at C-8 while a hydroxyl was still present at C-9 and that the C-9 hydroxyl was removed (and the C-6 hydroxyl introduced) after the coupling. No conclusion can be reached regarding the order of these events (C-9 hydroxyl removal, C-6 hydroxyl introduction) but actinorhodine, which is oxygenated at both C-6 and C-9 (present numbering), is coupled to a dimer at the same positions as crisamicin (corresponding to the present C-8)[10].

The absolute configurations of the quinone antibiotics nanaomycin D (**7**)[11] and kalafungin (**6**)[10] have been studied by ORD[11]. Nanaomycin D showed a negative Cotton effect and its enantiomer, kalafungin, showed a positive Cotton effect. Since the ORD curve of crisamicin A shows a negative Cotton effect with a trough, $[\phi]$ −7,841 at 353 nm, and a peak, $[\phi]$ +10,977 at 280 nm ($c$ $5.72\times10^{-3}$, $CH_3CN$), as shown in Fig. 4, it can be concluded that the absolute configuration of crisamicin A is 1*S*, 3*S*, 4*S*, the same as nanaomycin D, as shown in **1**.

## Experimental

### General

UV spectra were recorded with a Perkin-Elmer spectrophotometer, Model Lambda 3. IR spectra were determined (in KBr) with a Nicolet 5-MX FT-IR spectrometer. NMR spectra were obtained with a Nicolet NT-360 spectrometer; chemical shifts are expressed in ppm from TMS as an internal standard. Mass spectrometry was carried out using a Finnigan MAT 731 spectrometer and ORD

curves were recorded with a Jasco spectropolarimeter, Model J-40A. Liquid chromatography was carried out with a Waters Associates pump, Model M-6000A, an Altex Ultrasphere-ODS column (particle mesh size 5 $\mu$m, 250 mm $\times$ 10 mm i.d.), a Rheodyne 7125 injector, a Waters variable wavelength UV detector (254 nm, sensitivity 0.1 AUFS), and a Linear recorder (chart speed, 40 cm/hour). The flow rate of the mobile phase ($CH_3CN$ - $H_2O$, 70: 30) was 3.0 ml/minute.

Crisamicin A (**1**)

Crude material (70 mg), obtained from a fermentation medium of *M. purpureochromogenes* subsp. *halotolerans* (provided by Dr. C. P. SCHAFFNER, Rutgers University), was extracted repeatedly with $CH_3CN$ (20 ml portions) and each supernatant layer was analyzed by HPLC. The retention time of crisamicin A was 8 minutes and 10 seconds. When the area of the crisamicin A peak exceeded 95% of all peaks in the HPLC trace, the extracts were collected, combined, and evaporated to dryness on a Büchi Rotavapor R110 to give a yellow powder which was washed twice with a little $CH_3CN$. The residue was dissolved in dioxane to make a saturated solution, then concentrated with a stream of nitrogen until crystals precipitated and a little mother liquor remained. The crystals were removed by filtration, washed with dioxane, and dried *in vacuo* to give **1** (45 mg): UV $\lambda_{max}^{CH_3CN}$ nm ($\varepsilon$) 215 (28,600), 231 (37,500), 267 (32,800), 435 (11,700); IR, see Fig. 3; NMR ($CD_2Cl_2$), see Figs. 1 ($^1H$) and 2 ($^{13}C$); ORD, see Fig. 4; MS (FD) 600 (M+2H), 598 (M), 556 (600$-CO_2$), 554 (M$-CO_2$), 538 (556$-H_2O$), 536 (554$-H_2O$), 510 (554$-CO_2$). MW Calcd for $C_{32}H_{22}O_{12}$: 598.1111. Found: 598.1124 (HRFD-MS).

Acid **2**

Purified crisamicin A (70 mg) was dissolved with stirring in 1 N sodium hydroxide (10 ml) to give a violet solution free of yellow particles. Dropwise addition of 1 N hydrochloric acid caused the yellow color to reappear and was continued to pH 2.5. $CHCl_3$ (20 ml) was added with vigorous shaking and the turbid liquid was centrifuged for 5 minutes to give compound **2** at the interface between $CHCl_3$ and $H_2O$. The water layer was decanted and the precipitate was washed with $H_2O$ (10 ml). The residue was dried *in vacuo* and purified by HPLC as described in the General section except for the solvent system ($CH_3CN$ - $H_2O$, 55: 45; pH 2.5, adjusted with formic acid) and wavelength (270 nm) to give an orange powder (**2**): UV $\lambda_{max}^{CH_3CN}$ nm ($\varepsilon$) 229 (26,400), 265 (22,200), 430 (7,900); IR (KBr) $\nu$ 3500~2900 (br), 1724, 1644, 1618 $cm^{-1}$; retention time, 4 minutes and 55 seconds; $^1H$ NMR, see Table 1. MW Calcd for $C_{32}H_{26}O_{14}$: 634. Found: 598 (M$-2H_2O$, FD-MS).

Methyl Esters **3** and **4**

A solution of compound **2** (30 mg) in 2 N methanolic hydrochloric acid (50 ml) refluxed for 30 minutes while the solution gradually changed from yellow to reddish-orange. After cooling, the solution was evaporated *in vacuo* to dryness to yield 33 mg of residue. Part of this residue (15 mg) was chromatographed on a Macherey-Nagel preparative pre-coated Sil G-200 $UV_{254}$ plate ($CHCl_3$ - $CH_3OH$, 10: 1) to give a reddish-orange powder (10 mg, Rf 0.7). The powder was separated by HPLC as described above except for the solvent system ($CHCl_3$ - $CH_3OH$ - $H_2O$, 20: 70: 10; pH 2.5, adjusted with 1 N $H_2SO_4$), flow rate (2.5 ml/minute), and wavelength (432 nm). The HPLC trace showed two main fractions — **3**, with retention time 13 minutes and 20 seconds, and **4**, with retention time 10 minutes and 48 seconds, which were collected, combined, and evaporated *in vacuo*, separately, to yield two methyl esters; compound **3** had UV $\lambda_{max}^{CH_3CN}$ nm ($\varepsilon$) 229 (36,500), 274 (25,200), 338 (14,400), 426 (15,800); IR (KBr) $\nu$ 3400, 1745, 1645, 1569 $cm^{-1}$; $^1H$ NMR, see Table 1. MW Calcd for $C_{34}H_{28}O_{12}$: 626. Found: 627 (M+H, FD-MS).

Compound **4** had UV $\lambda_{max}^{CH_3CN}$ nm ($\varepsilon$) 229 (38,400), 268 (28,900), 320 (sh), 427 (15,100); IR (KBr) $\nu$ 3400, 1740, 1638, 1564 $cm^{-1}$; $^1H$ NMR, see Table 1. MW Calcd for $C_{35}H_{30}O_{13}$: 658. Found: 658 (M, FD-MS).

Methyl Ester **5**

A solution of diazomethane was added to a solution of **2** (8.7 mg) in methanol (15 ml), dropwise with stirring in an ice bath, until the color changed. The mixture was evaporated *in vacuo* to dryness to give 11 mg of residue, which was chromatographed on a preparative plate as above using benzene -

EtOAc (2: 8) as developing solvent to yield 3 mg of residue (Rf 0.06). The residue was purified by HPLC as for **2** to give a yellow powder (**5**): UV $\lambda_{max}^{CH_3CN}$ nm ($\varepsilon$) 228 (33,600), 265 (29,600), 420 (10,000); IR (KBr) $\nu$ 3500 (br), 1736, 1640, 1618, 1597 $cm^{-1}$; $^1H$ NMR, see Table 1; retention time 8 minutes and 48 seconds. MW Calcd for $C_{35}H_{32}O_{14}$: 676. Found: 677 (M+H, FD-MS).

Acknowledgments

This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (AI 04769). NMR and mass spectra were obtained with instruments provided in part by grants from the National Science Foundation (CHE 79-16100 to the University of Illinois Regional Instrumentation Facility) and the National Institute of General Medical Sciences (GM 27029), respectively. The authors gratefully acknowledge a gift of kalafungin from The Upjohn Company.

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