CRISAMICIN C, A NEW ISOCHROMANEQUINONE ANTIBIOTIC

ISOLATION, STRUCTURE DETERMINATION, AND BIOSYNTHESIS

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Micromonospora purpureochromogenes subsp. *halotolerans* was found to produce crisamicin C, a novel antibiotic, together with crisamicin A. Crisamicin C was purified by silica gel column chromatography and its physico-chemical properties, structure and biosynthesis were studied. Crisamicin C, mp 260°C (dec), showed UV maxima at 392 (ε 9,497), 261 (ε 32,959) and 232 nm (ε 24,623) in CH_3CN, and gave an IR spectrum with absorbances at 1782 (lactone), 1705 and 1655 (quinone) cm^{-1}. Crisamicin C plasma desorption mass spectrometry (PD-MS) m/z 615.9 ($(M+H)^+$, hydroquinone) was 16 amu higher than crisamicin A PD-MS m/z 600 ($(M+H)^+$, hydroquinone) suggesting that the two antibiotics differ by one additional oxygen in crisamicin C. Analysis of ^1H and ^{13}C NMR spectra, in comparison with those of crisamicin A, indicated that crisamicin C was the 4′a, 10′a epoxide derivative of crisamicin A. Carbon-thirteen labeled acetate feeding experiments were used to confirm the positions of the epoxide and other structural features. Crisamicin C was a more potent antibiotic than crisamicin A, but shared the same spectrum of antimicrobial activity (Gram-positive only).

In a previous study it was reported that *Micromonospora purpureochromogenes* subsp. *halotolerans* was found to produce a complex of isochromanequinone antibiotics named the crisamicins[1]. The first major component of the complex, crisamicin A, was found to have antibacterial[2], antitumor, antiviral and immunomodulatory properties[3]. It was found to be a dimeric isochromanequinone[4] structurally similar to actinorhodin[5]. A second major component of this complex, crisamicin C, has now been characterized and is an epoxide derivative of crisamicin A. The original TLC of the crisamicins showed crisamicins A, B and C in order of decreasing Rf value. Crisamicin B has not been characterized due to irreproducibility of its formation during fermentation. The present report describes the isolation, physico-chemical properties, structure assignment, and biosynthesis of crisamicin C.

Production and Isolation

The crisamicin fermentation was carried out by the procedure previously reported[2] with the exception that the production medium (glucose 10%, potato dextrin 2%, NZ-Amine type A 1%, Ardamine Z 0.5% and $CaCO_3$ 0.1%) was modified to contain glucose 6% and soluble starch 2% in place of potato dextrin. The crude broth pH 7.0 was extracted with EtOAc until little more color could be removed from the aqueous phase. The EtOAc layer was concentrated *in vacuo* and the crude

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material precipitated by addition of hexanes to the concentrated solution. The crude, dark brown powder was washed repeatedly with MeOH (25 ml/100 mg) to remove abundant polar material prior to silica gel column chromatography and the remaining solids were then solubilized in $CHCl_3$ and loaded onto a silica gel column. Crisamicin A eluted first using a $CHCl_3$ - MeOH (99 : 1) mobile phase. Crisamicin C was then eluted using a mobile phase composed of $CHCl_3$ (98.975%), MeOH (1.000%) and HOAc (0.025%). The crisamicin C fraction was then concentrated *in vacuo* to a volume of 50 ml and the addition of hexanes precipitated the final product.

Physico-chemical Characteristics

Crisamicin C was obtained as an orange powder which decomposed at 260°C. It was soluble in DMSO and DMF, moderately soluble in $CHCl_3$, EtOAc, Me_2CO and CH_3CN, and insoluble in MeOH, EtOH, hexanes and water. In solution crisamicin C was yellow but became purple and water soluble at alkaline pH. During TLC crisamicin C gave an Rf of 0.17 compared to 0.31 for crisamicin A. In HPLC crisamicin C had a retention time of 2.75 minutes while crisamicin A had a retention time of 3.19 minutes. The UV-visible absorption spectra (Fig. 1), showed maxima at 392 (ε 9,497), 261 (ε 32,959) and 232 nm (ε 24,623) in CH_3CN; 390 (ε 9,711), 260 (ε 33,991) and 231 nm (ε 25,096) in 0.1 N HCl - CH_3CN; 527 (ε 6,918), 454 (ε 7,864) and 259 nm (ε 32,874) in 0.1 N NaOH - CH_3CN. The IR spectrum indicated the presence of three types of carbonyl groups, γ-lactone (1782), quinone (1705) and hydrogen

Fig. 1. UV-visible absorption spectra of crisamicin C.

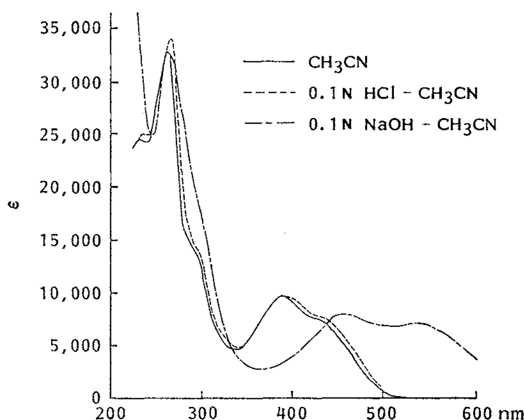
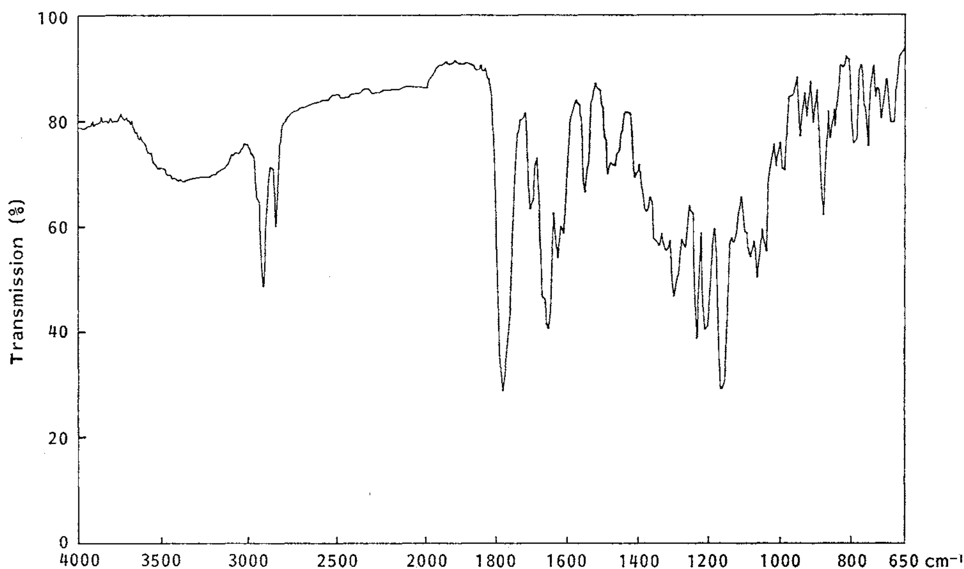


Fig. 2. The IR spectrum of crisamicin C.



bonded quinone (1655 cm^{-1}) (Fig. 2).

The elemental analysis was as follows:

Anal Calcd for $C_{32}H_{22}O_{13}$: C 62.55, H 3.61, O 33.84.
Found: C 61.72, H 3.65, O 30.42.

Crisamicin C did not yield a molecular ion when using electron impact mass spectrometry (EI-MS), chemical ionization mass spectrometry (CI-MS), fast atom bombardment mass spectrometry (FAB-MS) and field desorption mass spectrometry (FD-MS). When crisamicin C was subjected to Californium-252 plasma desorption mass spectrometry (PD-MS) it gave molecular ions in both the positive (*m/z* 615.9 $(M+H)^+$) and negative (*m/z* 615.5 $(M-H)^-$) ion modes (Fig. 3). Crisamicin A (MW 598.1124) was used as a standard and yielded molecular ions of *m/z* 600.1 $(M+H)^+$ and 599.3 $(M-H)^-$. The mass difference between the two crisamicins was 16 atomic mass units, suggesting that crisamicin C possessed one additional oxygen atom relative to crisamicin A. The crisamicin A molecular ion (positive mode) was two mass units higher than its known MW of 598.1124[4] which suggested that the PD-MS spectra may have reflected a charged hydroquinone moiety. Hence the MW of crisamicin C, assuming it behaved in a similar way in PD-MS relative to crisamicin A, should be 614 in the oxidized, quinone form. A high resolution mass spectrum of crisamicin C was not obtained.

The ^{13}C and ^{1}H NMR data are presented in Table 1 and Fig. 4, respectively.

Structure Assignment

The physico-chemical properties of crisamicin C were quite similar to those of crisamicin A suggesting that the structure of crisamicin C differed only slightly from that of crisamicin A. The mass spectrometry results indicated a molecular formula of $C_{32}H_{22}O_{13}$ for crisamicin C based on the observation that the MW of crisamicin C was 16 atomic mass units higher than that of crisamicin A ($C_{32}H_{22}O_{12}$).

In the IR spectrum of crisamicin C (Fig. 2), there was an absorbance at 1705 cm^{-1} corresponding

Fig. 3. ^{252}Cf Plasma desorption mass spectra for crisamicins C and A.

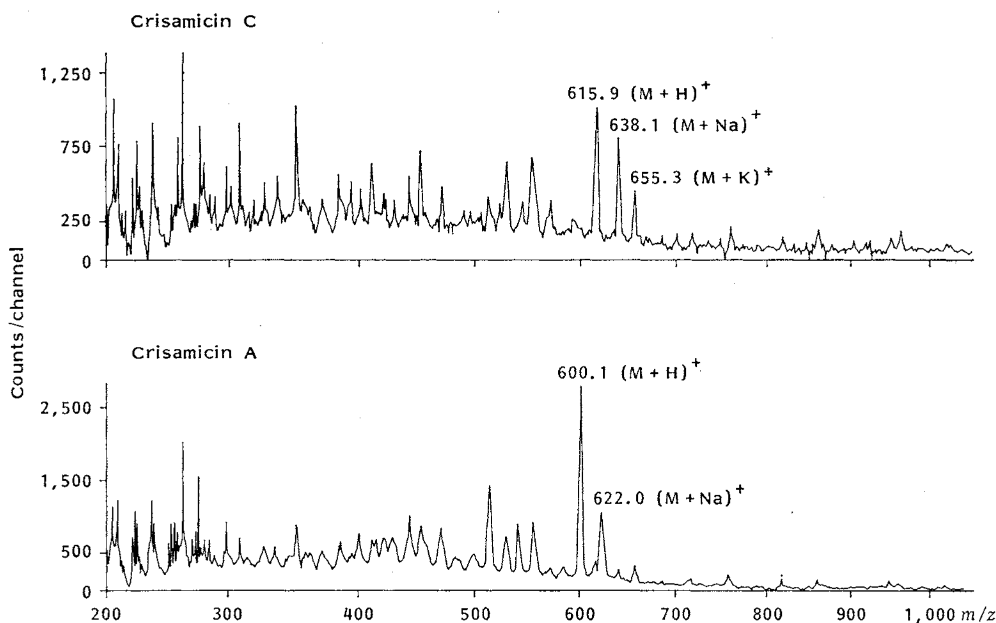


Table 1. ^{13}C NMR data for crisamicin C in comparison with crisamicin A, including data for labeling with [1-^{13}C]- and [2-^{13}C]acetate.

Carbon atom	Crisamicin C	Crisamicin A[a]	Carbon atom	Crisamicin C	Crisamicin A[a]
1	66.08[b], d[c], C-1[d]	66.05, d, C-1	8	144.74[b], s[c], C-2[d]	144.76, s, C-2
1′	64.21, d, C-1		8′	144.66, s, C-2	
3	66.65, d, C-1	66.61, d, C-1	9	117.02, d, C-1	116.88, d, C-1
3′	64.94, d, C-1		9′	117.02, d, C-1	
4	68.58, d, C-2	68.58, d, C-2	9a	132.31, s, C-2	132.26, s, C-2
4′	69.59, d, C-2		9′a	132.44, s, C-2	
4a	134.39, s, C-1	134.35, s, C-1	10	181.57, s, C-1	181.49, s, C-1
4′a	60.76, s, C-1		10′	187.94, s, C-1	
5	186.28, s, C-2	186.19, s, C-2	10a	149.52, s, C-2	149.46, s, C-2
5′	191.03, s, C-2		10′a	63.80, s, C-2	
5a	114.50, s, C-1	114.48, s, C-1	11	14.21, q, C-2	17.95, q, C-2
5′a	114.63, s, C-1		11′	17.97, q, C-2	
6	160.26, s, C-2	160.38, s, C-2	12	36.46, t, C-2	36.49, t, C-2
6′	160.42, s, C-2		12′	35.27, t, C-2	
7	122.13, d, C-1	122.08, d, C-1	13	174.90, s, C-1	174.83, s, C-1
7′	122.44, d, C-1		13′	174.97, s, C-1	

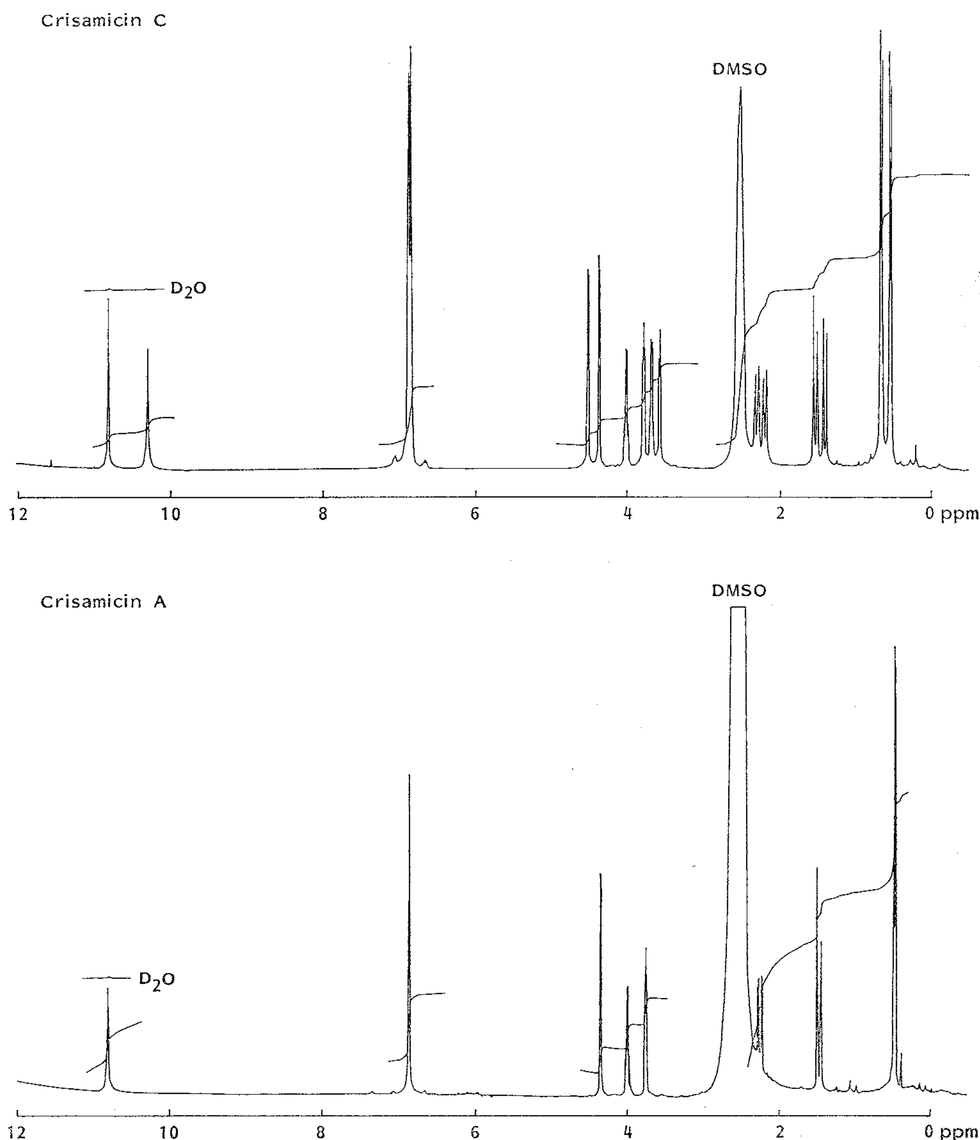
[a] Chemical shifts for crisamicin A carbons labeled n and n′ are identical.
[b] δ_C (ppm) relative to TMS in DMSO-d_6.
[c] Multiplicity.
[d] Acetate precursor; [1-^{13}C]- or [2-^{13}C]acetate.

to a non-chelated quinone carbonyl group. This was shifted from the analogous absorbance of crisamicin A (1650 cm^{-1}), suggesting a similar phenomenon to that observed for lactoquinomycin B where the absorbance was at 1700 cm^{-1} rather than 1665 cm^{-1} for lactoquinomycin A[6]. The difference between the two lactoquinomycins was the presence of an epoxide between carbons C-4a and C-10a (Fig. 5). The chelated quinone carbonyl group of crisamicin C gave an absorbance near 1655 cm^{-1}. However, this absorbance was confused by multiplicity which one would expect if the additional oxygen in crisamicin C introduced asymmetry into the molecule relative to crisamicin A.

The ^{13}C NMR of crisamicin A was that of a symmetrical dimer, hence each shift represented two homologous carbon atoms, one from each monomeric unit, superimposed on each other. If one additional oxygen were introduced into one of the subunits, the molecule would beome asymmetric, and the ^{13}C NMR would show 32 shifts rather than 16 as seen for crisamicin A. Thirty two shifts, half of which were nearly identical to those of crisamicin A, were indeed observed for crisamicin C and they all came as pairs of shifts [(C-1, C-1′), (C-3, C-3′), *etc.*] (Table 1). The exceptions to this observation were the shifts for C-4a and C-10a where single shifts were observed at 134.39 and 149.52 ppm respectively. Two unpaired peaks also appeared at 60.76 and 63.80 ppm. These upfield shifts were nearly identical to those of the epoxide bearing lactoquinomycin B [(C-4a, 60.0 ppm), (C-10a, 64.4 ppm)][6] and nanaomycin E [(C-4a, 79.5 ppm), (C-10a, 82.6 ppm)][7] (Fig. 5). The shift at 117.02 ppm appeared as a broad single peak, but its unusual shape suggested that it was in fact two signals nearly superimposed onto each other (C-9, C-9′).

The splitting of signals into homologous pairs by the introduction of asymmetry was also observed in the ^{1}H NMR of crisamicin C (Fig. 4). Again the shifts came as homologous pairs, half of which were nearly identical to the proton shifts of crisamicin A. There were no missing shifts and no novel shifts. The aromatic protons (6.80 ppm) were observed as two fused peaks, while crisamicin A in

Fig. 4. ^{1}H NMR spectra of crisamicins C and A.



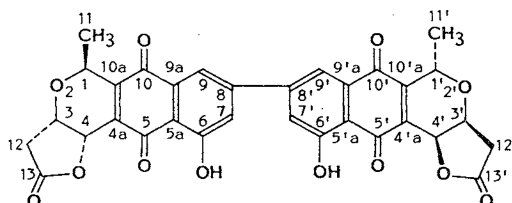
DMSO-d_6 had only one peak (6.89 ppm) for its aromatic protons. Integration of the aromatic proton peaks in addition to the ^{13}C NMR off-resonance decoupling results indicated that there were four aromatic protons. The two phenolic hydroxyl groups were demonstrated by shifts for exchangeable protons at 10.26 and 10.76 ppm. Integration of the ^{1}H NMR spectrum indicated that there were 22 protons on crisamicin C.

Based on the above data, the additional oxygen atom in crisamicin C was placed as an epoxide between carbons 4′a and 10′a. Therefore, the structure of crisamicin C is that depicted in Fig. 6. The stereochemistry of the epoxide ring remains to be determined.

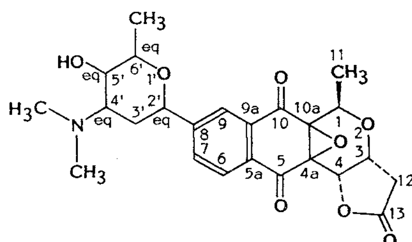
Biosynthesis

The biosynthesis of crisamicin C was investigated by feeding sodium [1-^{13}C]acetate and sodium

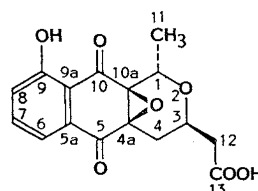
Fig. 5. The structures of crisamicin A, lactoquinomycin B and nanaomycin E.



Crisamicin A



Lactoquinomycin B



Nanaomycin E

Fig. 6. Crisamicin C.

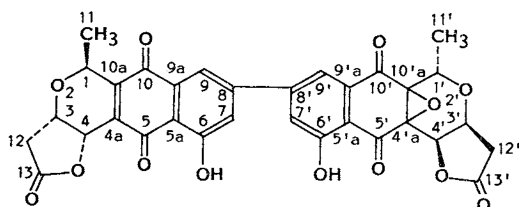
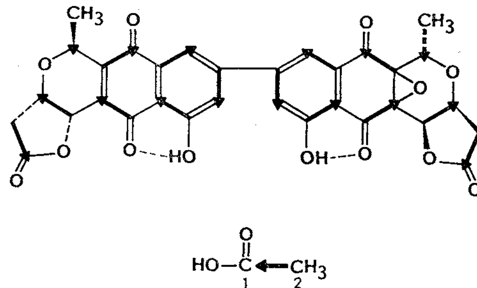


Fig. 7. Derivation of carbons in crisamicin C from acetate.



[2-^{13}C]acetate to separate fermentations and then observing the ^{13}C NMR of the resulting crisamicin C for enriched signals (Table 1). The data indicated that, like crisamicin A[8], crisamicin C was produced entirely from acetate derived carbon atoms *via* the polyketide pathway[9]. More importantly, the labeling data confirmed the position of the phenolic hydroxyl groups at C-6,6′. The shifts for carbons (C-6,6′) bearing hydroxyl groups were observed at 160.26 and 160.42 ppm respectively and were enriched by 2-^{13}C of acetate as expected. The shifts for the quinone carbonyl groups at C-5,5′ were down field (186.28 and 191.03 ppm) relative to the shifts for the quinone carbonyl groups at C-10,10′ (181.57 and 187.94 ppm). This was indicative of hydrogen bonding between the C-5,5′ carbonyl groups and their adjacent phenolic hydroxyl groups and thus comfirmed the position of the hydroxyl groups on C-6,6′. The cross link between subunits in crisamicin A was between C-8 and C-8′ both of which give shifts of 144.76 ppm, were enriched for 2-^{13}C of acetate, and gave singlets when off-resonance decoupled. Crisamicin C had shifts at 144.74 and 144.66 ppm with labeling and decoupling characteristics identical to the homologous carbons of crisamicin A. Thus, the cross link between the two subunits in crisamicin C is between carbons C-8 and C-8′, an identical cross link configuration relative

Table 2. Antimicrobial activity of crisamicin C compared to crisamicin A.

Test organisms	MIC (μg/ml)	
	Crisamicin A	Crisamicin C
Bacillus subtilis ATCC 7972	0.5	0.125
B. cereus ATCC 14579	0.5	0.25
Staphylococcus aureus ATCC 6538P	1	0.25
Streptococcus faecalis ATCC 19433	2	0.25
Micrococcus luteus ATCC 4698	0.125	0.125
Mycobacterium vaccae IMRU 282	>64	>64
M. smegmatis IMRU 607	>64	>64
Escherichia coli ATCC 11303	>64	>64
Pseudomonas aeruginosa ATCC 27853	>64	>64
Serratia marcescens IMRU 70	>64	>64
Candida albicans IMRU 204	>64	>64
Saccharomyces cerevisiae ATCC 9763	>64	>64
Penicillium chrysogenum ATCC 12690	>64	>64
Mucor rouxii IMRU 80	>64	>64

IMRU stands for the culture collection of the Waksman Institute of Microbiology, Rutgers-The State University of New Jersey, U.S.A.

to that of crisamicin A. This data supported the structure proposed for crisamicin C in Fig. 6.

Antimicrobial Activity

Crisamicin C was active only against Gram-positive bacteria and thus shared the same spectrum of antimicrobial activity as crisamicin A (Table 2). Crisamicin C was more potent than crisamicin A which was unusual considering the observation that the epoxides nanaomycin E[7] and lactoquinomycin B[6] were less potent than nanaomycin A and lactoquinomycin A, respectively. It is likely that the dimeric nature of the crisamicins accounts for this difference.

Experimental

General

UV-visible absorption spectra were recorded with a Beckman spectrophotometer, model UV5270. IR spectra were determined in KBr using a Perkin-Elmer Model 283 IR spectrometer. Mass spectrometry was attempted in EI, CI (with *iso*-butane) and FAB (glycerol or DMSO matrix) ionization modes using a VG Analytical Instruments Model ER mass spectrometer. FD-MS was attempted with a Finnigan MAT 731 mass spectrometer. Californium-252 PD-MS was performed on an instrument constructed by R. D. MACFARLANE of Texas A and M University[10,11]. A 15-μCi ^{252}Cf primary ion source was employed in a time of flight method. The samples were dissolved in MeOH - $CHCl_3$ - Me_2CO with TFA and electrosprayed onto an aluminized Mylar to form a 0.3-μm film. The instrument was maintained at 10^{-7} Torr. A Fluke model 410 high voltage power supply was used at 10 keV for accelerating voltage. The polarity of the accelerating voltage was adjusted to record positive and negative ion spectra.

The ^{1}H and ^{13}C NMR spectra were recorded using a Varian 400 NMR spectrometer at 400 and 100 MHz respectively. The samples were prepared in DMSO-d_6 due to insufficient solubility of crisamicin C in most organic solvents. TFA (0.5 μl/ml) was mixed with the samples to prevent the formation of multiple tautomeric forms which had resulted in shift broadening in the initial experiments.

Chromatography

TLC was done on E. Merck Silica gel 60 F_{254} plates (250 μm) using a solvent system of $CHCl_3$ - HOAc (97.5 : 2.5). HPLC analysis was carried out with a Varian Liquid Chromatograph, Model 5000 with Laboratory Data Control Spectromonitor II Model 1202 UV-visible detector. An E.

Merck, Lichrosorb RP-18 (10 μm) reversed phase column was used with a flow rate of 2.0 ml/minute and a solvent system composed of CH_3CN - H_2O (60:40). Column chromatography was performed in a glass column (3.5 cm i.d.) packed with 150 g of E. Merck Silica gel 60 (70~230 mesh).

Carbon-thirteen Labeling

To enrich crisamicin C with carbon-thirteen for improved ^{13}C NMR analysis and to investigate its biosynthesis *via* the polyketide pathway, a fermentation was performed in 2-liter, baffled, shake flasks, using 400 ml of the production medium and the conditions mentioned in the production and isolation section of this paper. After 70 hours of fermentation, sodium [1-^{13}C]acetate or sodium [2-^{13}C]acetate were introduced to their respective flasks (62.5 mg/100 ml of medium). At 180 hours, labeled crisamicin C was isolated *via* the solvent extraction and chromatographic methods mentioned above.

Antimicrobial Activity

Stock solutions of the crisamicins were prepared in DMSO and diluted serially in agar to give a final DMSO concentration of 1.0% in each test well. Final drug concentrations ranged from 64 to 0.031 μg/ml. The bacteria were grown on antibiotic medium 12 (Difco) at 37°C and the fungi were grown on Sabouraud maltose agar (Difco) at 28°C.

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