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Cytosporacin, a Highly Unsaturated Polyketide: Application of the ACCORD-ADEQUATE Experiment to the Structural Determination of Natural Products

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Cytosporacin (**1**), a novel antibacterial polyketide containing naphthopyranone and isochromandione moieties, was isolated from the fermentation broth of the fungus *Cytospora rhizophorae*. A ^{1}H-detected ACCORD-ADEQUATE pulse sequence that distinguished $^{2}J_{CH}$ from $^{3}J_{CH}$ correlations provided critical information for structural determination. NOE studies established the relative configuration and revealed the presence of two rotamers. A biosynthetic ^{13}C-labeling experiment indicated that cytosporacin was derived from acetate origin.

Introduction

The lack of information to distinguish between $^{2}J_{CH}$, $^{3}J_{CH}$, and $^{4}J_{CH}$ connectivities in the usual NMR data set complicates the structural characterization of natural products containing condensed systems. Therefore, a number of experiments, including the INEPT-INADEQUATE,[1] DEPT-INADEQUATE,[2] and 1,1-ADEQUATE,[3] have been designed to differentiate $^{2}J_{CH}$ from $^{3}J_{CH}$ correlations. Recently, an improved version of the refocused 1,1-ADEQUATE experiment has been described that incorporates an accordion-type delay for the evolution of ^{13}C−^{13}C couplings.[4] This experiment, termed the ACCORD-ADEQUATE pulse sequence, has been shown to be superior to the previously reported methods in providing both the number and the quality of $^{2}J_{CH}$ correlations. It does this by allowing optimization for a wide range of ^{13}C−^{13}C scalar coupling constants with little or no decrease in overall sensitivity. In this paper, the utility of the ACCORD-ADEQUATE NMR experiment is demonstrated by its application to the structural characterization of cytosporacin (**1**), a novel antibiotic containing highly condensed aromatic systems.

In the course of our continuing search for promising lead compounds for development as novel antibiotics[5] and anticancer drugs,[6] we isolated a red compound designated cytosporacin from the fermentation broth of *Cytospora rhizophorae* ATCC38475. Cytosporacin possesses an unprecedented carbon skeleton, which consists of two condensed heteronuclear aromatic systems linked by a C−C bond. The structure of cytosporacin was characterized by analysis of an ACCORD-ADEQUATE spectrum, in combination with typical HMBC and HSQC spectral data. A biosynthetic ^{13}C-labeling experiment helped to confirm the structure and established the polyketide origin.

Results and Discussions

Cytosporacin (**1**) was produced by fermentation of strain ATCC38475 in potato dextrose broth at 22 °C for 12 days. Repeated chromatography of the organic extract of the fermentation broth by reversed-phase HPLC on a C18 column afforded pure product (**1**) as a red amorphous powder. Cytosporacin showed modest activity in vitro against Gram-positive bacteria. The minimum inhibitory concentrations (MICs) obtained by the broth dilution method[7] were 32 μg/mL for *Staphylococcus aureus* (two strains, including a methicillin-resistant strain), 16 μg/mL for *Enterococcus faecium*, and 32 μg/mL for *Bacillus subtilis*.

The molecular formula of **1** was determined by high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry to be $C_{30}H_{28}O_{11}$. The UV spectrum displayed an absorption peak at 360 nm and an extended shoulder centered at about 470 nm. The HPLC analysis showed two close peaks with a ratio of 6:5 and identical UV spectra. Upon separation, each of

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TABLE 1. ^{1}H and ^{13}C NMR Spectral Data of Cytosporacin (1) in DMSO-d_6

	^{1}H (400 MHz, mult)		^{13}C (100 MHz)		
no.	rotamer A	rotamer B	rotamer A	rotamer B	HMBC (J = 8 Hz)
1			164.90	164.75	
2			97.20	97.31	
3			162.41	162.13	
4			106.87	106.61	
5			158.56[a]	158.59[a]	
6	6.65 (s)	6.65 (s)	101.58	101.40	C-4, C-5, C-7, C-8, C-16[b]
7			158.46	158.82	
8			107.41	107.57	
9			139.06	139.36	
10	6.81 (s)	6.70 (s)	111.24	110.19	C-1,[b] C-2, C-3,[b] C-4, C-5,[b] C-8, C-9, C-11, C-12
11			136.97	136.60	
12	5.29 (br s)	5.29 (br s)	64.62	64.71	C-2, C-10, C-11, C-13, C-14
13			156.19	155.97	
14	4.85 (2H, m)	4.85 (2H, m)	95.11	94.53	C-1,[b] C-12, C-13
15			162.02	162.05	
16			126.25	126.25	
17			139.47[a]	139.46[a]	
18	5.89 (s)	5.89 (s)	107.28	107.28	C-16, C-17, C-19, C-20, C-21,[b] C-22
19			151.33	151.42	
20			176.41	176.41	
21			144.59	144.59	
22			115.25[a]	115.24[a]	
23	5.65 (m)	5.65 (m)	75.17[a]	75.18[a]	C-15, C-17, C-21, C-22, C-24, C-25
24	1.95 (2H, m)	1.90 (2H, m)	36.18	36.59	
25	1.53 (2H, m)	1.45 (2H, m)	24.81	24.30	
26	1.30 (2H, m)	1.30 (2H, m)	28.67	28.81	
27	1.25		31.28	31.27	
28			28.58	28.56	
29			22.14	22.11	
30	0.82 (3H, t, 6.5 Hz)	0.81 (3H, t, 6.5 Hz)	14.04	14.02	C-28, C-29
OH	10.50 [c]	10.50 [c]			

[a] Assignments for rotamers A and B may be reversed. [b] Weak correlations. [c] Very broad signals, exchanged when D_2O was added.

the components reequilibrated to the original mixture in the HPLC solvent at ambient temperature. This phenomenon was attributed to the presence of two interconvertible isoforms of **1**, eventually demonstrated to be rotamers A and B.

An analysis of the ^{1}H and ^{13}C NMR spectral data for **1** in DMSO-d_6 (Table 1), which displayed two sets of signals for the majority of protons and carbons, revealed the presence of a 7-carbon fatty chain from C-24 to C-30. Several phenolic OH protons that showed no correlations to any carbon signals in the HMBC and HSQC spectra were observed as a broad hump centered at δ 10.50 in the ^{1}H NMR spectrum. The H-23 proton at δ 5.65, coupled to H_2-24 at ca. δ 1.95 and 1.90 in the COSY spectrum was correlated to C-24 and C-25 of the fatty chain, and C-15, -17, -21, and -22 of a polycyclic alkene system in the HMBC spectrum. The HMBC data also displayed strong correlations from the H-18 signal at δ 5.89 to C-16, -17, -19, -20, and -22, and a weak correlation to C-21. However, these data were not sufficient to identify an exclusive structure for the C-15 to C-30 moiety.

The ACCORD-ADEQUATE NMR data for **1** were acquired on a 30 mM sample dissolved in 600 μL of DMSO-d_6 on a 500-MHz Bruker instrument equipped with an inverse-detected 5-mm Bruker Cryoprobe. The acquisition was carried out with use of 256 scans per increment for a total of 180 (90 × 2) hypercomplex points in the F_1 dimension. These data were processed with an exponential weighting function (LB = 10 Hz) in both F_1 and F_2.

Several important pieces of information could be obtained from this ACCORD-ADEQUATE spectrum. As

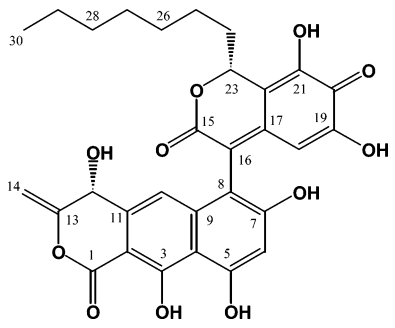

FIGURE 1. Structure of cytosporacin (**1**).

shown in Figure 2, $^2J_{CH}$ correlations from H-18 at δ 5.89 to C-17 at ~139.5 ppm and C-19 at ~151.4 ppm were observed, forcing C-17 and C-19 to flank C-18. Moreover, H-23 at δ 5.65 was found to correlate to C-22 at ~115.2, which required that C-22 be connected to C-23. The HMBC correlations ($^nJ_{CH}$) and ACCORD-ADEQUATE correlations ($^2J_{CH}$) fit well to an isochroman-6,8-dihydroxy-3,7-dione moiety (notice the different numbering from **1**). The ^{13}C NMR chemical shift data for C-19, -20, and -21 were consistent with those observed for a similar moiety.[8]

Among the four unassigned ^{1}H NMR signals, the broad H_2-14 at δ 4.85 showed strong cross-peaks to C-12 and C-13, and a weak one to C-1 in the HMBC spectrum.

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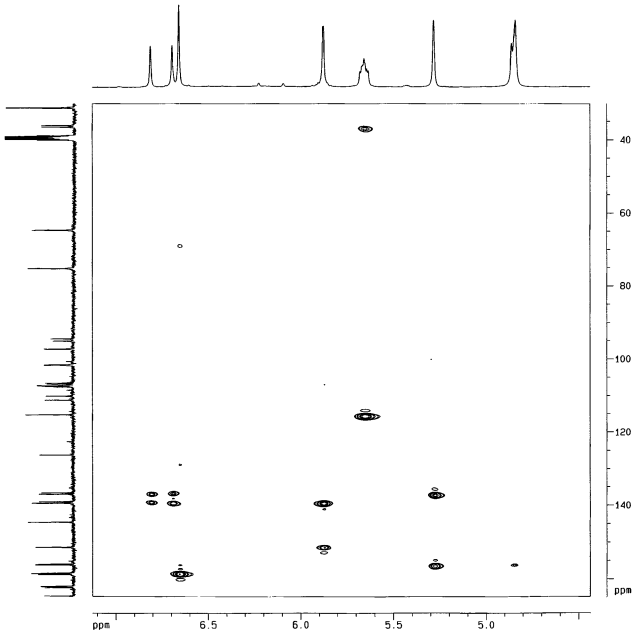

FIGURE 2. Low-field region of an ACCORD-ADEQUATE spectrum of **1**.

Although the chemical shift values for H_2-14 and C-14 could not differentiate between a dioxomethylene or an exocyclic olefin, the $^2J_{CH}$ correlation between H_2-14 and C-13 in ACCORD-ADEQUATE clearly indicated the connection between C-13 and C-14, and thereby the olefinic nature of C-14. The carbinyl proton, H-12 at δ 5.29, showed $^2J_{CH}$ correlations to C-11 and C-13 in ACCORD-ADEQUATE, and $^nJ_{CH}$ correlations to C-2, C-10, C-11, C-13, and C-14 in the HMBC. These correlations, together with chemical shift data, were indicative of a benzopyranone structure. In addition, H-6 at δ 6.65 displayed correlations to C-4, C-5 ($^2J_{CH}$), C-7 ($^2J_{CH}$), and C-8; H-10, with dual signals at δ 6.81 and 6.70, showed strong correlations to C-2, C-4, C-8, C-9 ($^2J_{CH}$), C-11 ($^2J_{CH}$), and C-12, and weak correlations to C-1, C-3, and C-5.

On the basis of these data, the tricyclic naphthopyranone moiety, with an open position at C-8, was defined for the partial structure from C-1 to C-14. Finally, evidence for linkage between the naphthopyranone and isochromandione moieties through C-8 and C-16 was found in a weak 4-bond correlation from H-6 at δ 6.65 to C-16 at 126.25 in the HMBC spectrum.

A ^{13}C-labeling experiment was carried out for biosynthetic studies and structural confirmation. Compound **1**, isolated from fermentation broth supplemented with $^{13}CH_3{}^{13}COONa$ (12 mM at 20 h after inoculation), contained ^{13}C isotope in approximately 1.8% incorporation yield. Carbon signals could be paired by analysis of an INADEQUATE spectrum[9] and the homonuclear $^{13}C-^{13}C$ coupling constants between each carbon pair were measured by a ^{1}H-decoupled ^{13}C NMR spectrum. This led to the identification of two condensed aromatic systems of

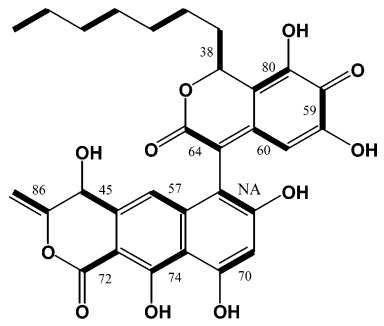

FIGURE 3. Acetate incorporation pattern in cytosporacin (**1**), identified by a ^{13}C-labeling experiment. Selected coupling constants between carbons derived from the same acetate molecules are indicated (in Hz; NA = not available due to signal overlap).

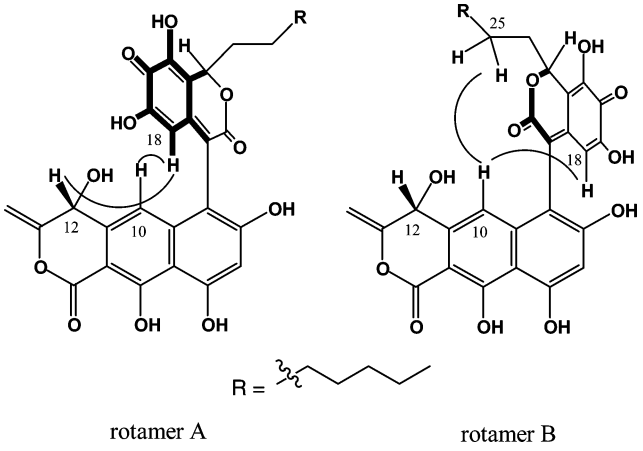

FIGURE 4. The relative stereochemistry of two rotamers of cytosporacin (**1**); NOEs are indicated by arcs.

acetate origin coupled to each other through a C−C bond. The naphthopyranone moiety (bottom) from C-1 to C-14 was found to derive biosynthetically from 7 acetate molecules, whereas the isochromandione moiety (top) from C-15 to C-30 was found to derive from 8 acetate molecules. The pairing of carbon signals provided further support for structure **1**. The incorporation pattern of doubly labeled acetate, together with one-bond $^{13}C-^{13}C$ coupling constants for carbons in the cyclic systems, is shown in Figure 3.

The relative stereochemistry of the two chiral centers, C-12 and C-23, was determined based on the observation that the molecule adopted a conformation with the naphthopyranone and isochromandione moieties oriented perpendicular to each other. The partially restricted rotation along the C-8−C-16 bond owing to steric hindrance allowed the presence of two interconvertible rotamers A and B.[10] These two rotamers were indicated by two discrete peaks in the HPLC chromatogram. In addition, these two peaks interconverted to each other upon separation, and provided two sets of ^{1}H and ^{13}C

(9) The INADEQUATE data for ^{13}C-labeled cytosporacin were acquired on a 30 mM sample in 500 μL of DMSO-d_6 on a 400-MHz Bruker instrument equipped with a 3-mm broadband probe. The acquisition was carried out with 1000 scans per increment for a total of 100 increments.

(10) The presence of discrete rotamers has been observed in natural products when cyclic systems hinder the rotation of the C−C bond that links them. In some instances the rotation is completely blocked at ambient temperature and the rotamers can be isolated as separate compounds, such as michellamines A and B. Manfredi K. P.; Blunt J. W.; Cardellina J. H.; McMahon, J. B., II; Pannell L. L.; Cragg G. M.; Boyd M. R. *J. Med. Chem.* **1991**, *34* (12), 3402−5.

NMR spectral data. As indicated in Figure 4, NOE cross-peaks displayed between H-10 at δ 6.70 and H_2-25 at δ 1.45 for rotamer B in a ROESY spectrum (DMSO-d_6), whereas such cross-peaks were not observed for rotamer A. In a mixed solvent of 1:2 $CDCl_3/CD_3OD$, where the H-12 and H-18 could be differentiated respectively for two rotamers, a weak NOE was observed for rotamer A between H-18 at δ 6.014 and H-12 at δ 5.251 in the 1-D NOE spectrum, but the corresponding NOE for rotamer B was not observed between H-18 at δ 6.008 and H-12 at δ 5.233. These spatial relationships could only be established when configurations of C-12 and C-23 were both *R′*, and the fatty chain was facing outside in rotamer A, as opposed to inside in B.

In summary, cytosporacin (**1**), a novel antibiotic that contains condensed aromatic moieties, was isolated from the fermentation broth of the fungus *Cytospora rhizophorae*. Some important structural information was obtained from a ^{1}H-detected ACCORD-ADEQUATE pulse sequence that emphasized $^2J_{CH}$ correlations. The relative configuration of cytosporacin (**1**) and the presence of two rotamers were revealed by NOE studies. Biosynthetically, cytosporacin was indicated to derive from acetate origin by a ^{13}C-labeling experiment.

Experimental Section

Organism. This fungus was obtained from the American Type Culture Collection (ATCC) in Manassas, Virginia as ATCC38475 *Cytospora rhizophorae*. ATCC38475 was isolated from the roots of two species of the mangrove tree *Rhizophora mangle* and *R. racemosae* growing in Florida, Bahamas, Liberia, Hawaii, Mexico, and Guatemala. ATCC38475 grew well on potato dextrose agar (Difco). After 7 days growth at 22 °C colonies were felty to lightly floccose to fuzzy and some mycelia were submerged. The margin was lobate to irregular. The surface color was buff to cream and the reverse was yellowish to tan to white. After longer incubation pycnidia formation occurs. They were less than 1 mm in diameter and oozed a yellow spore mass.

Inoculum Preparation and Fermentation. ATCC38475 was plated onto Bennett's agar medium (10 g/L of Sigma D-glucose, 1 g/L of Difco beef extract, 1 g/L of Difco yeast extract, 2 g/L of N-Z amine A, 20 g/L of Difco agar) from a Difco potato dextrose agar slant and incubated at 22 °C. Mycelia were scraped from the agar plate and inoculated into five 25 × 150 mm glass tubes each containing 11 mL of potato dextrose broth. This liquid seed culture was shaken at 160 rpm at 22 °C for 4 days and then used to inoculate the production medium of potato dextrose (1 L) in a 2.8-L Fernbach flask. The inoculated medium was incubated at 200 rpm and 22 °C for 12 days.

Isolation and Purification. The whole broth (1 L) was centrifuged at 3800 rpm for 30 min. The supernatant and the cell mass were respectively extracted by *n*-butanol (2 × 0.5 L) and methanol (2 × 0.5 L). The combined extract, upon evaporation, was separated by reversed-phase HPLC on a C18 column (YMC ODS-A, 5 μm particle size, 70 × 500 mm in size), using a gradient solvent from 40 to 100% acetonitrile in water (35 min) and acetonitrile (10 min), with both solvents containing 0.01% in volume of trifluoroacetic acid. The UV peak with a retention time of 36 min, monitored at 360 nm, was collected. Upon evaporation, the red powder was re-purified by HPLC (same condition) to afford the cytosporacin (32 mg). High-resolution FTICRMS (negative): *m*/*z* 563.15374 [$(M - H)^-$, $C_{30}H_{27}O_{11}$ requires 563.15319]; UV (1:1 MeCN/H_2O) λ_{max}(log ϵ) 225 (4.29), 266 (4.48), 360 (4.41), 470 (sh) nm; for ^{1}H and ^{13}C NMR data see Table 1.

Biosynthetic ^{13}C Labeling. For the first stage seed, an agar grown culture of ATCC38475 was transferred into a 25 × 150 mm tube containing 10 mL of Difco potato-dextrose broth. After 4 days of incubation at 22 °C and shaking at 160 rpm, the first stage seed was inoculated into production fermentation: 5% v/v inoculum into 50 mL of potato-dextrose broth in a 250-ml Erlenmeyer flask, at 22 °C, with shaking at 200 rpm. $^{13}CH_3{}^{13}COONa$ was fed after 20 h of incubation at a final concentration of 12 mM. The fermentation was harvested on the seventh day and the ^{13}C-labeled cytosporacin was purified by an analogous method.

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Supporting Information Available: Spectroscopic data for **1**, including UV spectrum, circular dichroism spectrum, 1-D ^{1}H and ^{13}C NMR spectra, and 2-D HMBC, HSQC, and ACCORD-ADEQUATE spectra, together with an INADEQUATE spectrum of the ^{13}C-labeled **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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