BIOSYNTHESIS OF THE ANTIBIOTIC GRANATICIN: ASSIGNMENT OF THE CARBON-13 MAGNETIC RESONANCE SPECTRUM

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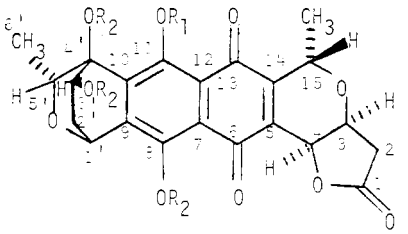
ABSTRACT.—As part of a biosynthetic study using ^{13}C-labeled precursors, the ^{13}C-nmr spectrum of dihydrogranaticin methyl ester, prepared from the antibiotic granaticin, has been analyzed. The resonance signals were assigned based on comparison with model compounds and derivatives, proton single-frequency decoupling and ^{13}C-^{1}H long-range coupling analysis.

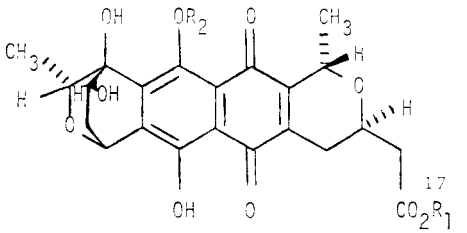
The antibiotic granaticin (**1a**) has been isolated from a number of actinomycetes (1–3). It often co-occurs with the glycoside granaticin B (**1b**) and dihydrogranaticin (**2a**). The biosynthesis of granaticin in *Streptomyces violaceoruber* has been studied by use of radioactive and ^{13}C-enriched precursors (4). The locations of enriched carbons in the product were determined by carbon-13 nmr spectroscopy following the complete assignments of the carbon-13 resonance signals. This paper reports the assignments of the cmr spectra of dihydrogranaticin methyl ester (**2b**), 3'-oxodihydrogranaticin methyl ester (**3**).



1a, $R_1=R_2=H$

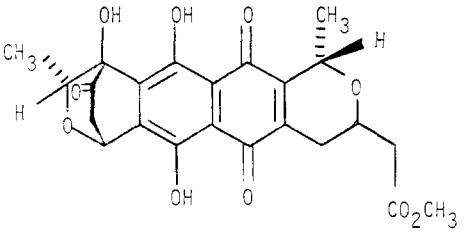
1b, $R_1=CH_3$, $R_2=H$

1c, $R_1=R_2=OAc$



2a, $R_1=R_2=H$

2b, $R_1=CH_3$, $R_2=H$



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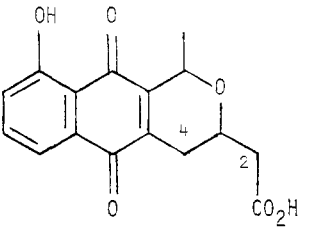
RESULTS AND DISCUSSIONS

Attempts to obtain the ^{13}C natural abundance spectrum of granaticin were not entirely successful due to its strong intermolecular aggregation and instability in solution. Thus, for ^{13}C analysis, **1a** was chemically transformed into dihydrogranaticin methyl ester (**2b**) by catalytic hydrogenolysis. The chemical shifts for the aliphatic carbon signals can be directly assigned based on simple chemical shift theory (5, 6) and on a series of proton single-frequency decoupling experiments [δ_H (irradiation): 0.99, 1.59, 3.79, 4.36, 5.21, 12.60 and 13.11 ppm]. In order to substantiate these resonance assignments, 3'-oxodihydrogranaticin methyl ester was prepared. The $C_{2'}$ and $C_{4'}$ signals undergo predictable downfield shifts (5, 7, 8, 9).



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Further comparison with the ^{13}C spectral data published for nanaomycin A (10) (**4**) reveals that the C_2 and C_4 resonance designations for nanaomycin A should be interchanged.

In the naphtharazin moiety, the quinone carbon resonances (C_6 and C_{13}) appear furthest downfield, followed by the two hydroquinone carbon signals (C_8 and C_{11}). The most upfield aromatic carbon peaks may be assigned to C_7 and C_{12} because of the adjacent carbonyl groups and the shielding effect of the phenolic hydroxy groups. The 142.1 (doublet of triplet) and 162.9 (triplet) ppm peaks become a triplet and a doublet, respectively, when the $H_{1'}$ (5.20 ppm) is irradiated, identifying them as the resonance signals of C_9 and C_8. Then the signals at 168.5, 144.5, and 136.2 ppm can be assigned to C_{11}, C_{14}, and C_{10}, respectively, by comparison with the chemical shift assignments of nanaomycin A.

In the proton single-frequency decoupled spectrum, the ^{13}C-^{1}H splittings, called reduced coupling constants (Jred), are smaller than the actual coupling (J) (11, 12).

$$Jred \approx \Delta f_H \cdot J / \gamma H_2$$

where γH_2 is the intensity of the decoupling field, $\Delta f_H = f_H^{resonance} - f_H^{irradiation}$. The magnitude of Jred depends on the Δf_H at the constant decoupling power.

We have earlier used the reduced long-range coupling patterns in the chemical shift determinations of sulfonamide drugs (13). A similar approach can also be applied to distinguish C_7 from C_{12} and C_6 from C_{13} in **2b**. In a series of proton single-frequency decoupled spectra, the C_7 and C_{12} signals display unique variations of the splitting patterns. The actual ^{13}C–^1H three-bond coupling constants for C_7–H_8 and C_{12}–H_{11} are almost identical (ca. 5 Hz), but the reduced three-bond coupling constants vary because of the different Δf_H. Therefore the C_7 peak (110.0 ppm) appears as a doublet, whereas the C_{12} peak (110.2 ppm) is a singlet when the irradiation frequency is at 5.06 ppm (figure 1).

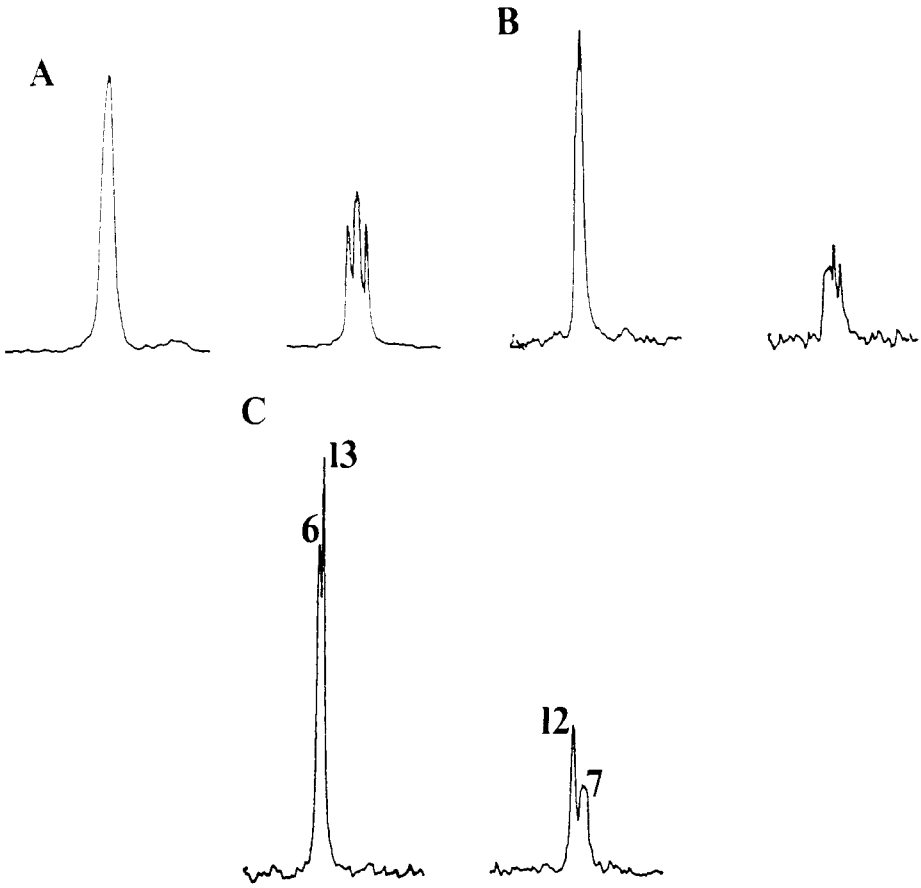
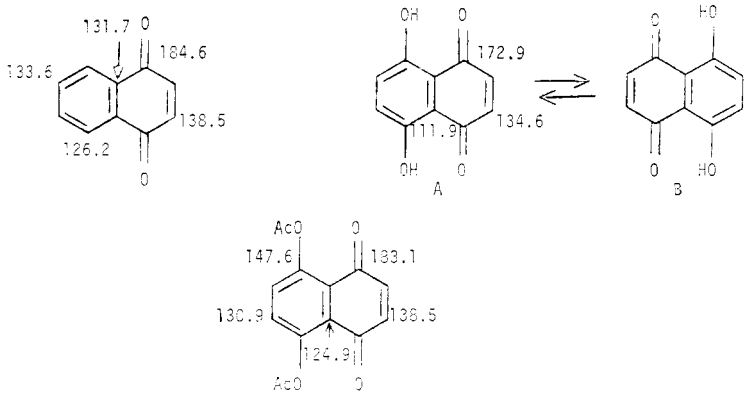


FIG. 1. ^{13}C Spectra of the C_7, C_{12}, C_6 and C_{13} Resonance Signals (A) Proton-Coupled; (B) Single-Frequency Proton-Decoupled at $\delta H = 3.90$; (C) Single-Frequency Proton-Decoupled at $\delta H = 5.06$.

The C_6 and C_{13} signals can theoretically be differentiated by measuring the ^{13}C–^1H long-range coupling patterns since C_6 can couple with two protons (H_4), while C_{13} can couple with only one proton (H_{15}). Unfortunately, none of these splittings can be clearly measured in the proton-coupled spectrum (figure 1). However, the upfield portion of this broad peak becomes a sharper singlet when the H_{15} (5.06 ppm) is irradiated. Thus the C_6 (174.9 ppm) and C_{13} (174.8 ppm) signals can be distinguished.



The chemical shifts of naphthoquinone and its hydroxy derivatives (14) indicate the dynamic equilibrium of the two tautomers (A and B) for 5,8-dihydroxynaphthoquinone. This equilibrium may be perturbed by acetylation of the hydroxy groups. A similar phenomenum has also been observed in the granaticin system (table 1). It is interesting to note the unusual effect on the tautomeric

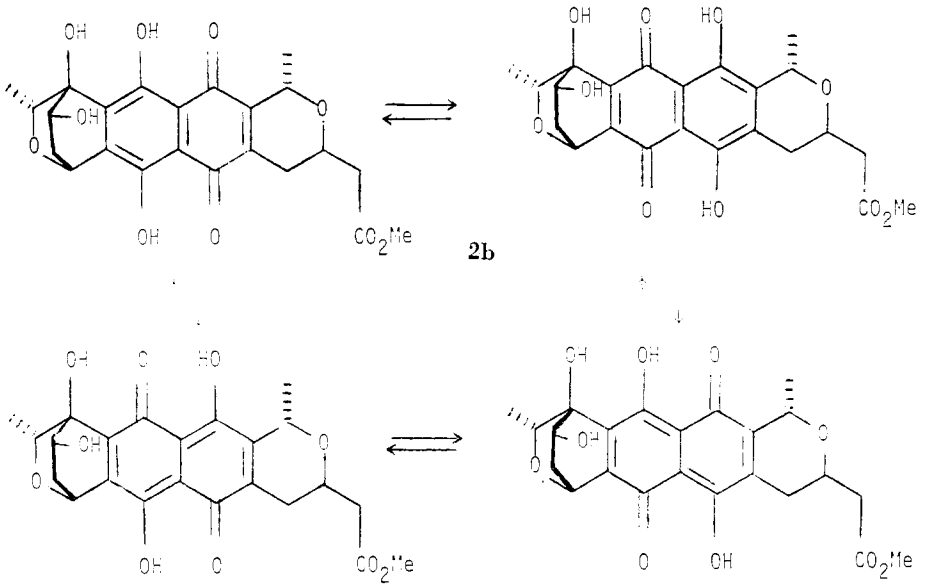
TABLE 1. Carbonyl carbon chemical shifts of granaticin derivatives.

	2a	3	1b
C_{13}.....	174.8	180.0	180.3
C_6.....	174.9	182.9	181.2
C_1.....	170.8	170.7	173.7

TABLE 2. ^{13}C Chemical shifts (δ) and ^{13}C–^1H splittings of methyl esters of 3'-oxodihydrogranaticin (3).

Carbon No.	Compound 2b	Compound 3
1	170.8, m	170.7, dt, 7.0 (H_3), 3.5 (H_2)
2	40.3, bt, 129.0 (H_2)	40.2, bt, 129.3 (H_2)
3	63.0, bd, 144.1 (H_3)	63.1, dt, 150.5 (H_3), 5.0 (H_4)
4	27.5, bt, 130.0 (H_4)	27.3, bt, 130.0
5	140.1, m	141.7, td, 8.0 (H_4, H_{15}), 4.0 (H_3)
6	174.9, bs	182.9, bs
7	110.0, d, 6.0 (H_8)	110.0, d, 2.4 (H_8)
8	162.9, t, 3.0 (H_8, $H_{1'}$)	154.0, dd, 4.3 (H_8), 2.4 ($H_{1'}$)
9	142.1, dt, 6.2 (H_{2R}), 3.1 (H_8, $H_{1'}$)	139.8, dt, 9.0 (H_{2R}), 4.0 (H_8, $H_{1'}$)
10	136.2, m	131.5, bs
11	168.5, m	159.0, d, 4.3 (H_{11})
12	110.2, d, 5.0 (H_{11})	111.5, d, 2.5 (H_{11})
13	174.8, bs	180.0, bs
14	144.5, m	146.9, m
15	67.4, bd, 149.6 (H_{15})	67.3, bd, 148.9 (H_{15})
16	19.0, qd, 128.8 (H_{16}), 4.3 (H_{15})	19.1, bq, 128.8 (H_{16})
17	51.8, q, 147.1 (H_{17})	51.8, q, 147.1 (H_{17})
1'	61.8, bd, 160.2 ($H_{1'}$)	64.1, bd, 162.1 ($H_{1'}$)
2'	35.6, bt, 132.8 ($H_{2'}$)	38.6, bt, 132.4 ($H_{2'}$)
3'	70.7, bd, 151.4 ($H_{3'}$)	190.6, bs
4'	80.4, bs	82.6, bs
5'	72.6, bd, 150.2	72.9, bd, 135.6
6'	16.4, q, 127.0	16.6, q, 127.5

equilibrium of the subtle intramolecular hydrogen-bonding alterations in **3**. This change is manifested not only in the strong downfield shift of the C_6 and C_8 resonances ($\Delta\delta = 8.9$ and 5.2 ppm), but also by the large upfield shift of the C_9 and C_{10} resonances ($\Delta\delta = -2.3$ and -4.7 ppm) (table 2).



After we completed our spectral assignments, we became aware of another, independent investigation of the cmr spectra of this class of compounds, (17) which arrives at assignments identical to ours.

EXPERIMENTAL[1]

ISOLATION.—The isolation of granaticin (**1a**) and dihydrogranaticin (**2a**) from *Streptomyces violaceoruber* fermentations, the incorporation of sodium ^{13}C-acetate into the latter, and chromatographic procedures have been described previously (4).

DIHYDROGRANATICIN METHYL ESTER (**2b**).—Dihydrogranaticin (**2a**) was converted to its methyl ester in quantitative yield by treatment with methanolic HCl as reported by Pyrek and coworkers (**15**). After evaporation of the solvent and removal of residual HCl under a stream of nitrogen gas, the residue was dissolved in a minimum volume of chloroform and precipitated with petroleum ether. It was characterized as follows: m.p. 185–189°; visible absorption, λ (log ϵ): 565 nm (shoulder), 550 (3.25), 518 (3.40), 490 (3.37); ir (KBr): 2.8–2.9 μ (OH), 5.7 (ester), 6.3 (quinone); ^{1}H-nmr ($CDCl_3$): δ 13.20 (s, H_8), 12.76 (s, H_{11}), 6.20 (s, OH), 5.21 (dd, H-1', $J_{1',2'} = 1.5$ and 3.1 Hz), 5.06 (q, H-15, $J_{15,16} = 6.6$ Hz), 4.36 (m, H-3), 4.00 (dd, H-3', $J_{3',2'S} = 2.0$ Hz, $J_{3',2'R} = 8.6$ Hz), 3.79 (q, H-5', $J_{5',6'} = 6.2$ Hz), 3.75 (s, ester CH_3) 3.2–2.1 (multiplets for CH_2-2', 2, 4), 1.59 (d, CH_3-16, $J_{15,16} = 6.6$ Hz), 0.99 (d, CH_3-6', $J_{5',6'} = 6.2$ Hz); ms: *m/e* 461·(M+1)$^+$ for $C_{23}H_{24}O_{10}$, MW = 460.14.

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[1]^{13}C-nmr spectra were primarily recorded on a Jeol PFT-100 system interfaced to an EC-100 Fourier transform computer with 20K memory. ^{1}H-nmr and some of the ^{13}C-nmr spectra were recorded on a Varian FT-80 system. $CDCl_3$ was used as the solvent, and chemical shifts are reported in ppm downfield from TMS for all nmr spectra. Mass spectra were recorded on a Dupont 21-492 BR mass spectrometer using chemical ionization with isobutane as ionizing gas. Uv-visible spectra were recorded on a Perkin-Elmer Coleman 124 instrument with chloroform as solvent. Infrared spectra were recorded on a Beckman IR-33 spectrometer. Melting points were determined on a Fisher-Johns hot stage apparatus and are not corrected.

3'—OXODIHYDROGRANATICIN METHYL ESTER (**3**).—Dihydrogranaticin (250 mg) (**29**) was dissolved in 1.5 ml of dimethyl sulfoxide and 1.5 ml of benzene. Dicyclohexylcarbodiimide (230 mg) and pyridinium trifluoroacetate (40 mg) were added with stirring, and the reaction was allowed to proceed at room temperature for one hour. The reaction was approximately 90% complete, as determined by tlc on oxalic acid treated silica gel plates in chloroform-ethyl acetate (60:40) (4), the major product having an R_f of 0.30 and one minor product having an R_f of 0.35. After 40 ml of ethyl acetate were added to the solution, dicyclohexylurea was removed by filtration. The filtrate was washed three times with water; the first two aqueous washes were washed with ethyl acetate; and the ethyl acetate extract was washed twice with water. The combined ethyl acetate solutions were dried over sodium sulfate and evaporated to dryness. The residue was dissolved in a minimum volume of chloroform and purified by preparative tlc in chloroform-acetone (9:1). The major product was eluted from the silica gel with chloroform-ethyl acetate (4:1), and the solution was washed with water, dried over sodium sulfate, and evaporated to dryness. The product was converted to its methyl ester as described for **2a**. One hundred forty-eight mg (58%) of pure **3** (mp 260–262°) were obtained. It gave an R_f of 0.52 on oxalic acid treated silica gel plates in chloroform-ethyl acetate (60:40); visible absorption, λ (log ϵ): 554 nm (3.24), 518 (3.44), 490 (3.40); ir (KBr): 2.9 μ (OH), 5.6 (ester), 6.3 (quinone); ^{1}H-nmr ($CDCl_3$): δ 13.11, 12.60 (s, hydroquinone OH), 5.67 (dd, H-1', $J_{1',2'}$= 2.0 and 3.1 Hz), 5.08 (q, H-15, $J_{15,16}$=6.7 Hz), 4.96 (s, 4'-OH), 4.34 (m, H-3), 4.17 (q, H-5', $J_{5',6'}$=6.0 Hz), 3.75 (s, ester CH_3), 3.3–2.2 (multiplets for CH_2-2', 2, 4), 1.58 (d, CH_3-16, $J_{15,16}$ =6.7 Hz), 1.08 (d, CH_3-6', $J_{5',6'}$=6.0 Hz).

Granaticin tetraacetate (**1c**) was prepared from **1a** as reported by Keller-Schierlein and coworkers (16), and its identity was confirmed by comparison of its physical and spectral properties with the published data.

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