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# 4. $^{13}$C-NMR. Analysis of Dihydrogranaticin Methyl Ester. A Case of Mixed Biogenesis[1])

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*Summary*

The $^{13}$C-NMR. spectrum of dihydrogranaticin methyl ester has been completely analyzed. Feeding experiments of $CH_3$ $^{13}$COONa to a culture of *Streptomyces olivaceus* have shown that only a sixteen carbon moiety of the antibiotic granaticin is of polyketide origin, thus confirming the hypothesis of a mixed biogenesis for this natural substance.

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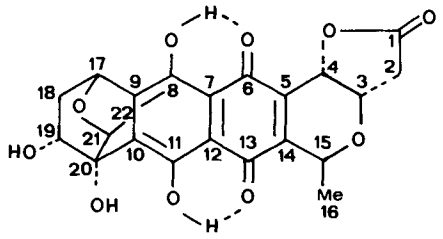
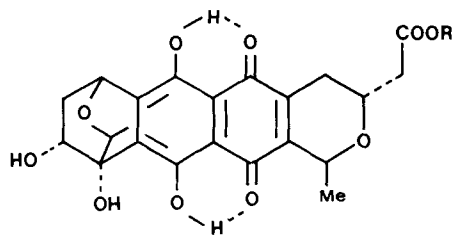
Granaticin (**1**) is an antibiotic isolated for the first time from *Streptomyces olivaceus* [2] and then from other strains of *Streptomyces* [3] [4], whose structure and absolute configuration were established by *Keller-Schierlein et al.* in 1968 [5]. It also shows antiprotozoal [6] and antileukemic [4] activity. Recently one of us [7] has reported the isolation from *S. thermoviolaceus* of dihydrogranaticin (**2**), the direct precursor of granaticin.



**1**



**2** R = H

**3** R = Me

Although a biogenetic origin of **1** from polyketide precursors only could be envisaged, a more attractive hypothesis [5] [7] suggests that the chain of carbon atoms C(1) to C(16) of **1** and **2** can be formed from an octaketide, whereas the 'left' moiety C(17) to C(22) could be originated from a hexose, *via* a hypothetic *C*-glycoside analogous to aquayamycin [8] and further cyclization. This communication reports the complete assignment of the $^{13}$C-NMR. spectrum of dihydrogranaticin methyl ester (**3**), a derivative [7] particularly suitable for NMR. analysis, and preliminary incorporation experiments, which support the hypothesis of a mixed biogenesis.

The attribution of all the $^{13}$C-signals of **3** *(Table 1)*, including those of carbonyl and quaternary carbon atoms, was uniquely defined by $^{1}$H-SFSD. experiments. As all the quaternary carbon atoms show long range coupling constants, the same decoupling technique was used, as already successfully experienced for a series of anthraquinone models [9] and for other antibiotics [10]. Only a low decoupling power was needed since the interactions with protons are small. The analysis of the $^{1}$H-NMR. spectrum of **3** has already been reported [7].

Table 1. *$^{13}$C chemical shift assignments and enrichment level of dihydrogranaticin methyl ester*[a])

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C(6) | 175.2[b]) | C(9) | 142.2 (3.97) | C(20) | 80.5 (1.27) | OMe | 51.8 (1.11)[c]) |
| C(13) | 175.1[b]) | C(5) | 140.3 (5.34) | C(21) | 72.7 (1.02) | C(2) | 40.4 (0.92) |
| C(1) | 170.9 (3.56) | C(10) | 136.3 (1.35) | C(19) | 70.9 (0.96) | C(18) | 35.8 (0.84) |
| C(11) | 168.4 (3.62) | C(12) | 110.4 (1.18) | C(15) | 67.5 (3.44) | C(4) | 27.7 (0.90) |
| C(8) | 162.9 (1.01) | C(7) | 110.1 (3.87) | C(3) | 63.2 (3.20) | C(16) | 19.2 (0.87) |
| C(14) | 144.7 (1.20) | | | C(17) | 61.8 (1.01) | C(22) | 16.5 (0.99) |

[a]) The chemical shifts (ppm from internal TMS) are obtained from a 80 mg/ml $CDCl_3$ solution and are accurate to 0.05 ppm; for SFSD. experiments a 300 mg/ml solution was used. Numbers in parentheses are atom% $^{13}$C obtained by the intensity ratio between the enriched and not enriched spectrum, whose values are normalized using the OMe as a standard (arbitrarily assumed error ± 10%).

[b]) The intensities could not be measured separately, because of overlap.

[c]) Natural abundance value, as this atom is not of biosynthetic origin.

Table 2. *$^1H$ chemical shifts of dihydrogranaticin methyl ester*[a])

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| H–C(17) | 5.20 | H–C(19) | 4.01 | $H_{eq}$–C(4) | 2.87 | $H_{exo}$–C(18) | 2.62 | $H_3C(22)$ | 0.98 |
| H–C(15) | 5.03 | H–C(21) | 3.78 | $H_{ax}$–C(4) | 2.38 | $H_{endo}$–C(18) | 1.54 | HO–C(11) | 13.15 |
| H–C(3) | 4.30 | OMe | 3.74 | $H_2C(2)$ | 2.67 | $H_3C(16)$ | 1.57 | HO–C(8) | 12.75 |

[a]) The chemical shifts, in ppm relative to internal TMS, are obtained from the same solution used for SFSD. experiments (300 mg/ml $CDCl_3$).

The assignment of the hydrogen bearing carbon atoms by selective irradiation of the directly bonded protons was straightforward. In the case of $H_2C(2)$ and $H_{exo}$–C(18), which have very close chemical shifts, the irradiation at 2.67 ppm led to a singlet (C(2)) and to a doublet (C(18), residual $J_{C(18),Hendo-C(18)}$). The quaternary carbon atoms presented major difficulties, due not only to the close vicinity of some signals, but also to symmetrical interactions. They were grouped by their chemical shift, *e.g.* C=O and =C–OH, 160–170 ppm region, C(5), C(9), C(10), C(14) from 130 to 145 ppm, and C(7), C(12) at 110 ppm. The large shielding of these two latter carbon atoms can be explained on the basis of a study of the substituent effects in a series of model anthraquinones [9]. The assignment of C(20) at 80.5 ppm was obvious since it is the only aliphatic quaternary carbon atom. C(1) was located at 170.9 ppm by SFSD. of the methoxy protons, and C(13) at 175.1 ppm by decoupling of H–C(15). The irradiation of H–C(4) to confirm the other quinone carbonyl signal at 175.2 ppm was unsuccessful, but this assignment followed indirectly after the attribution of C(8) and C(11). SFSD. of H–C(17) affected only the C(8) signal, which collapsed into a doublet ($^2J_{C(8),OH}$ = 2.5 Hz). Then the selective irradiation of each of the chelated hydroxyl protons at 13.15 and 12.7 ppm decoupled C(11) and C(8) respectively. C(11) collapsed into a singlet, C(8) into a doublet ($J_{C(8),H-C(17)}$ = 2.0 Hz). The combination of these three experiments allowed the assignment of the aromatic hydroxyl protons, and consequently the irradiation of each of them led also to the identification of C(7) *vs.* C(12). In the coupled spectrum C(7) is a doublet ($J_{C(7),OH}$ = 5 Hz), whereas C(11) is broad; as HO–C(11) is also broad some intramolecular exchange with the close hydroxyl proton at C(20) must occur.

The remaining four carbon atoms in the 130–145 ppm region were attributed by the following SFSD. experiments: *i)* upon irradiation at 2.65 ppm ($H_2C(4)$ plus $H_{exo}$–C(18)) only the signal at 136.3 ppm was unaffected; *ii)* on the contrary the same signal was decoupled by irradiation at 3.75 ppm (H–C(21) plus H–C(19)), thus confirming the attribution of C(10); *iii)* the irradiation of H–C(3) at 4.30 ppm located C(5) (*t*, $J_{C(5),H-C(4)}$ = 6 Hz); *iv)* upon irradiation at 1.56 ppm ($H_3C(16)$ plus $H_{endo}$–C(18)) only the signal at 144.7 ppm was affected (C(14) or C(9)); *v)* upon irradiation of the OH at 12.75 ppm the signal at 142.2 ppm collapsed into a double doublet as a *J* of *ca.* 3 Hz vanished; this identified C(9) ($J_{C(9),H-C(17)}$ = 2, $J_{C(9),Hexo-C(18)}$ = 7 Hz) and consequently also C(14).

The advantage of such a complete and unambiguous assignment of all the carbon atoms performed by proton decoupling and not by comparison with models is that only one incorporation experiment with monolabelled acetate is sufficient to give the labelling pattern.

In a preliminary experiment [1-$^{14}$C]acetate (52.5 μCi/mmol) was added to a submerged culture of *Streptomyces olivaceus* (CBS 417.59) after five hours of growth on potato-glucose medium. Fortyfive hours later the mycelium was filtered off, the culture broth acidified to pH 5.5 with AcOH, and extracted with AcOEt. Purification by TLC. (benzene/ether/HCOOH 1:3:0.5%) on silica gel impregnated with $KH_2PO_4$ gave a 7:3 ratio of **1** and **2**. Hydrogenation of **1** in presence of $PtO_2$ [7] gave **2**, which was joined to the other part and crystallized to constant molar radioactivity (11.28 μCi/mmol), corresponding to an incorporation of 0.65%.

Therefore [1-$^{13}$C]acetate (90%, 0.4 g/l) was fed to another culture under the same conditions, and the thus obtained dihydrogranaticin was converted into the methyl ester **3**. The $^{13}$C-NMR. spectrum of this sample *(Table 1)* showed clearly the enrichment only of carbon atoms 1, 3, 5, 7, 9, 11, 13 and 15, with an average enrichment of 2.7% $^{13}$C over natural abundance[5]).

This first result indicates that only the C(1) to C(16) moiety of granaticin (**1**) is derived from an octaketide precursor, and is therefore an unambiguous proof of the mixed biogenesis of this antibiotic.

*Note added in proof:* Similar and further results have been obtained by *H.G. Floss* (J. Amer. chem. Soc., in press). We thank Professor *Floss* for kindly sending a preprint of his paper.

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[5]) The use of $Cr(acac)_3$ as a relaxation agent was not necessary because the enriched and not enriched spectra were performed in rigorously constant conditions, and thus with such a high incorporation level the clear enhancement of all the enriched signals could be observed. Only the quinone carbonyl carbon atoms were not distinguished in the enriched spectrum, but the labeling of C(13) followed from the biosynthetic pathway.