Further Studies on the Biosynthesis of Granaticin*

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

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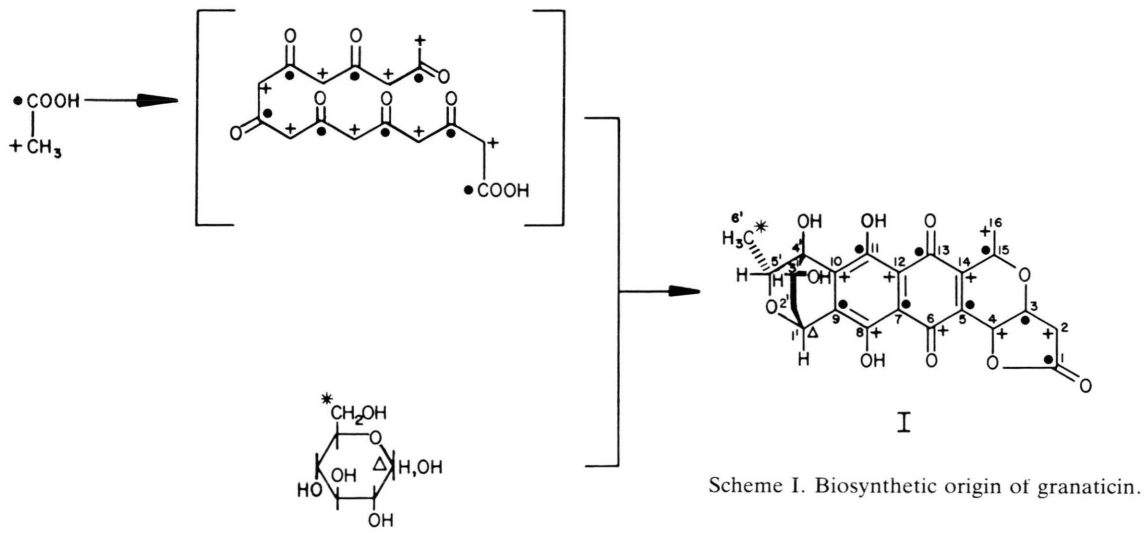
Experiments with cerulenin-inhibited cultures of *S. violaceoruber* showed conversion of dihydrogranaticin (**II**) into granaticin (**I**), but not *vice versa,* confirming an earlier conclusion that **II** is the biosynthetic precursor of **I**. Feeding of $CH_3{}^{13}C^{18}O_2Na$ followed by ^{13}C-NMR analysis of the product by the ^{18}O shift method indicated the expected incorporation of ^{18}O at carbons 1, 11 and 13 of **I** and showed that the oxygen of the pyran ring originates from C-3 and not from C-15. Analysis of **I** biosynthesized from $^{13}C^2H_3COONa$ by $^{13}C\{^1H, ^2H\}$ triple resonance NMR spectroscopy showed the incorporation of one atom of deuterium each at C-2 and C-4. C-16 carried a maximum of 2, not 3, atoms of deuterium. These results are discussed in terms of biosynthetic mechanisms.

The antibiotic granaticin [1, 2] (**I**), a metabolite of a number of *Streptomyces* species [3–6], is a member of the broader class of benzoisochromane quinone microbial metabolites, which also includes actinorhodin [7], kalafungin [8], the nanaomycins [9, 10] and the naphthocyclinones [11, 12]. Its biosynthesis has been studied in feeding experiments with radioactive and stable isotope-labeled precursors [13, 14]. In agreement with the origin of other members of this class of compounds [9, 15, 16], the 16-carbon benzoisochromane framework is formed via the polyketide pathway from 8 acetate/malonate units [13, 14]. The additional 6 carbon atoms of the bicyclic moiety of **I** (C-1′–C-6′) [17] represent a 2,6-dideoxyhexose which is derived from the intact carbon chain of glucose [13] (Scheme I). Details of the mode of conversion of glucose into the dideoxyhexose moiety have been elucidated and the relationship of **I** to its co-



Scheme I. Biosynthetic origin of granaticin.

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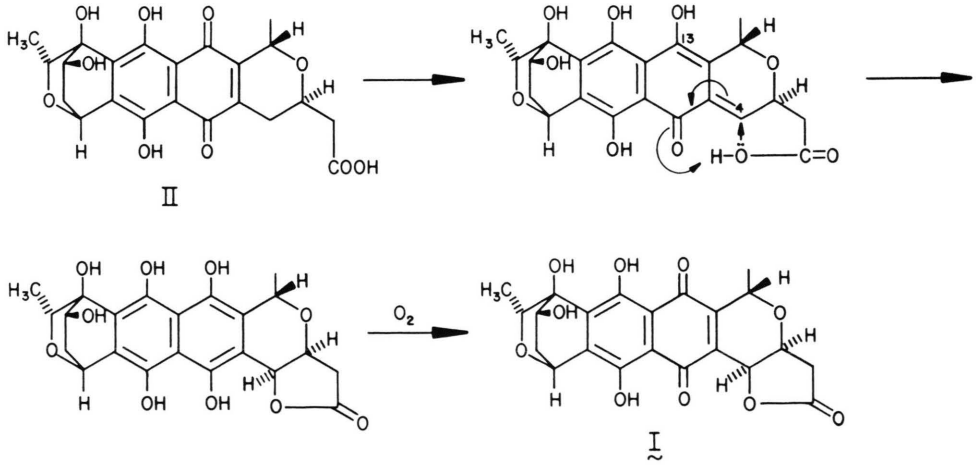
metabolite, dihydrogranaticin (**II**), has been examined [13].

In the present communication we report some additional results which confirm earlier conclusions and provide further insights into the biosynthesis of **I**.

Results

In our earlier work [13] we had concluded that **I** is formed via **II** as an intermediate. This conclusion was based on the observation that a cell-free extract of the granaticin-producing organism, *S. violaceoruber* strain Tü 22, catalyzed the conversion of **II** into **I** in the presence of air, and on the earlier finding of Pyrek *et al.* [6], confirmed by us, that the appearance of **I** in the fermentation is preceded by that of **II**. Despite the dependence of the enzymatic conversion **II** → **I** on air oxygen, no ^{18}O was incorporated into **I** when the reaction was carried out in the presence of $^{18}O_2$ [13]. This points to a direct cyclization mechanism involving Michael addition of the carboxylate group to the 4,13-conjugate enol tautomer of **II**, followed by air oxidation of the resulting hydroquinone of **I** (Scheme II). Subsequently, Ōmura and coworkers [18] demonstrated that in the nanaomycin series the biosynthetic reaction sequence proceeds in the reverse direction, *i.e.*, the lactone nanaomycin D is reduced to the ring-open dihydro compound, nanaomycin A. They purified the enzyme, nanaomycin D reductase, from the nanaomycin producer, *S. rosa* var. *notoensis,* and showed that it operates quite unidirectionally, probably by a reverse reaction sequence as that shown in Scheme II for the oxidation of **II** to **I** [19]. A reductive reaction sequence was also observed in the biosynthesis of the naphthocyclinones, where we demonstrated [21] the unidirectional conversion of the lactone, γ-naphthocyclinone, the earliest compound in the biosynthetic sequence, into its ring-open dihydro derivative, β-naphthocyclinone.

The results in the nanaomycin and the naphthocyclinone series obviously raised questions about the validity of our ealier conclusion on the biosynthetic interrelationship of **I** and **II**, and caused us to reexamine the issue. We used for this purpose the technique, pioneered by Ōmura [18], of studying metabolite interconversions in cultures in which *de novo* metabolite synthesis was blocked by the addition of the antibiotic cerulenin, a potent inhibitor of fatty acid and polyketide synthesis [21]. Cultures of *S. violaceoruber* Tü 22 tolerated up to 15 μg/ml of cerulenin without apparent effects on the general appearance and behavior of the cells. At that concentration of cerulenin the production of **I** and **II** was not completely inhibited, but was sufficiently reduced, to 10–15 μg/ml, to allow observation of the transformation of added **I** or **II**. Pure samples of **I** and of **II** were prepared chromatographically from normal culture extracts of *S. violaceoruber.* Five mg of either **I** or **II** dissolved in 1 ml ethanol were added to 25 ml cultures of *S. violaceoruber,* containing 15 μg/ml cerulenin, 24 hr after inoculation. Control flasks received no addition of **I** or **II** and others contained **I** or **II** but no cells and cerulenin. Samples of one ml were



Scheme II. Proposed mechanism of the interconversion of dihydrogranaticin and granaticin [13].

Table I. Interconversion of granaticin (**I**) and dihydrogranaticin (**II**) in cerulenin-inhibited cultures of *S. violaceoruber* strain Tü 22.

Time (h) after addition of **I** or **II** (24 h after) inoculation)	Concentration [μg/ml] of **I** or **II** in Expt.									
	1 (No addition)		2 (Addition of **I**)[b]		3 (Addition of **II**)[b]		4[a] (Addition of **I**)[b]		5[a] (Addition of **II**)[b]	
	I	**II**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
0	0	0	69	0	0	77	67	0	0	73
3	0	0	69	0	0	76	67	0	7	72
6	0	0	69	0	7	75	69	0	10	71
12	0	0	69	0	23	70	66	0	14	69
24	10	10	65	0	50	55	63	0	19	66
36	10	12	63	0	51	53	60	0	20	64
48	11	13	61	0	52	52	60	0	21	63

[a] Control flasks containing medium without cells or cerulenin.
[b] 5 mg per 25 ml culture.

drawn from each culture at various times and analyzed for the amounts of **I** and **II** present. The results, summarized in Table I, clearly indicate that under the experimental conditions, which except for the presence of cerulenin are identical to the normal fermentation conditions for the production of **I** and **II**, **II** is converted into **I** (Expt. 3), but no conversion of **I** into **II** is observed (Expt. 2). The amount of **I** formed exceeds the amount of added **II** consumed, probably reflecting some residual *de novo* formation of **I** and **II**. The enzymatic formation of **I** from **II** is significantly (2.5 times) faster than the nonenzymatic conversion in the control without cells (Expt. 5). These results support our earlier conclusion that **II** is the biosynthetic precursor of **I**.

In order to obtain further insight into mechanistic aspects of the biosynthesis of granaticin, we traced the fate of the oxygen and the hydrogen atoms of the precursor, acetic acid, in the conversion into the polyketide moiety of **I**. To determine which oxygen atoms are incorporated into the antibiotic, sodium [1-^{13}C,$^{18}O_2$]acetate (315 mg, 99.9% ^{13}C, 91,5% ^{18}O) was fed to ten 100 ml cultures of *S. violaceoruber* and the resulting dihydrogranaticin (26 mg) was derivatized by methylation with methanolic HCl as described earlier [6, 13] to give dihydrogranaticin methyl ester for NMR analysis. The ^{18}O-isotopic shift method [22] was used to detect ^{18}O nuclei directly bonded to a ^{13}C-enriched carbon atom. An upfield shift of the ^{13}C-NMR signal of a ^{13}C atom bonded to ^{18}O, relative to the corresponding ^{13}C-^{16}O assembly, indicates, in the context of the present experimental arrangement [23], that the bond between this carbon and the oxygen has not been broken during the conversion of the precursor into the product. In other words, the two isotopes originate from the same precursor molecule. Spectra were recorded in $CDCl_3$ and in CD_3CN because in each solvent some signals were poorly resolved or broadened. The spectra (Table II) show the expected [13] ^{13}C enrichment at

Table II. NMR analysis by the $^{13}C\{^{18}O\}$ shift method of dihydrogranaticin methyl ester, a derivative of granaticin, biosynthesized from $CH_3{}^{13}C^{18}O_2Na$.

^{13}C-Enriched carbon atom[a]	Spectrum recorded in				
	$CDCl_3$			CD_3CN	
	δ [ppm][b]	$\Delta\delta^{18}O$ [Hz]	% $^{13}C^{18}O$	$\Delta\delta^{18}O$ [Hz]	% $^{13}C^{18}O$
1	170.8	3.7	32	3.6	33
3	63.1	2.2	75	[c]	
5	140.3	no ^{18}O shift		no ^{18}O shift	
7	110.1	no ^{18}O shift		no ^{18}O shift	
9	142.0	no ^{18}O shift		no ^{18}O shift	
11	168.5	6.0	70	5.0	62
13	174.8	[c]		0.9	65
15	67.4	no ^{18}O shift		no ^{18}O shift	

[a] Average enrichment per carbon 11%; all other signals showed no significant enrichment.
[b] Assignments based on ref. [24].
[c] Signal broadened, but not resolved into discrete peaks.

carbons 1, 3, 5, 7, 9, 11, 13 and 15. Of these, the signals of all the oxygen-carrying carbons except C-15, *i.e.*, those for C-1, C-3, C-11 and C-13, show ^{18}O isotope satellites. An average of 70% of the ^{13}C is ^{18}O shifted, half as much for C-1 due to loss of half of the ^{18}O during esterification. After correction for the natural abundance ^{13}C contribution, this indicates that most, if not all the ^{13}C of the precursor incorporated into these positions retains one of the original ^{18}O atoms of the precursor. The finding that C-3, but not C-15, shows an ^{18}O shift indicates that the oxygen of the pyran ring is derived from the acetate/malonate unit which gives rise to C-3 and C-4, not from the starter unit which provides C-15 and C-16.

To trace the fate of the methyl hydrogens of acetate, sodium[2-^{13}C,$^{2}H_3$]acetate (956 mg, 93 atom % ^{13}C, 98 atom % ^{2}H) was fed to twenty 100 ml cultures of *S. violaceoruber* Tü 22, and granaticin (51 mg) was isolated as described earlier. A part of the material was converted to dihydrogranaticin methyl ester, and both samples were analyzed by NMR spectroscopy. The $^{13}C\{^{1}H,^{2}H\}$ triple resonance technique [25] was used to locate ^{2}H bonded to ^{13}C. Observation of the ^{13}C-NMR spectrum with simultaneous proton and deuterium broad-band decoupling shows ^{2}H-isotope shifted signals for carbons carrying directly attached ^{2}H which disappear upon removal of the ^{2}H broad-band decoupling. The deuterium isotope shifts are additive, *i.e.*, two atoms of ^{2}H shift twice as much as one, three about three times as much. Hence the fractions of mono-, di- and trideuterated species of a methyl group, and mono- and dideuterated species of a methylene group can be discerned. The results, summarized in Table III, indicate the expected [13] ^{13}C labeling of carbons 2, 4, 6, 8, 10, 12, 14 and 16, at an average enrichment of about 9–10%. Of these, all three hydrogen-carrying carbons displayed ^{2}H isotope-shifted satellites. The degree of deuterium retention relative to ^{13}C was low, averaging only about 10%. As expected, C-4 showed the presence of $^{13}C^{1}H$ and $^{13}C^{2}H$ species, but the observation of only $^{13}C^{1}H_2$ and $^{13}C^{1}H^{2}H$, but no $^{13}C^{2}H_2$ species at C-2 was unexpected. It suggests that in the course of the biosynthetic transformations, C-2 must pass through the intermediate stage of a methine group. Equally surprising is the presence of only $^{13}C^{1}H_3$, $^{13}C^{1}H^{2}H_2$ and $^{13}C^{1}H_2{}^{2}H$, but no $^{13}C^{2}H_3$ species at the methyl group of the starter unit, C-16. This may, of course, simply reflect very rapid interconversion of acetyl-CoA and malonyl-CoA relative to the use of acetyl-CoA as a starter unit for the polyketide synthesis, although one would expect to see far more $^{13}C^{1}H_2{}^{2}H$ than $^{13}C^{1}H^{2}H_2$ species as the result of such a process. Alternatively, C-16 may obligatorily have to pass through the intermediate stage of a methylene group in the course of the biosynthesis.

Discussion

The demonstrated unidirectional conversion of **II** into **I** in cerulenin-inhibited cultures of *S. coelicolor* confirms our earlier conclusion [13] that the biosynthetic pathway proceeds from the ring-open dihydrogranaticin to the lactone granaticin. This aspect of the biosynthesis of **I** thus differs distinctly

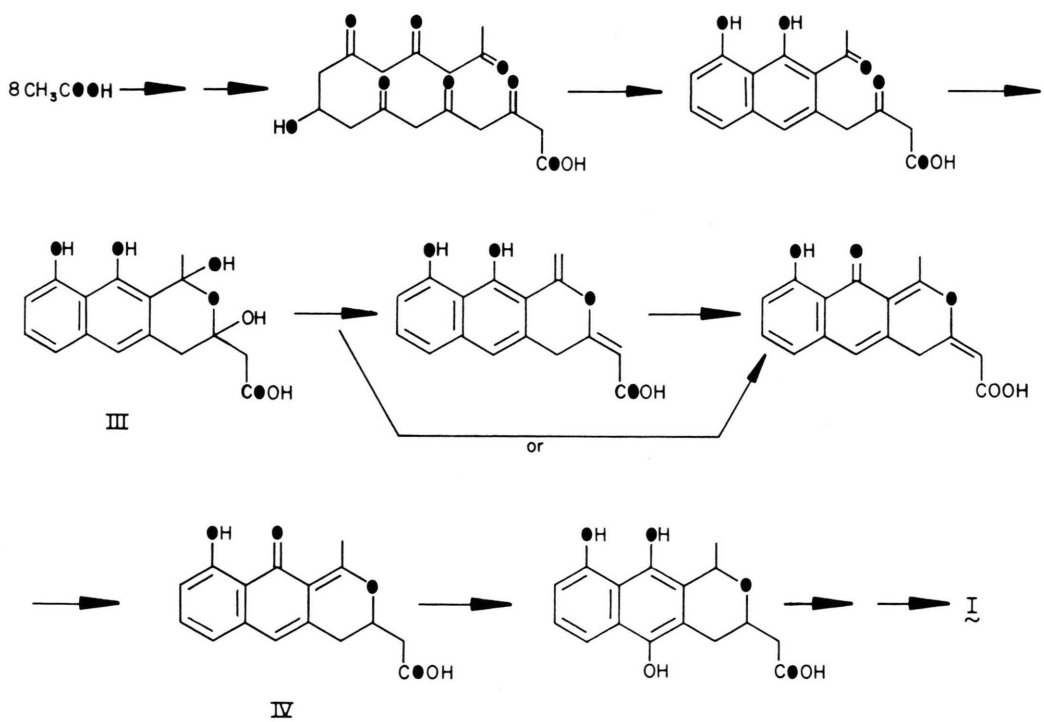
Table III. NMR analysis by the $^{13}C\{^{1}H, ^{2}H\}$ triple resonance method of granaticin and its derivative dihydrogranaticin methyl ester biosynthesized from $^{13}C^{2}H_3COONa$.

Enriched[e] carbon atom	Compound analyzed: Granaticin δ [ppm][a]	Granaticin Δδ [ppm]	Granaticin % $^{13}C^{2}H_x$	Dihydrogranaticin methyl ester δ [ppm][b]	Dihydrogranaticin methyl ester Δδ [ppm]	Dihydrogranaticin methyl ester % $^{13}C^{2}H_x$
2	38.9	0.27	13	40.3	0.28	12
4	73.6	0.30	11 (x = 1)[c]	27.6	0.30	12 (x = 1)[c]
6	168.1			174.9		
8	174.4			162.5		
10	149.9			136.1		
12	114.0			110.3		
14	137.2			144.7		
16	18.5	0.23	10 (x = 1)	19.1	0.25	8 (x = 1)
		0.49	8 (x = 2)[d]		0.50	7 (x = 2)[d]

[a] Average ^{13}C-enrichment 9.3 ± 1.0%.
[b] Average ^{13}C-enrichment 10.1 ± 0.8%.
[c] No $^{13}C^{2}H_2$ species detectable.
[d] No $^{13}C^{2}H_3$ species detectable.
[e] All other carbons showed no significant enrichment.

from the pathways established for the related nanaomycins [18] and naphthocyclinones [20]. Consistent with this observation Ōmura and coworkers [19] observed that granaticin is not a substrate for nanaomycin reductase. In the case of actinorhodin, another dimeric benzoisochromane quinone, no conversion of the lactone, γ-actinorhodin, into the dihydro compound, actinorhodin, or *vice versa* was observed with cultures of the producing organism, *S. coelicolor* [26, 27]. However, both compounds are rather poorly soluble and it is not clear whether they were taken up by the bacterial cells. The reasons for these differences in the otherwise apparently very similar pathways leading to granaticin on the one hand and to the nanaomycins and naphthocyclinones on the other are not at all obvious. Since the step in question is a redox process, one might suspect a difference in the redox potential of the substrates to be the cause. However, it is likely that the redox potentials of **I** and the naphthocyclinones are similar since they share the naphthazarin structure and different from that for the nanaomycins which contain the 5-hydroxynaphthoquinone system. More likely, therefore, the reason for the differences in the biosynthetic pathway lie in a different enzymatic make-up of the producing organisms. While the producers of the nanaomycins and the naphthocyclinones contain a reductase, the granaticin producer presumably has an enzyme which by its mode of action, and thus redox potential, is designed to carry out the oxidative conversion of **II** into **I**.

The finding that the ether oxygen of the pyran ring of **I** is derived from C-3 rather than C-15 suggests that the process of pyran ring formation may involve addition of a C-3 OH-group to a double bond, either C = O or C = C, at C-15. Mechanistically related may be the observation that C-2 may go through the intermediate stage of a methine group, since only one deuterium atom from the precursor, $^{13}C^{2}H_3COOH$, is retained, and perhaps even the fact that C-16 retains only two rather than three atoms of deuterium from the methyl group of acetic acid. Scheme III shows a hypothetical pathway for the formation of the pyran ring which accounts for the above observations. While this scheme is obviously quite speculative, it is supported by some recent results on the biosynthesis of actinorhodin [27]. Compound **IV** has been isolated as a biosynthetic inter-



Scheme III. Hypothetical pathway for pyran ring formation in the biosynthesis of granaticin.

mediate from an *act* mutant of *S. coelicolor*. Ring closure between C-3 and C-15 via a C-3 hydrate rather than the C-3 secondary alcohol is suggested by the isolation of another intermediate in actinorhodin biosynthesis whose tentative structure shows a C-3/C-15 cyclic ether with two methylene groups separated by a quaternary carbon, *i.e.*, a structure resembling **III** [27].

The fact that only a maximum of two, not three atoms of deuterium from $^{13}C^{2}H_3COONa$ are retained at C-16, if it is not merely due to rapid exchange via repeated interconversion acetyl-CoA/malonyl-CoA, can be explained by the occurrence of a C-16 exo-methylene intermediate as shown in Scheme III. Another possible explanation is suggested by some earlier results on the biosynthesis of α-naphthocyclinone [15]. It was found that both acetate and malonate labeled uniformly the starter as well as the chain extension units of the polyketide moiety. *A priori* this would suggest that not only can acetyl-CoA be readily converted to malonyl-CoA, but also malonyl-CoA to acetyl-CoA. However, only acetate, but not malonate labeled an acetoxy group also present in the molecule, which clearly comes directly from acetyl-CoA. Hence, malonyl-CoA is apparently not converted readily into acetyl-CoA, and yet labels the polyketide starter unit. As an explanation for this paradox, the possibility was suggested that malonyl-CoA may also serve as the polyketide starter unit, leading to an enzyme-bound symmetrical octaketide with an extra carboxyl group attached to C-16. This carboxyl group is then selectively removed while the polyketide is still attached to the enzyme matrix [15]. Such a mechanism would also account for the absence of $^{13}C^{2}H_3$ species at C-16 of **I** derived from $^{13}C^{2}H_3COONa$. It may not be possible to examine this possibility definitively until an enzyme system catalyzing this reaction becomes available for study.

Experimental Section

Materials

$CH_3{}^{13}C^{18}O_2Na$ was prepared from the ^{13}C-labeled compound by exchange with $H_2{}^{18}O$/HCl for 48 h at 95 °C, and $^{13}C^{2}H_2COONa$ was purchased from Prochem Ltd. Cerulenin was obtained as a gift from Prof. S. Ōmura, Kitasato University, Tokyo. All other chemicals were procured from commercial sources and were used without further purification.

Fermentations

The maintenance and fermentation of *S. violaceoruber* Tü 22, the isolation of granaticin and the derivatization to dihydrogranaticin methyl ester were carried out as described earlier. Granaticin and dihydrogranaticin for the conversion experiments were purified from extracts of *S. violaceoruber* by preparative layer chromatography on silica gel with chloroform/methanol 9:1 containing 1% oxalic acid. The same solvent system was used in the quantitative analysis of granaticin and dihydrogranaticin from the conversion experiments. The 1-ml samples of the cultures drawn at different times were acidified to pH 3 with 1 N HCl, extracted twice with 5 ml $CHCl_3$ and the extracts dried over Na_2SO_4 and evaporated. The residues were chromatographed on 2 mm silica gel plates, the bands of **I** (R_f 0.50) and **II** (R_f 0.20) were scraped off and extracted with 2 ml methanol. The absorbances of the extracts at 532 nm were measured in a Gilford 250 spectrophotometer and compared to those of standard solutions of **I** or **II**, respectively.

NMR spectroscopy

Spectra of the ^{18}O-labeled sample were recorded on a Bruker WH-400 spectrometer operating at 9.5 Tesla at a ^{13}C frequency of 100.6 MHz, using a 45° flip angle and acquisition times of 0.66 sec (full width spectra) and 16.38 sec (expanded spectra). The spectra of the deuterated compounds were recorded on a modified Varian XL100 spectrometer with internal fluorine lock operating at a ^{13}C frequency of 25.16 MHz, using 1.6 sec acquisition time and flip angles of 30° (**I**) and 40° (**II** methyl ester), 44 μsec 90° pulse, with 100.0 MHz and with or without 15.4 MHz broadband decoupling. Samples of **I** were measured in CF_3COOH/H_2O 1:1 and those of **II** methyl ester in $CDCl_3$ with or without added chromium[tris acetylacetonate] [$Cr(acac)_3$] as relaxing agent.

Acknowledgements

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