STUDIES DIRECTED TOWARDS THE BIOSYNTHESIS OF THE C_7N-UNIT OF RIFAMYCIN B: INCORPORATION OF [^{14}C(G)]QUINIC ACID AND [1,2-$^{13}C_2$]GLYCEROL

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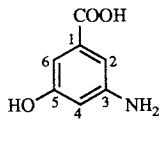
The synthesis of racemic [1,2-$^{13}C_2$]glycerol (**13**) starting from [1,2-$^{13}C_2$]malonic acid (**10**) and results of the incorporation experiments of [^{14}C(G)]quinic acid (**5**) and racemic [1,2-$^{13}C_2$]glycerol (**13**) with the double mutant P14 of *Nocardia mediterranei* are described.

The rifamycins represent one family in the class of antibiotics known as the ansamycins. Rifamycin B (**1**), the main fermentation product from cultures of *Nocardia mediterranei*, displays very poor biological activity, but upon standing in aqueous solution, is transformed into the highly active rifamycin S (**2**) which represents the most important starting material for the production of semi-synthetic derivatives used in the treatment of tuberculosis[1]. Although all ansamycins have been tested for their antibacterial, antiviral and antitumor activity, the only substances suitable for pharmaceutical application were members of the rifamycin family[2,3] and as a result, most of the biosynthetic and fermentation studies have dealt with this family of antibiotics.

Although the biosynthesis of the ansaymcins have been subjected to extensive studies[3~34] the origin of part of the chromophore *i.e.*, the C_7N-unit, still remains unclear. Incorporation studies conducted by WHITE and MARTINELLI using [1-^{13}C]glycerate and [1-^{13}C]glucose led to the hypothesis that the C_7N-unit of rifamycin S (**2**) was derived from an intermediate of the shikimic acid pathway[18] *i.e.*, a somewhat



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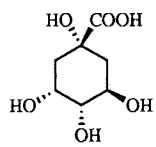

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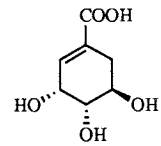

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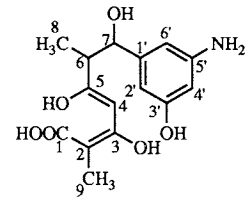

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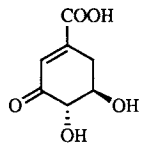

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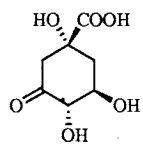

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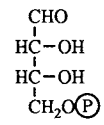

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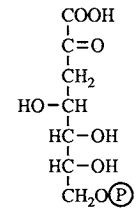

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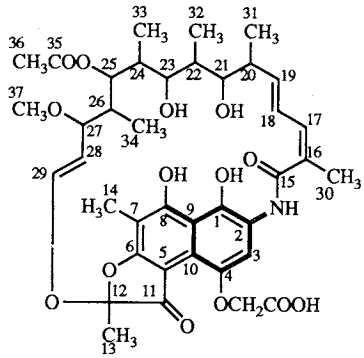
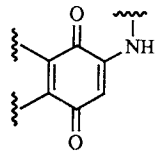


Rifamycin B (1)



Rifamycin S (2)

stronger enrichment on C-1 and C-8 of rifamycin S (**2**) suggested that this C_3-unit was derived from phosphoenolpyruvate and consequently C-1 in rifamycin S (**2**) could not be derived from C-4 of erythrose 4-phosphate (**3**). This interpretation was somewhat speculative owing to the use of singly-labeled precursors. However, incorporation attempts with [*U*-^{14}C]shikimate were unable to substantiate this statement in that ^{14}C-shikimate was found neither in the ansa chain nor in the C_7N-unit of rifamycin S (**2**)[7].

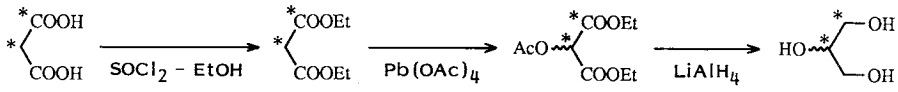
In an attempt to identify the precursor of 3-amino-5-hydroxybenzoic acid (AHBA) (**4**), an incorporation experiment with the mutant *N. mediterranei* P14 and ^{14}C-quinic acid (**5**) was conducted by GYGAX[21]. Identification of the radioactive products on the basis of their Rf values led to the recognition of quinic acid (**5**), shikimic acid (**6**), 3-dehydroshikimic acid (**7**) and a product exhibiting a very similar Rf value to that of an earlier metabolite of rifamycin B (**1**) identified as product P8/1-OG (2,6-dimethyl-3,5,7-trihydroxy-7-(3′-amino-5′-hydroxyphenyl)-2,4-heptadienoic acid) (**8**)[35]. These results suggested that the biosynthesis of AHBA (**4**) most likely occurs *via* a cyclic intermediate which is then transaminated to AHBA (**4**).

With this work we attempted to identify the precursor of AHBA (**4**) and to determine in which position of the precursor, in comparison to the planar AHBA molecule (**4**) that the nitrogen atom is incorporated. It was our intention to reach these goals using incorporation experiments with specifically labeled ^{13}C- or ^{14}C-compounds and the double mutant P14 from *Nocardia mediterranei* in an attempt to reduce the distribution of labeled material into the various intermediates leading to rifamycin B (**1**) which, in place of rifamycin B (**1**), accumulated product P8/1-OG (**8**)[35]. Here we report the synthesis of racemic [1,2-$^{13}C_2$]glycerol and results of our incorporation experiments with [1,2-$^{13}C_2$]glycerol and [^{14}C(G)]quinic acid into P8/1-OG (**8**).

Results and Discussion

Classical incorporation studies of shikimic acid (**6**) with [3,4-^{14}C]glucose, led to a labeling of C-4 and C-5 in shikimic acid (**6**) as a result of the production of the intermediate [1,2-^{14}C]erythrose 4-phosphate[36]. Subsequently we considered an incorporation experiment using ^{13}C-doubly labeled glycerol in order to recognize in which position, in comparison to the planar AHBA (**4**) molecule, that the N-atom is incorporated. Because D-glycerate is established as a specific precursor of the C-atoms of phosphoenolpyruvate (**9**) and erythrose 4-phosphate (**3**), we synthesized racemic [1,2-$^{13}C_2$]glycerol (Scheme 1) using a somewhat modified procedure[37] from GIDEZ and KARNOVSKY[38]. [1,2-$^{13}C_2$]Malonic acid (**10**) was esterified with thionylchloride in EtOH to yield diethyl[1,2-$^{13}C_2$]malonate (**11**). Oxidation of **11**

Scheme 1.



10 11 12 13

with $Pb(OAc)_4$ gave racemic diethyl 2-acetoxy-[1,2-$^{13}C_2$]malonate (**12**). Subsequent reduction of **12** with $LiAlH_4$ resulted in [1,2-$^{13}C_2$]glycerol (**13**) with an overall yield of 73% starting from **10**[38]. According to Amersham International plc the ^{13}C-enrichment for [1,2-$^{13}C_2$]malonic acid (**10**) corresponded to 90~99% isotopic abundance. Through comparison of the signal intensity in the mass spectrum of the labeled and unlabeled glycerol, the proportion of unlabeled to singly labeled to doubly labeled glycerol was calculated to be 6 : 6 : 88 (HÄDENER, A., Institut für organische Chemie der Universität Basel; personal communication).

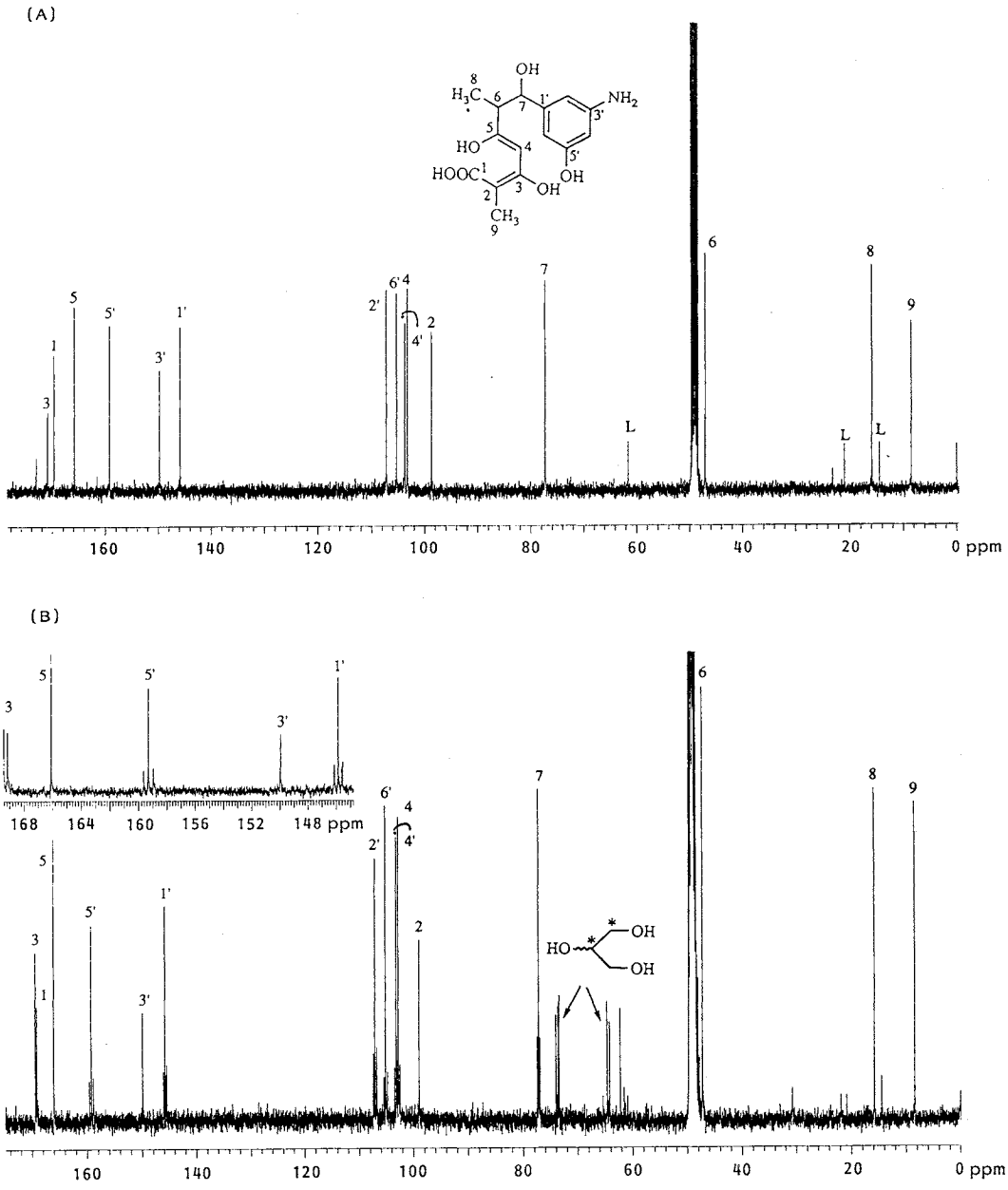
[1,2-$^{13}C_2$]Glycerol (**13**) was administered to growing cultures of *Nocardia mediterranei* P14. In the proton-noise-decoupled ^{13}C NMR spectrum of P8/1-OG (**8**) (Fig. 1) traces of racemic [1,2-$^{13}C_2$]glycerol (**13**) were also identified. Two incorporation patterns were possible. In the first case (Fig. 2), **8a**, phosphoenolpyruvate (**9**) would label C-7, C-1′, C-2′ and erythrose 4-phosphate (**3**) would label C-4′, C-5′, and C-6′ with C-3′ remaining unlabeled, or in the second case (Fig. 2), **8b**, phosphoenolpyruvate (**9**) would label C-7, C-1′, and C-6′ and erythrose 4-phosphate (**3**) would label C-2′, C-3′, C-4′, with C-5′ remaining unlabeled. This would suggest that C-3 of erythrose 4-phosphate (**3**) was aminated or in the preceding case, that C-1 of erythrose 4-phoshate (**3**) was aminated. The coupling pattern in the enriched sample of **8** (Fig. 2) revealed satellites resulting from the incorporation of intact C_2-units from racemic [1,2-$^{13}C_2$]glycerol (**13**) showing two labeled C_3-units and an additional C_2-unit in the side chain of **8**. The fact that no satellites were observed for C-3 indicated that this C-atom was not labeled from a C_2-unit of racemic [1,2-$^{13}C_2$]glycerol. The missing satellites for C-3′ were a clear confirmation of the first case, **8a**, with C-8, C-1′, and C-2′ originating from phosphoenolpyruvate (**9**) and C-3′, C-4′, C-5′ and C-6′ originating from erythrose 4-phosphate (**3**) with C-1 of erythrose 4-phosphate (**3**) carrying the N-atom or in the case of a cyclic intermediate, C-5 would be aminated.

GHISALBA and co-workers isolated 1,272 mg/liter of P8/1-OG (**8**)[35]. We were able to isolate no more than 367 mg/liter of **8**. This difference could be explained by the additional purification step required here using column chromatography. Although an attempt was made to isolate P8/1-OG (**8**) as its lactone as described in the literature[35], in our hands we isolated only its acid. Interestingly, the fast atom bombardment (FAB) mass spectrum of **8** indicated the signal of highest mass as the protonated glycerol addition product of the lactone ($(M+2Gly+H)^+$) at *m/z* 476, ($(M+Gly+H)^+$) at *m/z* 384 and the protonated molecular ion of the lactone at *m/z* 292. As reported by GHISALBA, and here verified, all attempts to observe the molecular ion at *m/z* 309 or the protonated molecular ion of the free acid at *m/z* 310 were futile[35]. Apparently a spontaneous lactonization of the acid occurred in the mass spectrometer.

Along with product P8/1-OG (**8**) an additional metabolite identified as 2-amino-benzamide (**14**) was isolated from the culture broth of *N. mediterranei* P14 for the first time (Fig. 3). The isolation of 2-amino-benzamide (**14**) although unexpected, since the mutant strain *N. mediterranei* P14 accumulates shikimic acid (**6**) due to a block probably at the level of shikimate kinase and as a result no products after shikimic acid (**6**) should be formed, indicated that the block was not 100%, but rather "leaky". This strongly fluorescent material was first isolated by a Japanese group from cultures of *Streptomyces*

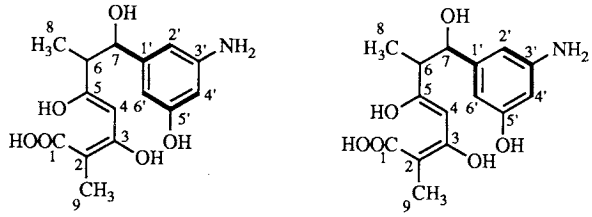
Fig. 1. Proton-noise-decoupled ^{13}C NMR spectra of P8/1-OG (**8**) in CD_3OD.

(A) Reference spectrum, (B) after incorporation of [1,2-$^{13}C_2$]glycerol (**13**).



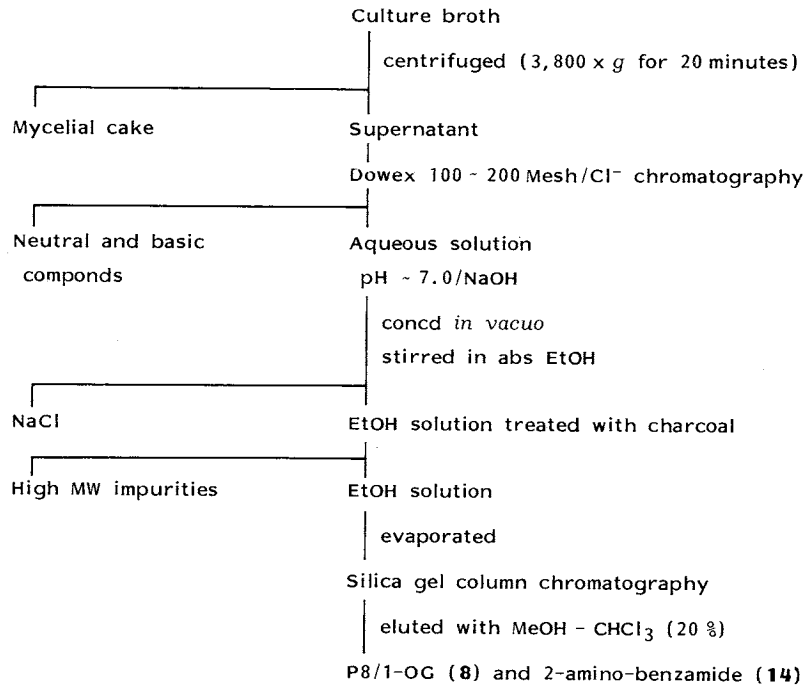
sioyaensis[39]. 2-Amino-benzamide (**14**) is formed biogenetically from 2-amino-benzoic acid (*i.e.* anthranilic acid), an intermediate of the shikimic acid pathway originating from chorismic acid[40]. The identity of **14** was verified by mass spectrometry (EI) with the molecular ion (*m/z*) representing the highest mass at 136 (79%) and the *m/z* at 119 (100%) representing the first fragment after loss of ammonia being observed. These results verified the *ortho*-disubstitution of the benzene ring so that the proton transfer from the amide to amino group would be unlikely to occur, and as a result no loss of ammonia ($(M-NH_3)^+$)

Fig. 2. Possible coupling patterns in P8/1-OG (**8**) after incorporation of racemic [1,2-^{13}C]glycerol.



8a **8b**

Fig. 3. Isolation procedure for P8/1-OG (**8**) and 2-amino-benzamide (**14**).



could take place[41]. In the ^{1}H NMR both protons H-2 and H-5 appeared as a doubled doublet due to the existance of *ortho* as well as long-range coupling. H-3 and H-4 experienced long-range in addition to vicinyl coupling.

For our incorporation experiment using [^{14}C(G)]quinic acid (**5**), we transformed the commercially available [^{14}C(G)]shikimic acid (**7**) into [^{14}C(G)]quinic acid (**5**) *via* an epoxide on positions 1 and 2 of shikimic acid (**7**)[42]. [^{14}C(G)]Quinic acid (8.63 μCi, 82.1 μCi/mmol) was administered to growing cultures of *N. mediterranei* P14 at 72 hours after innoculation in 50 ml of industrial fermentation medium 151b[43]. After 8 days the antibiotics P8/1-OG (**8**) and 2-amino-benzamide (**14**) were isolated and purified, as shown in Fig. 3, to yield 30.2 mg of P8/1-OG (**8**) and 6.6 mg of 2-amino-benzamide (**14**). The specific activity of **8** and **14** was determind by liquid scintillation counting and is shown in the accompanying table (Table 1).

P8/1-OG (**8**) and 2-amino-benzamide (**14**) display both low absolute incorporation rates and relatively high dilution factors. The fact that 2-amino-benzamide (**14**) is derived from anthranilic acid, an intermediate

Table 1. Incorporation results obtained for P8/1-OG (**8**) and 2-amino-benzamide (**14**).

	Activity	Specific activity	% Incorporation[a]	Dilution factor[b]
P8/1-OG (**8**)	3,430 dpm/mg	0.4775 μCi/mmol	0.54	172
2-Amino-benzamide (**14**)	17,469 dpm/mg	1.0721 μCi/mmol	0.60	77

[a] % Incorporation or absolute incorporation is a ratio of the activities of the product to precursor, *i.e.*, 0.0467 μCi/8.63 μCi for P8/1-OG (**8**) to quinic acid (**5**).

[b] Ratio of specific activities of the precursor to product, *i.e.*, 82.1 μCi/mmol/0.4775 μCi to mmol for quinic acid (**5**) to P8/1-OG (**8**).

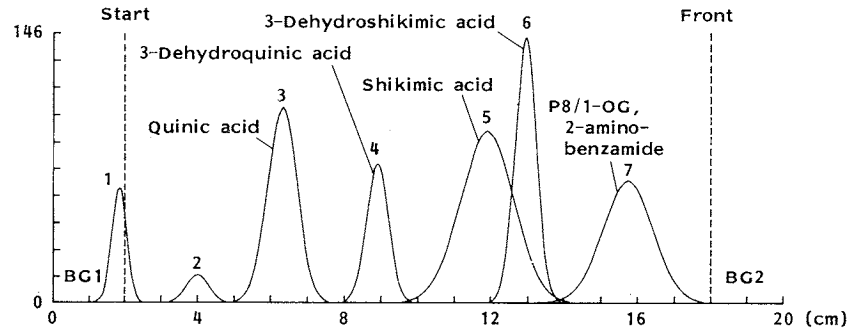
Fig. 4. TLC-scan of the culture filtrate from *Nocardia mediterranei* after incorporation of [^{14}C(G)]quinic acid (**5**).

The numbers refer to the peaks	Rf	Center (cm)	From (cm)	To (cm)	Net (cts)	Net (cpm)	S.D. (%)	ROIs (%)	All (%)
1	0.01	1.83	1.10	2.55	1,655.6	9.20	3.08	5.35	2.63
2	0.12	3.99	2.91	5.06	601.3	3.34	12.26	1.94	0.95
3	0.27	6.28	4.86	7.70	5,437.5	30.21	3.74	17.58	8.63
4	0.43	8.87	7.79	9.95	2,997.0	16.65	4.72	9.69	4.76
5	0.62	11.86	9.26	14.47	8,798.1	48.88	22.78	28.44	13.96
6	0.68	12.93	11.86	13.99	5,526.7	30.70	12.64	17.87	8.77
7	0.86	15.72	13.28	18.16	5,916.3	32.87	17.71	19.13	9.39

Solvent: BuOH - acetone - acetic acid - ammonium hydroxide (25%) - H_2O (70 : 50 : 18 : 1.5 : 60).
Background from 0 to 1.5 and 18.5 to 20.0.
Measurement time: 3 hours.
Position: 12.0 (cm), Rf-start = 2.00 (cm), front = 18.00 (cm), gain = 3, significance = 3.0, peak-reject = 20 (cts), HW-width = 0.8/0.0 (cm).

	(cts)	(cpm)
Total gross:	63,026,	350.14
Gross in ROIs:	65,678,	364.87
Net in ROIs:	30,933,	171.85

Total gross: Total activity for region from 1.5 to 18.5.
ROI: Region of interest.
ROIs (%): % of ROI activity to ROI total activity.
Gross in ROIs: ROI brutto total activity (including background counts).
Net in ROIs: ROI net total activity (background is subtracted).
S.D. (%): Standard deviation expressed in %.
All (%): % of ROI activity to total activity.



of the shikimic acid pathway[40], should be proof that, ^{14}C-quinic acid (**5**) was utilized for the biosynthesis of anthranilic acid (**14**). As a result, a relatively high incorporation rate and low dilution would be expected for 2-amino-benzamide (**14**). The low incorporation rate obtained most likely signifies that the labeled

^{14}C-quinic acid (**5**) was utilized to a large extent for the synthesis of other metabolites. The same explanation could hold for the slightly lower incorporation rate shown for P8/1-OG (**8**).

On account of the irreversible transformation of 3-deoxyarabinoheptulosonate-7-phosphate (DAHP) (**15**) into 3-dehydroquinic acid (3-DHQ) (**16**), the radioactivity should not be incorporated into DAHP (**15**)[40]. As a result of a block, in the mutant P14 after shikimic acid (**6**), **6** should be accumulated. This was confirmed by a TLC-scan of the culture filtrate (Fig. 4), showing that the majority of the radioactivity was contained in quinic acid (**5**), 3-DHQ (**16**), 3-dehydroshikimic acid (3-DHS) (**7**) and shikimic acid (**6**), with these substances accounting for nearly 74% of the activity of the signals $1 \sim 7$ (Fig. 4). Quinic acid (**5**) and shikimic acid (**6**) were the predominant radioactive peaks.

A further explanation for the observed weak activity in P8/1-OG (**8**) is the possible transformation of (−)-quinic (**5**) or (−)-shikimic acid (**6**), by certain bacteria, *via* protocatechuic acid to succinic acid and acetate (β-ketoadipic acid pathway)[40,44]. Many mono- and 1,2-disubstituted aromatic rings (*i.e.* phenylalanine, phenol, benzol and anthranilic acid) can also be catabolized to catechol which is particularly interesting here considering that 2-amino-benzamide (**14**) was isolated from the culture medium of *N. mediterranei* P14. Catechol or protocatechuate are then transformed to succinyl-CoA and acetyl-CoA *via* the ketoadipic acid pathway[44]. Succinyl-CoA is incorporated *via* methylmalonyl CoA, which along with acetyl-CoA participates in the construction of the side chain in P8/1-OG (**8**). The existence of such ^{14}C-labeled propionate and acetate precursors could possibly explain the low activity observed for P8/1-OG (**8**). A further problem through the occurence of ^{14}C-labeled acetate is its incorporation in the citric acid cycle[44], whereby ^{14}C-labeled oxaloacetate would be produced which could then be decarboxylated with oxaloacetate-decarboxylase leading to a C_3-acid, either pyruvate or phosphoenolpyruvate which could then be incorporated into the C_7N-unit of P8/1-OG (**8**). This would imply that a part of the radioactivity observed for P8/1-OG (**8**) was indirectly incorporated into the C_7N-unit without incorporation of [^{14}C(G)]quinic acid (**5**) as an intact unit. As a result, the radioactivity would be spread over the whole side chain as well as a portion of the ring in P8/1-OG (**8**).

In comparison to the recently published data from STALEY and RINEHART[33], our data exhibit comparably low incorporation rates. In order to make any predictions as to whether an open chain or a cyclic precursor is first aminated, an incorporation experiment using ^{13}C doubly-labeled quinic acid is necessary.

Experimental

General

Water- and air-sensitive reactions were carried out in a N_2- or Ar-atmosphere. All organic extracts were dried (Na_2SO_4) and evaporated below 40°C. TLC: Silica gel 60 F_{254} (Merck). Column chromatography (CC): silica gel ($60 \sim 200\ \mu$M, Chemische Fabrik Uetikon, Switzerland). MP: Kofler block; corrected. $[\alpha]_D$: Perkin-Elmer-141 polarimeter. IR spectra (cm^{-1}): Perkin-Elmer-781 IR spectrometer. NMR: Varian-EM-360 (^{1}H, 60 MHz), Varian-EM-390 (^{1}H, 90 MHz), Bruker WH-90-FT (^{1}H, 90 MHz; ^{13}C, 22.63 MHz) and Varian-VXR-400 spectrometer (^{1}H, 400 MHz; ^{13}C, 101 MHz, correlation of the signals was accomplished by BB and APT experiments); chemical shifts in ppm relative to internal TMS. Digital resolution for the coupling constants (J) was ± 0.2 Hz/point. MS: VG-70-250 spectrometer. Shikimic acid (**6**) and quinic acid (**5**) were purchased from Fluka Chemie AG, Buchs, Switzerland. [^{14}C(G)]Shikimic acid was purchased from Du Pont de Nemours International S. A., Biotechnology Division, Regensdorf, Switzerland and [1,2-$^{13}C_2$]malonic acid from Amersham International plc, Buckinghamshire, England.

Isolation of Product P8/1-OG (2,6-Dimethyl-3,5,7-trihydroxy-7-(3′-amino-5′-hydroxyphenyl)-2,4-heptadienoic Acid) (**8**) as Its Free Acid

A 300-ml shake flask with 60 ml of liquid complex medium 148[19] was inoculated with mycelium of *Nocardia mediterranei* P14 (60 mg total dry weight) and fermented for 4 days (250 rpm, 28°C). 10 ml of this culture were transferred into each of 3 × 500-ml flasks containing 100 ml industrial fermentation medium 151b[44] and fermented for another 10 days. At the end of this period of time, the mycelium was separated by centrifugation (20 minutes, 3,800 × *g*). The supernatant (300 ml) was passed over a Dowex column (500 g Dowex 2X8, 100~200 mesh, Cl^-) to absorb the acidic compounds and washed with 2 liters of deionized water to remove the neutral and alkaline compounds. The acidic compounds were then eluted (360 ml/hour) with 0.05 N HCl (1 liter) followed by 0.1 N HCl (3 liters). Fractions of 150 ml were collected and tested by TLC (20% MeOH - $CHCl_3$). Fractions containing product P8/1-OG (**8**) were combined and neutralized (pH 7.0) with 2 N NaOH and evaporated under reduced pressure to yield a beige precipitate which was stirred for 60 minutes with 500 ml of absolute ethanol. The insoluble NaCl-fraction was separated by filtration and the operation was repeated twice with 250 ml of abs ethanol. The yellow filtrates were combined, treated with 200 mg of charcoal to remove high molecular weight impurities, dried with molecular sieves (3 A) and, after filtration, evaporated to dryness under reduced pressure to yield 480 mg of a beige precipitate which was further purified by CC(20% MeOH - $CHCl_3$). A light yellow oil was obtained which upon addition of a few drops of methanol and ethyl acetate, precipitated out. After drying *in vacuo*, 110 mg of chromatographically pure product P8/1-OG (free acid) (**8**) was obtained as a light beige amorphous powder. IR (KBr) cm^{-1} 3370, (OH, NH), 2980, 2930, 2660 (OH of carboxylic acid), 1675 (α,β-unsaturated ketone), 1600 (aromatic and vinylic C=C), 1465, 1420, 1380 (CH_3), 1340, 1310, 1255, 1175 (C–O), 1140, 1085, 1040, 1000, 985, 960, 935, 845, 760, 727, 700. ^{1}H NMR (400 MHz, CD_3OD) δ 8.12 (<1H, br s, NH), 8.03 (<1H, br s, NH), 6.25 (1H, t, J=1.6 Hz, 2′-H), 6.18 (1H, t, J=1.8 Hz, 6′-H), 6.15 (1H, t, J=2.1 Hz, 4-H), 6.06 (1H, s, 4′-H), 4.54 (1H, d, J=9.0 Hz, 7-H), 2.79 (1H, qdd, J=1.8 Hz, J=8.9 Hz, J=7.0 Hz, 6-H), 1.87 (3H, s, 9-CH_3), 1.00 (3H, d, J=7.1 Hz, 8-CH_3). ^{13}C NMR (101 MHz, CD_3OD) δ 169.3 (C-3), 168.2 (C-1), 166.2 (C-5), 159.3 (C-5′), 149.8 (C-3′), 145.9 (C-1′), 107.2 (C-2′), 105.3 (C-6′), 103.3 (C-4′), 102.3 (C-4), 99.2 (C-2), 77.3 (C-7), 47.3 (C-6), 15.8 (C-8), 8.3 (C-9). FAB-MS *m/z* 476 (M+2Gly+H), 384 (M+Gly+H), 292 (M+H from lactone), 154 ($C_8H_9O_3$+H), 139 ($C_7H_8NO_2$+H).

Isolation of 2-Amino-benzamide (**14**)

The same culture medium, from which P8/1-OG (**8**) was isolated, afforded after CC with MeOH - $CHCl_3$, an additional nonpolar, strongly fluorescent material. Further CC(40% EtOAc - hexane) yielded a colorless oil, that after addition of a few drops of hexane, followed by evaporation under reduced pressure yielded a colorless precipitate (5 mg). MP: (85~89.5°C, changes of the crystal form), 109.5~111.5°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (1H, dd, J=7.9 Hz, J=1.5 Hz, 2-H), 7.23 (1H, td, J=7.8 Hz, J=1.4 Hz, 3-H), 6.69 (1H, dd, J=8.4 Hz, J=1.1 Hz, 5-H), 6.65 (1H, td, J=7.6 Hz, J=1.1 Hz, 4-H), 5.69 (1H, br s, NH). EI-MS (70 eV) *m/z* 136 (M, $C_7H_8N_2O$), 119 (M−NH_3), 92 (M−$CONH_2$), 65, 59, 43.

Incorporation Experiment with Racemic [1,2-$^{13}C_2$]Glycerol (**13**)

A 300-ml shake flask with 60 ml of liquid complex medium 148[19] was inoculated with mycelium of *Nocardia mediterranei* P14 (60 mg total dry weight) and fermented for 5 days (250 rpm, 28°C). 6 ml of this culture were transferred into each of 3 × 500 ml shake flasks with 50 ml industrial fermentaion medium 151b-modified[45] and fermented. After one day, 310 mg of racemic [1,2-$^{13}C_2$]glycerol (**13**) was added *via* "pulse-feeding" in 5 portions every 10~14 hours. After a further 10 days, the mycelium was separated by centrifugation (20 minutes, 3,800 × *g*) and purified as described above to yield 4.9 mg of P8/1-OG (**8**). ^{13}C NMR (101 MHz, CD_3OD) δ 168.7 (C-1), 168.7, 168.5 168.2 (C-3), 166.1 (C-5), 159.6, 159.3, 159.0 (C-5′), 150.0 (C-3′), 146.1, 145.9, 145.6 (C-1′), 107.5, 107.2, 106.8 (C-2′), 105.5, 105.2, 104.8 (C-6′), 103.5, 103.2, 102.8 (C-4′), 103.1, 102.8, 102.5 (C-4), 99.0 (C-2), 77.5, 77.3, 77.0 (C-7), 47.3 (C-6), 15.8 (C-8), 8.4 (C-9).

Incorporation Experiment with [^{14}C(G)]Quinic Acid (**5**)

A 300-ml shake flask with 60 ml of liquid complex medium 148[19] was inoculated with mycelium of *Nocardia mediterranei* P14 (60 mg total dry weight) and fermented for 4 days (250 rpm, 28°C). 5 ml of

this culture were transferred into a 500-ml shake flask with 50 ml industrial fermentation medium 151b[44] and fermented. After 3 days, 8.86 μCi [^{14}C(G)]quinic acid (**5**)[43] were added in one portion *via* a sterile filter and the bacteria were fermented for a further 8 days. At the end of this time period, the bacteria were harvested and the product P8/1-OG (**8**) (30.2 mg) and 2-amino-benzamide (**14**) (6.6 mg) were isolated.

Liquid Complex Medium 148[19]

22 g glucose, 5 g Lab-Lemco Beef-extract (Oxoid), 5 g Peptone C (Cudahy-Laboratories, Omaha, U.S.A.), 5 g Beef yeast extract (Vitaminhefe AG, Rheinfelden, Switzerland), 3 g Bacto-casitone (Difco), 1.5 g NaCl and 0.01% amino acids[c] *ad* 1,000 ml with deionized water. Sterilization: pH before sterilization was adjusted to 7.0 with 2 N NaOH. pH 6.5 after sterilization for 30 minutes at 120°C.

Industrial Fermentation Medium 151b[44]

70 g glucose, 20 g glycerol, 30 g protanimal, 10 g soybean meal, 8 g $CaCO_3$, 3 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 10 ml trace-element solution[d] and 0.01% amino acids[c] *ad* 1,000 ml with deionized water. Sterilization: pH 7.1~7.3 after sterilization for 20 minutes at 120°C.

Industrial Fermentation Medium 151b-modified[45]

As described above for the industrial fermentation medium151b, but in this case 35 g glucose and no glycerol were used. c) Amino acids: 0.2 g L-tyrosine *ad* 60 ml deionized water, 0.2 g L-tryptophan *ad* 20 ml with deionized water and 0.2 g L-phenylalanine *ad* 20 ml with deionized water were sterilized separately, combined and under sterile conditions added to the fermentation medium. d) Trace-element solution: 1 g $FeSO_4 \cdot 7H_2O$, 0.33 g $CuSO_4 \cdot 5H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $MnSO_4 \cdot 4H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$ and 0.1 g $(NH_4)_6Mo_7O_2 \cdot 4H_2O$ *ad* 1,000 ml with deionized water. The trace-element solution was added to the sterile fermentation medium using a sterile filter.

Synthesis of Racemic [1,2-$^{13}C_2$]Glycerol (**13**)

Diethyl[1,2-$^{13}C_2$]malonate (**11**)

To a solution of 0.50 g (4.80 mmol) [1,2-$^{13}C_2$]malonic acid (**10**) in 10 ml abs EtOH, under argon, 0.5 ml (6.87 mmol) thionylchloride was added dropwise. The pale yellow colored solution was left to stir at room temperature for a period of 5 hours. The mixture was then diluted with ether (3 × 50 ml), washed with water (3 × 40 ml), and satd $NaHCO_3$ soln (2 × 40 ml) dried and evaporated to afford **11** as a pale yellow oil in quantitative yield. ^{1}H NMR (60 MHz, $CDCl_3$) δ 4.2 (4H, q, J=6.0 Hz, $CH_2(COOCH_2CH_3)_2$), 3.3 (2H, s, $CH_2(COO-CH_2CH_3)_2$), 1.2 (6H, t, J=6.0 Hz, $CH_2(COOCH_2CH_3)_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.6 (d, J=59.4 Hz, $^{13}CH_2{}^{13}COOCH_2CH_3$ and s, $CH_2{}^{13}COOCH_2CH_3$), 61.5 (s, $CH_2(COOCH_2-CH_3)$), 41.7 (d, J=59.3 Hz, $^{13}CH_2{}^{13}COOCH_2CH_3$ and s, $^{13}CH_2(COOCH_2CH_3)_2$), 14.1 (s, $CH_2COOCH_2CH_3$). CI-MS (NH_3) m/z 180 ($(M+NH_4)^+$), 163 ($(M+H)^+$).

Racemic Diethyl 2-Acetoxy-[1,2-$^{13}C_2$]malonate (**12**)

To a solution of **11** (762 mg, 4.80 mmol) in 15 ml abs benzene, 3.4 g $Pb(OAc)_4$ (*ca.* 1.5% AcOH; 6.52 mmol) were added under a steady stream of argon. After refluxing for 3 hours 15 minutes the mixture was diluted with 40 ml of ether, washed with 1.0 N HCl soln (4 × 40 ml), satd Na_2CO_3 soln (3 × 40 ml), with water until the pH was neutral (4 × 40 ml) and with brine (2 × 40 ml), dried and evaporated under reduced pressure to yield 949 mg (91%) of **12** as a pale yellow oil. ^{1}H NMR (60 MHz, $CDCl_3$) δ 5.45 (1H, s, $AcOCH(COOCH_2CH_3)_2$), 4.25 (4H, q, J=7.0 Hz, $AcOCH(COOCH_2CH_3)_2$), 2.15 (3H, s, $-OCOCH_3$), 1.30 (6H, t, J=7.0 Hz, $AcOCH(COOCH_2CH_3)_2$. ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.5 (d, J=65.4 Hz, $AcO^{13}CH^{13}COOCH_2CH_3$ and s, $AcOCH^{13}COOCH_2CH_3$ and s, $-OCOCH_3$), 71.9 (d, J=65.3 Hz, $AcO^{13}CH^{13}COOCH_2CH_3$ and s, $AcO^{13}CHCOOCH_2CH_3$), 62.6 (s, $-COOCH_2CH_3$), 20.4 (s, $-OCOCH_3$), 14.0 (s, $-COOCH_2CH_3$). CI-MS (NH_3) m/z 238 ($(M+NH_4)^+$), 221 ($(M+H)^+$), 150, 85, 83.

Racemic [1,2-$^{13}C_2$]Glycerol (**13**)

Reduction of 949 mg (4.35 mmol) of **12** in 16 ml abs ether with 610 mg (16.10 mmol) of $LiAlH_4$ was carried out according to GIDEZ and KARNOVSKY[38] with some modications in the work-up. The excess of $LiAlH_4$ decomposed at 0°C through the careful addition of 2.5 ml of 2 N HCl followed by 3.0 ml of cold

concd HCl instead of water. The ether was then removed on a steam bath and the aq phase transferred to a Kutscher-Steudel apparatus and continuously extracted with 250 ml ether over a period of 2.5 weeks to deliver, after evaporation, 322 mg (80%) of **13**. ^{1}H NMR (60 MHz, D_2O) δ 3.90~3.45 (3H, m CH_2OD and CHOD). ^{13}C NMR (101 MHz, D_2O; internal std CH_3CN=1.30 ppm) δ 72.5 (d, J=41.1 Hz, $^{13}CH_2OH^{13}CHOH$ and s, $CH_2OH^{13}CH$-OH), 62.9 (d, J=41.2 Hz, $^{13}CH_2OH^{13}CHOH$ and s, $^{13}CH_2OHCHOH$). CI-MS (NH_3) m/z 112 ($(M+NH_4)^+$), 95 ($(M+H)^+$).

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