URDAMYCINS, NEW ANGUCYCLINE ANTIBIOTICS FROM *STREPTOMYCES FRADIAE*

IV. BIOSYNTHETIC STUDIES OF URDAMYCINS A~D

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The biogenetic origin of the angucycline antibiotics urdamycins A~D was studied by feeding experiments with isotope labeled precursors and by NMR analysis. Feeding experiments with [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate show that the chromophores of urdamycins A and B and the angucycline 4-ring skeleton of the urdamycins C and D chromophores are formed from a single decapolyketide chain. The chromophores of the urdamycins C and D contain additional structural elements which derived from the amino acids tyrosine and tryptophan, respectively. The latter was shown by feeding deuterium-labeled tyrosine and ^{13}C-labeled tryptophan derivatives. Feeding of [1-^{13}C]glucose and of [*U*-$^{13}C_3$]glycerol proved that the *C*-glycosidic moiety and the three sugars (2×L-rhodinose, 1×D-olivose each) of the urdamycins arise from glucose. Experiments with ^{14}C-labeled urdamycin A, obtained by biosynthesis from [^{14}C]acetate, showed this compound to be a late precursor of the urdamycins C and D.

Angucycline antibiotics[1,2] are a growing group of biologically active compounds with interesting activities, *e.g.*, bacteriostatic, enzyme inhibitory and antitumor activity[3~5]. The urdamycins, produced by *Streptomyces fradiae* (strain Tü 2717), were the first members of this group of antibiotics found to contain enlarged aglycones. Exemplifying this aglycone structure are urdamycins C (**3**) and D (**4**). The urdamycins are also the first members of the angucyclines with (A (**1**), C (**3**), D (**4**), E and F) and without (B (**2**)) directly adjacent angularic hydroxy groups. While many groups of glycoside antibiotics differ in the amount and kind of their sugar units, the urdamycins differ only with respect to their aglycones. The biosynthetic origin of these unusual variations in aglycone structure was of interest to us, as was the structure elucidation of urdamycins C (**3**) and D (**4**). The complete structure elucidation was facilitated by compounds obtained from biosynthetic studies[6]. In this paper we report the results of our experiments on the biosynthesis of the urdamycins A~D (**1~4**). The complete structure details of urdamycins C (**3**) and D (**4**) were the subject of a previous paper[6].

Materials and Methods

[1-^{13}C]- and [1,2-$^{13}C_2$]acetate, the [1-^{13}C]glucose and [3,5-$^{2}H_2$]tyrosine (99 atom % ^{2}H) were obtained from Cambridge Isotope Laboratories (Cambridge, MA, U.S.A.). The [*U*-$^{13}C_3$]glycerol was synthesized in our laboratories from K^{13}CN and [1,2-$^{13}C_2$]acetic acid (99% ^{13}C, supplied by Los Alamos

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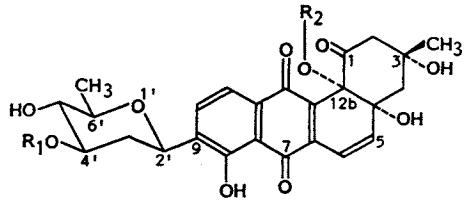

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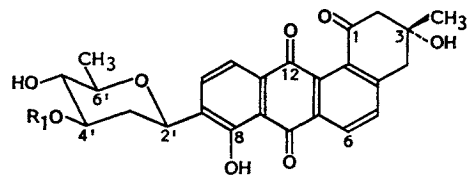

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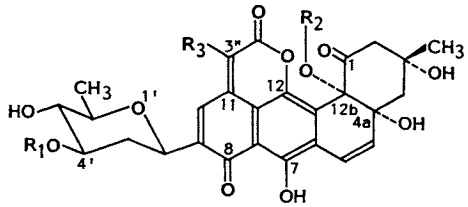

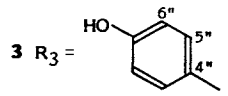

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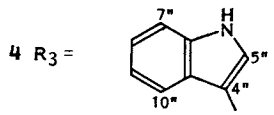

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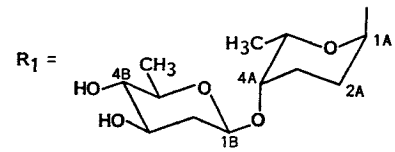

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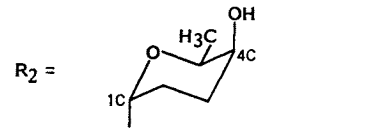


R_2 =

Stable Isotope Resource) *via* diethyl malonate and diethyl 2-acetoxymalonate. Sodium [1-^{14}C]acetate, 96 mCi/mmol, was obtained from New England Nuclear.

NMR and Mass Spectroscopy

^{13}C NMR spectra were recorded on a Bruker WM-300 spectrometer operating at a filed strength of 7.1 Tesla. The spectra of urdamycins A, C, and D were recorded in acetone-d_6; those of urdamycin B were recorded in DMSO-d_6. The chemical shifts (δ in ppm) are referenced relative to TMS. Signal assignments are based on chemical shift theory and comparisons among the urdamycin series, and on a variety of 1D and 2D heteronuclear and homonuclear correlations.

Fast atom bombardment (FAB)-MS were obtained on a Finnigan MAT 8230 instrument using triethanolamine as the matrix.

Fermentation and Isolation of Urdamycins

S. fradiae (Tü 2717) was cultivated in 500 ml triply-baffled Erlenmeyer flasks, each containing 100 ml of a soybean meal - glucose - Na_2HPO_4 (2%, 2%, 1%) medium, pH adjusted to 7.2, with rotary shaking (New Brunswick G-25 incubators) at 300 rpm and 28°C. The cultures were inoculated directly from slants. After incubating for 96 hours, the entire cultures were extracted with a mixture of ethyl acetate - acetone (1 : 1, 1 liter/liter culture) and filtered through Celite 545 (Fisher). Thecrude mixture of antibiotics was obtained by flash chromatography on Silica gel 60 (Merck), using first CH_2Cl_2, then CH_2Cl_2 - MeOH (9 : 1) as eluent. The four main compounds urdamycins A, B, C and D could be separated by reversed phase HPLC (Altech C-18/10 μm column, flow rate 3 ml/minute, H_2O - CH_3CN - MeOH, 50 : 25 : 25). The antibiotics were finally purified on Sephadex LH-20 (column 100×2.5 cm, MeOH). For feeding experiments with isotopically-labeled materials, fermentations were conducted as above and precursors were added as aqueous solutions sterilized by filtration.

Feeding Experiments with Labeled Precursors

Feeding of [1-^{13}C]Acetate and [1,2-$^{13}C_2$]Acetate: Labeled substrate (2 g) was dissolved in 50 ml sterile water and neutralized with 0.1 M HCl. Five ml of this solution was added 30 hours after

inoculation to each of ten Erlenmeyer flasks containing 100 ml each of the growing culture of *S. fradiae* (Tü 2717).

Feeding of [1-^{14}C]Acetate to Obtain ^{14}C-Labeled Urdamycin A: [1-^{14}C]Acetate (60 μCi, specific activity 96 mCi/mmol) was dissolved together with 1 g nonlabeled carrier acetate (sterile water, neutralized as above) and was fed to ten 100-ml flasks of *S. fradiae* (Tü 2717) 24 hours after inoculation. Harvesting after 72 hours and workup as described above yielded 40 mg ^{14}C-labeled urdamycin A (specific activity 150 μCi/mmol).

Feeding of [1-^{13}C]Glucose: Because glucose is the major carbon source of the microorganism, it was necessary to separate the mycelium from the glucose-containing culture filtrate after 32 hours and proceed with replacement culture techniques. The cells were reincubated in a medium containing soybean meal (10 g/liter), glucose (5 g/liter, 250 mg of which was ^{13}C-labeled), and Na_2HPO_4 (5 g/liter).

Feeding of [*U*-$^{13}C_3$]Glycerol: The [*U*-$^{13}C_3$]glycerol was dissolved in 50 ml sterile water and fed 32 hours after inoculation to ten 100-ml flasks (1 g/liter).

Feeding of [3'-^{13}C]-2-Methyltryptophan and of [3'-^{13}C]-4-Methyltryptophan: The amino acids (80 mg each) were dissolved in DMSO - water (1 : 1) filter-sterilized, and added each to ten 100-ml cultures 72 hours after inoculation, when the cultures had become light orange (signalizing urdamycin A production).

Feeding of [3,5-$^2H_2$]Tyrosine: The tyrosine (250 mg) was dissolved in 0.1 N sterilized HCl and added to 72 hours-old cultures (ten 100-ml flasks) of *S. fradiae* (Tü 2717), which showed evidence of initiation of production of urdamycin A (orange color).

Feeding of ^{14}C-Labeled Urdamycin A: ^{14}C-Labeled urdamycin A (40 mg/liter, specific activity: 150 μCi/mmol) was administered as a DMSO - water (1 : 1) solution to a growing culture of *S. fradiae* (Tü 2717) in two portions, 20 mg at 60 hours after inoculation, and 20 mg at 72 hours after inoculation. Incorporation of radioactivity into urdamycins B, C, and D was assessed by HPLC, using a United Technology Packard Trace-2 radiochemical detector, with DuPont 963 scintillation cocktail (1.8 ml/minute mixing rate), continuously with the HPLC eluent.

Results

[1-^{13}C]- and [1,2-$^{13}C_2$]Acetate Feeding

The absolute incorporation of ^{13}C-labeled acetate was about 10% as calculated from the intensities of the ^{13}C-satellites in the ^{1}H NMR spectra of the compounds. The broad-band decoupled ^{13}C NMR spectra of samples from the [1-^{13}C]acetate feeding experiment show 9 carbon atoms to be enriched, as was predicted for the angucycline 4-ring system. The remaining ambiguity, the orientation of folding of the polyketide chain, was resolved by the [1,2-$^{13}C_2$]acetate feeding experiment. This experiment showed nine spin-coupled (AB-type) pairs of carbon atoms (C-13/C-3; C-4/C-4a; C-5/C-6; C-6a/C-7; C-7a/C-8; C-9/C-10; C-11/C-11a; C-12/C-12a; C-12b/C-1) and C-2 as an enriched but non-coupled singlet in the broad-band decoupled ^{13}C NMR spectrum. Carbon-13 connectivities were also confirmed by 2D ^{13}C homonuclear double quantum spectroscopy, using the 'INADEQUATE'[7] pulse sequence (*e.g.* Fig. 1). In the case of urdamycin B (**2**), which could not be obtained in large amounts, a 2D *J*-resolved ^{13}C NMR spectrum was used to distinguish among all of the carbon atoms in the congested aromatic region. Table 1 shows all connectivities and C-H coupling constants of the acetate-derived moiety of the urdamycins.

[1-^{13}C]Glucose Feeding

The broad-band decoupled ^{13}C NMR spectra of urdamycins A~D (**1~4**) show enrichments (*ca.* 2% relative to natural abundance=1.0) of the carbons 2', 1A, 1B, and 1C. Thus, all sugars including the *C*-glycosidic-bonded olivose are derived from glucose.

Fig. 1. 2D INADEQUATE plot of the [1,2-$^{13}C_2$]acetate labeled urdamycin C at 300 MHz in acetone-d_6.

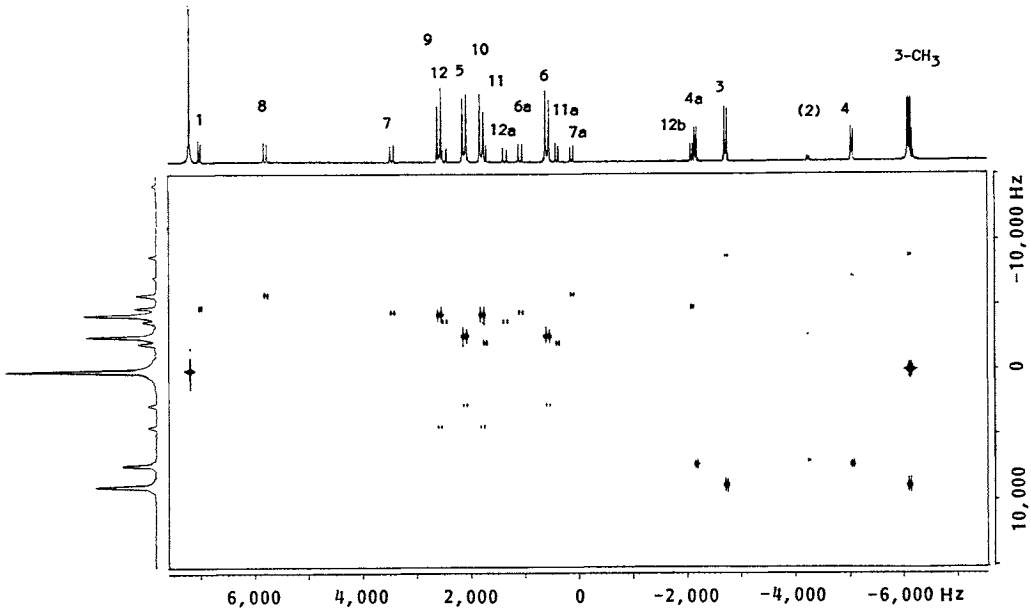


Table 1. C-C coupling constants (in Hz) and connectivities of the acetate-derived part of the urdamycins A~D from the broad-band decoupled ^{13}C NMR spectra of the [1,2-$^{13}C_2$]acetate labeled compounds at 300 MHz, δ in ppm.

C and coupling partner[a]	Urdamycin			
	A[b] (1)	B[c] (2)	C[b] (3)	D[b] (4)
1 →12b	202.6 (38)	196.7 (51)	203.8 (40)	203.9 (41)
2 s[d]	53.4 (—)	53.1 (—)	54.6 (—)	54.7 (—)
3 →13	75.7 (42)	71.5 (39)	74.9 (42)	74.8 (42)
4 →4a	43.2 (37)	43.4 (41)	44.0 (37)	44.1 (37)
4a →4	80.6 (37)	149.3 (41)	82.2 (37)	82.3 (37)
5 →6	145.6 (68)	134.1 (57)	138.8 (67)	138.3 (67)
6 →5	115.9 (68)	128.5 (57)	118.6 (67)	118.8 (67)
6a →7	130.7 (56)	132.7 (55)	125.2 (67)	123.9 (67)
7 →6a	188.4 (56)	187.3 (55)	156.6 (67)	156.7 (67)
7a →8	113.9 (65)	114.7 (64)	112.6 (56)	112.9 (56)
8 →7a	156.3 (65)	157.1 (64)	187.5 (56)	187.5 (56)
9 →10	140.3[†]	136.4 (60)	145.0 (65)	143.6[†]
10 →9	133.0 (64)	133.4 (60)	134.7[†]	136.1 (65)
11 →11a	118.9 (61)	118.4 (61)	133.9[†]	130.7 (52)
11a→11	136.2[†]	133.7 (61)	116.2 (51)	116.8 (52)
12 →12a	182.2 (54)	182.5 (54)	143.7 (75)	143.1[†]
12a→12	137.1[†]	135.4[†]	128.9 (75)	128.4 (76)
12b→1	81.1 (38)	135.5 (51)	83.1 (40)	83.3 (41)
13 →3	29.3[††]	29.6 (39)	29.4[††]	29.5[††]

[a] From 2D INADEQUATE-spectra.
[b] In acetone-d_6, [c] in DMSO-d_6. coupling constants also from the 2D *J*-resolved spectrum.
[d] Singlet, partner decarboxylated, [†] not detectable because of overlapping signals, [††] not detectable because of solvent overlapping.

[*U*-$^{13}C_3$]Glycerol Feeding

The broad-band decoupled ^{13}C NMR spectra show the same coupling pattern in the polyketide-derived part as obtained by labeling with [1,2-$^{13}C_2$]acetate, but incorporation of intact 3-carbon units from glycerol can be detected in the lower halves of the sugar units (C-5′/C-6′/C-7′; C-4A/C-5A/C-6A; C-4B/C-5B/C-6B; C-4C/C-5C/C-6C).

[3,5-$^2H_2$]Tyrosine Feeding

The ^2H NMR spectrum of urdamycin C labeled from this precursor shows a broad singlet at δ 7.1, indicating the presence of deuterium in the 6″/8″-positions. The ^1H NMR spectrum of the deuterated urdamycin C shows the 5″/9″ proton as a superimposed doublet (J=8 Hz) and singlet instead of the doublet (J=8 Hz) which is observable in the spectrum of nonlabeled urdamycin C. The singlet component results from the loss of *ortho*-coupling in the deuterated species. From the integral of the 6″/8″ ^1H signal, the enrichment of deuterium from [3,5-$^2H_2$]tyrosine in urdamycin C could be calculated as 15%.

[3′-^{13}C]Methyltryptophan Feedings

The sole product of the 2-methyltryptophan feeding was urdamycin D, enriched in C-3″-position (10-fold in comparison to natural abundance). That this was the only compound was proved by a single frequency proton decoupled carbon-13 spectrum, which, upon irradiation of the indole C-2 proton line, demonstrated collapse of the 4.3 Hz ^{13}C doublet to a singlet. Likewise, a FAB-MS clearly showed a molecular ion of one amu greater than reference urdamycin D, indicating the presence of one atom of ^{13}C. In the case of the sample from the 4-methyltryptophan feeding, analysis indicated the presence of two closely-spaced, enriched quaternary carbon signals whose chemical shifts (δ 129.7 and 130.1) were similar to, but not identical with that for C-3″ of urdamycin D (δ 127.9). FAB-MS analysis of the samples indicated that the one from the 2-methyltryptophan feeding was pure urdamycin D, whereas that from the 4-methyltryptophan experiment contained a mixture of a 10″-methylurdamycin D and a related compound presumed to be the 10″-hydroxymethylurdamycin D (the negative FAB-MS shows m/z 1,031 besides m/z 1,015, reference urdamycin D: m/z 1,001). The results thus suggest that 2-methyltryptophan was incorporated with concomitant (oxidative?) removal of the methyl group, whereas in the incorporation of 4-methyltryptophan the methyl group was retained either intact or as a hydroxymethyl group.

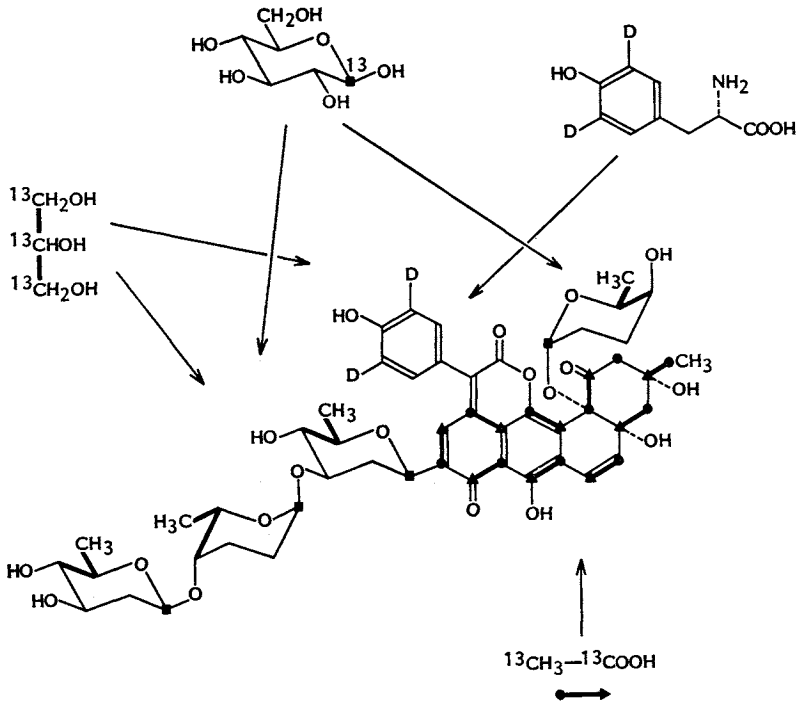
Incorporation Experiments with Radiolabeled Urdamycin A

Detection by HPLC of radioactivity from urdamycin A appearing in other urdamycins clearly demonstrated that there was no conversion of urdamycin A into urdamycin B. Urdamycins C and D were labeled, however; 14% specific incorporation was found into urdamycin C, and 11% into urdamycin D.

Discussion

The biosynthesis of urdamycins A (**1**) and B (**2**) follows pathways common to many glycoside antibiotics, *e.g.*, anthracycline formation. The labeling pattern from acetate, which forms the angucycline 4-ring-system *via* a single decaketide chain, follows the route which ŌMURA *et al.* described for the angucycline antibiotic vineomycin A_1[8]. Probably all of the angucyclines are assembled in this manner. The urdamycins C (**3**) and D (**4**) belong to the rare group of secondary metabolites, which are biosynthesized from building blocks from all three major biogenetic sources, *i.e.* sugars, fatty

Fig. 2. The biogenesis of urdamycin C, a conclusion of all feeding experiments.



acids and amino acids[9]. Fig. 2 summarizes the results of all feeding experiments on urdamycin C. The enzyme system which connects the amino acid part to the polyketide-derived chromophore is unusual and will be the subject of further investigations to establish its mechanism and its potential for utilization of amino acids or related compounds other than tyrosine or tryptophan. Also, the ability of the organism to carry out biotransformations with other hydroxyquinones might be a very interesting topic for further study.

The incorporation of radioactivity from urdamycin A into urdamycins C and D indicates that urdamycin A is the biogenetic precursor of both antibiotics. The position of urdamycin B in the biosynthetic relationships of the urdamycins is unclear at this time.

The fact that carbons 1, 2 and 3, of the deoxyhexoses are less highly enriched by [*U*-$^{13}C_3$]glycerol than carbons 4, 5, and 6 indicates that the "top" and the "bottom" halves of these sugars derive from different nonequilibrating triosephosphate pools during gluconeogenesis. This surprising phenomenon has already been observed during our biosynthetic studies of the α-glucosidase inhibitor acarbose[10] and has been discussed there.

The observation that [3′-^{13}C]-2-methyltryptophan is demethylated to tryptophan, probably by oxidation and decarboxylation either before or after its conversion into urdamycin D is unexpected and intriguing. Considering the loss of a methyl group from tryptophan is a biochemically implausible (although not impossible) step, we wondered whether the NMR spectra could result from a mixture of, *e.g.*, 90% unlabeled urdamycin D, giving rise to the natural abundance signals, and 10% of the (about 100% enriched) methyl analog, giving rise to the 10-fold more enriched signal for C-3″ (assuming that the chemical shift of C-3″ in the methyl analog is not significantly different from that in urdamycin D itself). Thus the full proton coupled ^{13}C NMR spectrum was irradiated with the single proton frequency of the indole C-2 proton line (5″-H) leading to the collapse of the 4.3 Hz $^3J_{C\text{-}H}$ coupling of the 10-fold enriched C-3″-signal. This could not have happened if the 10-fold enrichend signal was caused by the still methylated by-product. Thus a complete demethylation is indeed the only possible interpretation of this NMR experiment.

In the case of [3′-^{13}C]-4-methyltryptophan, the results are less clear, but indicate that the precursor may be incorporated into compounds related to urdamycin D without demethylation. Since the enrichment in the two urdamycin D relatives was approximately equal, it appears that the 4-methylindole unit of the precursor is incorporated intact, and that in approximately half of the molecules an oxidative biotransformation occurs at the C-10″ methyl group. While it is possible that the precursor could be oxidized at the methyl group prior to incorporation, it is unlikely that in such a case the incorporations of label into the C-10″ methyl and hydroxymethyl groups would be equal. The results indicate that the origin of the indole-δ-lactone moiety of urdamycin D (**4**) is tryptophan, and suggest the possibility that tryptophan derivatives may be fed to obtain additional urdamycin derivatives by precursor-directed biosynthesis. It is possible that the same enzyme is responsible for oxidation of the tryptophan methyl groups at C-2 and C-4. Substrate specifity constraints may favor the complete oxidation of the C-2 methyl, which is followed by decarboxylation, whereas the oxidation of the C-4 methyl group stops at the carbinol stage.

Acknowledgments

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