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Carbon-13 Nuclear Magnetic Resonance Study of the Biosynthesis of Daunomycin and Islandicin

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The biosynthesis of daunomycin (**1a**) and islandicin (**2a**) has been studied, utilizing both sodium [1-^{13}C]- and [2-^{13}C]acetate and sodium [1,2-^{13}C]acetate. The results establish the biosynthetic scheme for both compounds and indicate a pathway more complicated than a simple acetate connectivity pattern in the biosynthesis of daunomycin. The assignment of the ^{13}C resonance frequencies in the two compounds was based partially on a study of the ^{13}C frequencies in a series of diacetoxyanthraquinones.

Introduction

A ^{13}C nuclear magnetic resonance study using $^{13}CH_3{}^{13}CO_2Na$ was used to establish the connectivity pattern in the biosynthesis of the polyketide antibiotic daunomycin (**1a**) and the related compound islandicin (**2a**). Both daunomycin (**1a**) and adriamycin (**1b**) demonstrate therapeutic activity in the treatment of leukemias, especially acute lymphocytic leukemia in children. Adriamycin is a particularly potent anticancer antibiotic showing, in addition, chemotherapeutic effectiveness against many solid tumors.[1] The fluorescent properties of daunomycin had made possible the observance of the molecule's penetration into the cell and its fixation in the nuclear structure. It has been shown to bind strongly to DNA and current thinking regards its primary effect as one of interfering with template DNA function.[1]

A number of plausible connectivity patterns for daunomycin are possible (Figure 1) via an acetate–polymalonate biosynthetic route (a or b). We have applied the Tanabe[2] technique to this biosynthetic problem and have obtained results that are consistent with only route a, a propionate "starter" and nine successive malonate condensations with loss of the terminal carboxyl.[3]

This result for daunomycin is strikingly different from that determined for the similar polyketide islandicin (**2a**) which was originally considered a biosynthetic prototype for daunomycin and adriamycin. Gatenbeck[4] had established using $CH_3{}^{14}CO_2Na$ that islandicin is biosynthesized from a polyketide precursor, with the labeling pattern indicated in Figure 2, but this pattern could arise from one of two possible foldings of the polyketide chain (Figure 2). In this case, we have demonstrated that islandicin is biosynthesized via configuration a of Figure 2.

Results and Discussion

We have previously described the method used by Tanabe[2] and ourselves[6] of employing [1,2-^{13}C]acetate of high isotopic purity, which enables one to trace acetate incorporation patterns on a two-carbon basis and detect skeletal rearrangements and cleavages without integration of the carbon spectrum. We have also described[6] how excellent carbon spectra of anthraquinone and naphthaquinone type molecules can be obtained when these compounds are acetylated to increase their solubility in $CDCl_3$ and render them compatible with the tris(acetylacetonato)chromium(III)($Cr(acac)_3$) T_1 suppressor technique.[7] The use of this technique assures that all peaks, including the easily saturated quarternary carbons, are visible when minimal pulse delays (2–3 s) are employed, and that all peaks are distinct and of comparable height, which is crucial to the studies using monolabeled acetate. For ^{13}C NMR analysis, islandicin was converted to its corresponding triacetate (**2b**) and daunomycin was hydrolyzed and acetylated with the formation of daunomycinone tetraacetate (Figure 3). The spectra of islandicin triacetate and daunomycinone tetraacetate are shown in Figures 4 and 5, respectively.

Culture and Isolation. Islandicin was first isolated in purified form by Howard and Raistrick[8] when they sought to determine the coloring matter in *Penicillium islandicum* Sopp. This fungus was first discovered and named by Sopp[9] in 1912 when he isolated the organism from a moldy specimen of Skyr, a soured milk peculiar to Iceland. Islandicin is just one of several hydroxyanthraquinones produced by the organism and isolated and identified by Howard and Raistrick.

Since our initial attempts to reproduce the culture conditions of Howard and Raistrick[8] gave variable yields of islandicin, we decided to employ a Czapek–Dox–agar medium for the organism. The use of these agar plates provided a small, reasonably uniform surface on which the mold could grow and to which the potential precursors could be added.

Instead of the multiple extraction and recrystallization steps utilized by the initial authors, our much simpler isolation procedure involved only chromatography of the crude extract and sublimation of the islandicin-containing fractions. The major contaminant in these fractions was iridoskyrin, an is-

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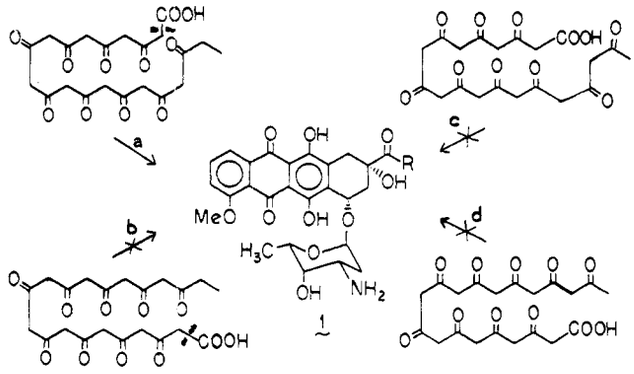


Figure 1. Plausible acetate connectivity patterns for the biosynthesis of daunomycin: **1a**, R = CH_3 (daunomycin); **1b**, R = CH_2OH (adriamycin).

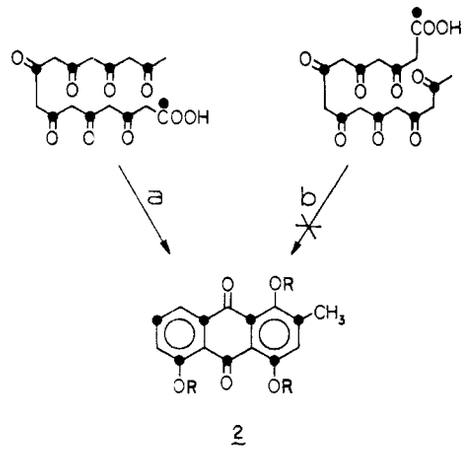


Figure 2. Acetate connectivity patterns for the biosynthesis of islandicin (**2a**, R = H). Islandicin triacetate (**2b**, R = $COCH_3$) was used for NMR analysis. Both indicated foldings of the polyketide chain would result in the labeling pattern observed by Gatenbeck using $CH_3{}^{14}CO_2Na$ (indicated with ●).

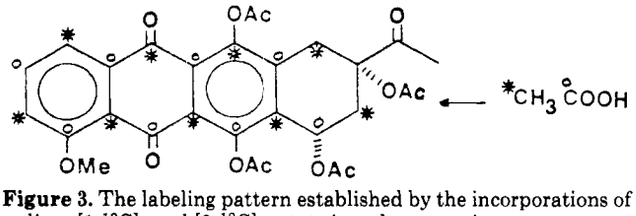


Figure 3. The labeling pattern established by the incorporations of sodium [1-^{13}C]- and [2-^{13}C]acetate into daunomycin.

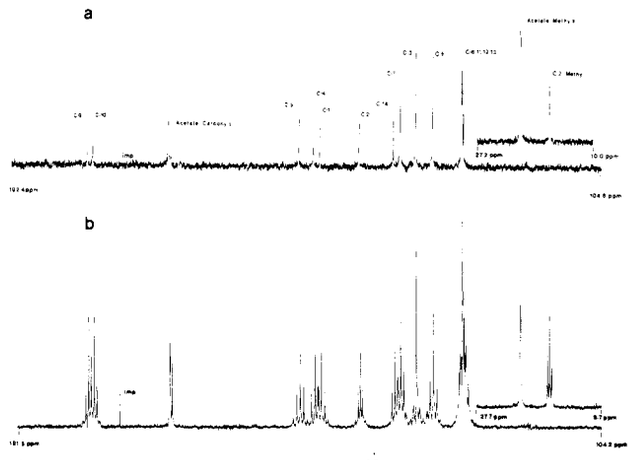


Figure 4. Proton noise-decoupled FT ^{13}C NMR spectrum of islandicin triacetate (20K transients, 3.0 s pulse delay): (a) from ^{13}C natural abundance (73 mg + 15 mg $Cr(acac)_3$); (b) from $^{13}CH_3{}^{13}CO_2Na$ enrichment (152 mg + 40 mg $Cr(acac)_3$).

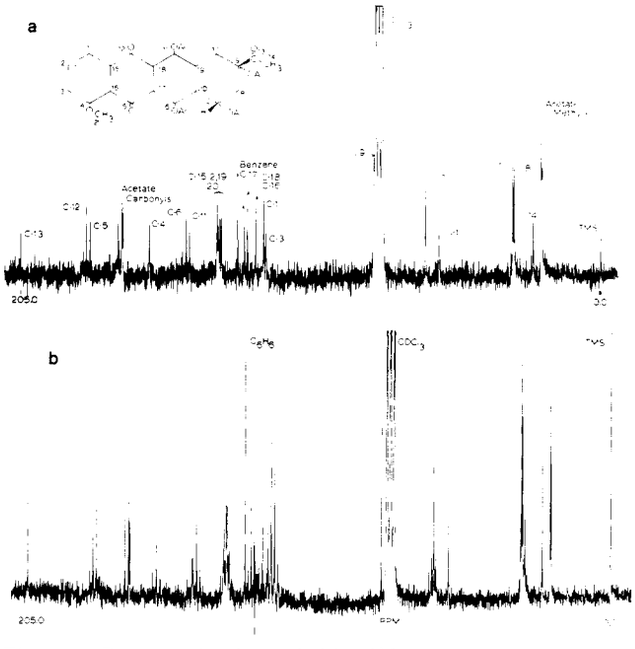


Figure 5. Proton noise-decoupled FT ^{13}C NMR spectrum of daunomycinone tetraacetate (11K transients, 1.27 s pulse delay): (a) from ^{13}C natural abundance (135 mg + 45 mg of $Cr(acac)_3$); (b) from $^{13}CH_3{}^{13}CO_2Na$ enrichment (30 mg + 6 mg of $Cr(acac)_3$).

landicin dimer, which could be recovered unchanged from the sublimation vessel. The yield of this material (<5% of the amount of islandicin isolated) was too low to warrant ^{13}C NMR analysis.

Daunomycin was first reported by Grien et al.[10] when they isolated this metabolite from the bacterium *Streptomyces peucetius*. Elucidation of the daunomycin structure was facilitated by its resemblance to several anthracyclines which have also demonstrated antibiotic activity.

Yields of daunomycin from submerged fermentations of *S. peucetius* have been reported[10] as high as 70 μg/mL, but this figure was based solely on spectral data. Actual isolation of the hydrochloride salt produced yields in the vicinity of 15 μg/mL, and this method of precipitating the material is not conducive to the small-scale fermentation necessary for ^{13}C incorporation studies.

Consequently, experimentation was directed toward elucidating the biosynthesis of daunomycinone (Figure 3), the aglycone portion of the molecule, which is much more adaptable to isolation and purification (chromatography) techniques than the glycoside itself. Both the aglycone and glycoside have been found in the mycelial extracts, and it was felt that acidic hydrolysis of the crude extract would liberate the aglycone portion of daunomycin while leaving the daunomycinone already formed unchanged. This procedure proved successful and yields of 25–30 μg/mL of daunomycinone were obtained.

The preparation of the tetraacetate has already been described,[11b] but in our hands the reaction also produced small amounts of the triacetate which had to be separated chromatographically. The triacetate could be recycled to produce more of the tetraacetate, but the reaction never effected complete conversion. Longer reaction times resulted in the formation of increasing amounts of low R_f material and a darkening of the reaction solution, so the reaction times were shortened and the recycling process used. This method rou-

Table I. Chemical-Shift Assignments for Carbons of Anthraquinone and Diacetoxyanthraquinones

Registry no.	Compd	Carbon no.	Obsd chemical shift[a]	Predicted/calcd chemical shift[b]
84-65-1	Anthraquinone	1, 4, 5, 8	127.3	126.4[c]
		2, 3, 6, 7	134.1	133.9[d]
		9, 10	183.1	184.9[e]
		11, 12, 13, 14	133.7	131.9[e]
2289-36-3	1,4-Diacetoxyanthraquinone	1, 4	148.3	148.0
		2, 3	131.0	129.0
		5, 8	127.0	127.3
		6, 7	134.2	134.1
		9, 10	181.5	183.1[g]
		11, 12	126.2	126.8[h]
		13, 14	133.4	133.7[h]
1747-93-9	1,5-Diacetoxyanthraquinone[f]	1, 5	150.1	150.3
		2, 6	129.7	127.7[i]
		3, 7	135.1	135.4
		4, 8	125.8	125.0
		9, 10	181.1	183.1[g]
		11, 13	124.4	127.3[h,i]
1963-82-2	1,8-Diacetoxyanthraquinone[f]	1, 8	150.1	150.3
		2, 7	130.3	127.7[h]
		3, 6	134.7	135.4
		4, 8	125.5	125.0
		9	181.0	181.1–181.5[j]
		10	182.0	183.1
		11, 14	125.7	127.3[h,i]
		12, 13	134.5	135.0[h]
1629-51-2	1,2-Diacetoxyanthraquinone	1	142.2	143.9
		2	148.5	150.7
		3	128.8	129.0
		4	126.6	126.3
		5, 8	127.3, 127.1	127.3
		6, 7	134.4, 134.2	134.1
		9	181.5	181.5[k]
		10	181.7	182.0[k]
		11	126.5	128.6[h,i]
		12, 13, 14	134.3, 132.7, 132.4	132.7–133.7[h]
		Acetate carbonyls	168.4, 167.7	
		Acetate methyls	20.8, 20.7	

[a] Chemical shifts were measured in $CDCl_3$ in ppm relative to internal Me_4Si. [b] The chemical shifts observed in anthraquinone formed the basis for the predicted/calculated chemical shifts of the diacetoxyanthraquinones, which were obtained by adding the substituent effect parameters for –OAc[12] to the basic anthraquinone shifts. 1,4-Naphthoquinone provided the model for the predicted shifts of 9,10-anthraquinone. [c] Broad doublet in undecoupled spectrum (J_{CH} = 163.7 Hz) with further long-range coupling apparent. [d] Doublet of doublets in undecoupled spectrum (J_{CH} = 162.8 Hz, J_{CCH} = 8.3 Hz). [e] Broad singlet in undecoupled spectrum. [f] The chemical shift of the acetate carbons are not separately recorded because they are consistent and characteristic; carbonyl carbon shift 169.3–169.5 ppm and methyl carbon shift 21.2 ppm. [g] Some upfield shift relative to anthraquinone expected due to the peri acetoxy group. [h] Sharp singlet in off-resonance experiments. For other carbons, signal appears as a broad singlet or multiplet. [i] The discrepancy between observed and calculated shifts was greatest for carbons ortho to an acetoxy substituent. [j] Predicted shift based on 1,4- and 1,5-diacetoxyanthraquinone. [k] Predicted shift of C-9 based on 1,4- model and of C-10 on 1,8- model; however, these two assignments could be interchanged.

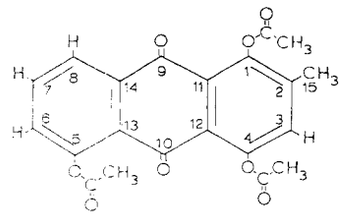
tinely gave yields of 60–70% of tetraacetate suitable for ^{13}C NMR analysis.

Chemical-Shift Assignment. Accurate chemical-shift assignment of each carbon of islandicin and daunomycin was essential in establishing which pairs of carbons originate from the same molecule of acetate, a prerequisite to determining the biosynthetic pathway. Because the literature had very few examples of model compounds for anthraquinone-type molecules, we undertook the determination of the ^{13}C chemical shifts of a series of model diacetoxy 1,4-anthraquinones (Table I), relating the proton and carbon chemical shifts to facilitate single-frequency decoupling experiments in daunomycin.

Diacetoxyanthraquinones. The basic method for assigning these chemical shifts was to compare a predicted/calculated chemical shift with the observed shift; the former was determined by adding parameters for ^{13}C substituent effects[12,13] to the chemical shifts observed for anthraquinone itself (Table I). Since these substituent effect parameters were determined for benzene, some systematic deviations from the calculated shifts were observed, in particular, the shifts of carbons ortho to an acetoxy group. For these ortho carbons, observed and calculated shifts agreed only to within 3 ppm, while for the other carbons agreement was almost always within 1 ppm. In addition to calculated shifts, other standard methods, mainly off-resonance decoupling, were useful in making the assignments detailed in Table I.

Islandicin Triacetate (2b). No single diacetoxyanthraquinone proved to be a "best model" for predicting the chemical shifts in islandicin triacetate (which, in turn, became the model for many of the carbons in daunomycinone tetraacetate). As might be expected, some carbons were best predicted from one model and some from another. Therefore, Table II gives a range of calculated chemical shifts for each carbon. The range represents the spread of values based on calculations using four model compounds; anthraquinone itself and 1,4-, 1,5-, and 1,8-diacetoxyanthraquinone. Obviously, the observed chemical shift could be even more closely matched by choosing a different "best model" for each indi-

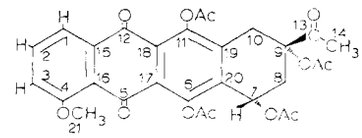
Table II. Chemical Shift Assignments for the Carbons of Islandicin Triacetate (tabulated by the numbering system shown below)



Carbon no.	Obsd chemical shift[a]	Predicted/calcd chemical shift[b]
1	146.4	148.5–149.0[c]
2	140.6	137.9–140.5
3	132.2	129.0–131.7
4	147.4	148.0–148.8[d]
5	149.6	150.0–150.3[d]
6	129.7	127.7–130.3
7	134.6	134.7–135.5[e]
8	125.3	124.6–125.8[f]
9	181.2	181.1–181.5[g]
10	180.4	181.0–181.1[g]
11	125.4	126.1–128.6[f,h]
12	125.1	123.3–126.5[f,h,i]
13	125.3	124.3–127.3[f,h,i]
14	135.5	134.5–136.0[e]
Acetate carbonyls		168.8–169.1
Acetate methyls		20.9–21.0

[a] Chemial shifts were measured in $CDCl_3$ in ppm relative to internal Me_4Si. [b] The chemical shifts observed in four model compounds, anthraquinone itself and 1,4-, 1,5-, and 1,8-diacetoxyanthraquinone, formed the basis for the range of predicted/calculated chemical shifts. As appropriate, substituent effect parameters[12,13] were added to the basic model compound shifts to obtain the predicted chemical shifts. [c] This carbon is distinguished from C-4 and C-5 on the basis of its derivation from the methyl carbon of acetate, while C-4 and C-5 are derived from the carbonyl carbon. [d] Calculated chemical shifts based on any one of the model compounds predict that C-4 will be at higher field than C-5. [e] C-7 distinguished from C-14 by off-resonance experiments where C-14 appears as a sharp singlet. [f] C-11, C-12 and C-13 distinguished from C-8 by off-resonance experiments where all except C-8 appear as a singlet. [g] Predicted chemical shift of C-9 is based on 1,4- and 1,5-diacetoxyanthraquinone, and of C-10 on 1,5- and 1,8-diaceoxyanthraquinone. These carbons distinguished primarily on the basis of singly labeled acetate studies which show C-9 as derived from the acetate methyl carbon and C-10 from the acetate carbonyl carbon. [h] C-11 distinguished from C-12 and C-13 by monolabeling experiments which show the former as derived from the carbonyl carbon of acetate and the latter two as derived from the acetate methyl. [i] C-12 distinguished from C-13 by experiments using doubly labeled acetate which clearly indicate that carbon at 125.1 ppm is coupled to C-4, while the carbon at 125.3 ppm is coupled to C-10 which, given the other data, is only internally consistent with the assignments given.

Table III. Chemical Shift Assignments for the Carbons of Daunomycinone Tetraacetate (tabulated by the numbering system shown below)



Carbon no.	Obsd chemical shift[a]	Predicted/calcd chemical shift[b]
1	119.3	118.4–119.6[c]
2	134.1	134.1–135.1[c]
3	118.3	118.6–119.7[c]
4	159.7	157.5–158.7
5	180.6	180.4[d]
6	146.7	147.4[d]
7	62.1	*c*
8	30.9	*c*
9	80.4	~81.2[e]
10	31.3	~29.3–38.5[c,f]
11	145.1	146.4[d]
12	182.0	181.2[d]
13	205.0	~205.0[g]
14	24.0	*c*
15	135.8	133.7–134.7
16	122.3	118.2–119.3
17	126.2	125.1[h]
18	125.2	125.4[h]
19	134.7	*h*
20	134.2	*h*
21	57.0	54.7[c,i]

[a] Chemical shifts were measured in $CDCl_3$ in ppm relative to internal Me_4Si. [b] The chemical shifts observed in the four model anthraquinones used to predict islandicin shifts (see Table II, *b*) also formed the basis for the predicted/calculated shifts of carbons 1–4 and carbons 15 and 16 of daunomycinone tetraacetate, while islandicin itself provided this basis for carbons 5, 6, 11, 12, 17, and 18. As appropriate, substituent effect parameters[12,13] were added to the basic model compound shifts to obtain the predicted chemical shifts. [c] Assignment of C-1, C-2, C-3, C-7, C-8, C-10, C-14, and C-21 was confirmed by single-frequency decoupling experiments. (H-1 = 7.70 ppm, H-2 = 7.59 ppm, H-3 = 7.24 ppm, H-7 = 6.34 ppm, H-8 protons, which are obscured, are best decoupled by irradiation at 2.40 ppm, H-10 is an AB quartet centered at 3.10 ppm, H-14 = 2.42 ppm, H-21 = 3.96 ppm.) The assignment of the proton spectrum was based on the previously reported assignments for daunomycinone[11a,f] and *N*-acetyldaunomycin.[11d] [d] The use of islandicin triacetate as a model predicts the given chemical shifts for C-5 vs. C-12 and for C-6 vs. C-11, but these assignments were also confirmed by the experiments using singly labeled acetate. [e] Predicted shift based on the model 1-acetoxy-1-methylcyclohexane: J. B. Stothers, ref 12, p 168. [f] Predicted shift range based on the shift of the carbon of alkylbenzenes: J. B. Stothers, ref 12, p 98. [g] Predicted shift based on acetone. [h] The pair C-17 and C-18 and the pair C-19 and C-20 were distinguished on the basis on the singly labeled acetate experiments. [i] Predicted shift based on anisole.

vidual islandicin carbon, but the range of values seems to be a less subjective approach in general, although as recorded in Table II, specific models were used in a few cases.

In addition to the standard carbon assignment technique, the islandicin assignments depended extensively on single-labeling experiments. The labeling pattern observed by Gatenbeck (Figure 2) and confirmed by experiments using sodium [1-^{13}C]- and [2-^{13}C]acetate establishes whether a given carbon is derived from the methyl or carbonyl carbon of acetate. Once most of the chemical-shift assignments have been made, one can use the results of the singly labeled acetate experiments to distingush between pairs of carbons, for example C-9 and C-10, which are close in chemical shift. Definitive assignment in such cases is easily made on the basis of the observed labeling pattern.

The doubly labeled acetate experiment permits final assignments of some carbons; C-12 and C-13 were assigned on the basis of their observed coupling to another carbon of known chemical shift. (The use of the doubly labeled results in making this assignment leads to completely internally consistent results.)

Daunomycinone Tetraacetate (Figure 3). Islandicin was the obvious basic model compound for predicting/calculating chemical shifts for C-5, 6, 11, 12, 17, and 18 of daunomycinone, and predicted values agree within 1.3 ppm with observed

Table IV. The Enrichment Levels at Various Carbons of Islandicin Triacetate from Sodium [1-^{13}C]- and [2-^{13}C]Acetate

Carbon	Peak intensity[a] Natural abundance	From [1-^{13}C]-NaOAc	From [2-^{13}C]-NaOAc
Acetate methyls (2 or 3)	1.00	1.00	1.00
C-1	0.39	0.26	1.10
C-2	0.38	0.76	0.30
C-3	0.79	0.60	2.04
C-4	0.50	1.08	0.41
C-5	0.43	1.05	0.39
C-6	0.77	0.52	2.15
C-7	0.65	1.73	0.52
C-8[b]	0.80	1.04	2.85
C-9	0.34	0.18	0.71
C-10	0.34	0.56	0.23
C-11[b]	0.80	1.04	2.85
C-12	0.39	0.30	0.84
C-13[b]	0.80	1.04	2.85
C-14	0.43	0.78	0.36
C-2 methyl	0.49	0.44	1.55

[a] Peak intensities were standardized to the peak representing two of three acetate methyls. The peak for the third methyl, being a partial shoulder, was not a suitable standard. [b]The peak at 125.3–125.4 ppm contains the resonances for C-8, C-11, and C-13. Since two of these are derived from the acetate methyl (C-8, -13) and one from the acetate carbonyl (C-11), the greatest enhancement is observed in islandicin grown from [2-^{13}C]acetate.

values (Table III). The shifts for C-1, 2, 3, 4, 15, and 16 were calculated from anthraquinone and appropriate substituent effect parameters as described above. The remaining carbons were assigned from various other models as indicated in Table III.

Since the predicted ^{13}C shifts for C-1 and C-3 were very close, the assignment of these carbons rests on single-frequency experiments (H-1 at 7.70 ppm and H-3 at 7.24 ppm), which were definitive due to the adequate difference in proton chemical shift. Single frequency experiments also distinguished C-8 and C-10 (by irradiation of the AB quartet for C-10 at 3.10 ppm).

The following pairs of carbons, which were not definitively distinguishable on the basis of chemical shift, were assigned from the results of the experiments using singly labeled acetate: C-5 and C-12, C-6 and C-11, C-17 and C-18, and C-19 and C-20 (see results in Table V).

Incorporation of Singly Labeled Acetates. Islandicin Triacetate. In order to confirm the acetate labeling pattern in islandicin observed by Gatenbeck[4] using $CH_3{}^{14}CO_2Na$, *P. islandicum* was cultured separately in the presence of sodium [1-^{13}C]- and [2-^{13}C]acetate (both 91% isotopic purity). In each case, the resulting islandicin was chemically acetylated for ^{13}C NMR analysis. Because conditions had been established[6] under which all of the carbon signals of the unlabeled islandicin were within a factor of 2.5 of the same peak height, the spectra of the singly labeled islandicin triacetates were easily analyzed. The results of these labeling experiments are summarized in Table IV, and clearly show that the labeling pattern in islandicin is as shown in Figure 2. Seven carbons originate from the carbonyl carbon of acetate (C-2, 4, 5, 7, 10, 11, and 14) and eight from the methyl carbon (C-1, 3, 6, 8, 9, 12, 13, and 15), indicating that one decarboxylation is involved in the biosynthesis.

Table IV also shows that the incorporation level of [1-^{13}C]acetate about three times the natural abundance level. The variation in the apparent incorporation level between

Table V. The Enrichment Levels at the Various Carbons of Daunomycinone Tetraacetate from Sodium [1-^{13}C]- and [2-^{13}C]Acetate

Carbon	Peak intensity[a] Natural abundance	From [1-^{13}C]-NaOAc	From [2-^{13}C]-NaOAc
C-1	1.68	1.00	3.89
C-2	1.07	2.31	1.02
C-3	0.97	0.76	3.10
C-4	1.21	1.84	0.68
C-5	1.21	1.71	1.02
C-6	1.39	1.53	0.91
C-7	2.21	3.25	1.70
C-8	2.54	1.12	5.61
C-9	2.60	1.04	2.00
C-10	2.57	1.31	6.20
C-11	1.43	0.86	2.96
C-12	1.43	0.71	2.87
C-13	1.07	0.55	1.05
C-14	1.25	0.73	2.28
C-15	1.50	1.98	0.75
C-16	1.36	0.90	2.82
C-17	1.50	0.64	2.60
C-18	1.68	1.84	0.84
C-19	1.82	1.84	1.04
C-20	1.32	0.51	2.34
C-21	1.00	1.00	1.00

[a] Peak intensities were standardized to the methoxy carbon.

carbons is due partly to the insufficient resolution of the computer on the Brucker WH-270 spectrometer, which possesses a 16 384 digit capacity for the real part of the spectrum. At the required spectral width of 13 500 Hz, the resolution is 0.92 Hz/point, while most of the carbon peaks were observed to be 2–3 Hz wide, so that highly accurate peak height/peak area definition could not be expected (although it is somewhat better than the 1.38 Hz/point resolution available with the XL-100 spectrometer, used for the daunomycinone tetraacetate spectra).

A further obvious cause for relative peak height variation between samples is intrinsic differences in carbon relaxation times. These differences could be caused by different sample concentrations, different $Cr(acac)_3$ concentrations (although a contant molar ratio was used throughout), and/or instrumental variation in spite of standard settings.

Daunomycinone Tetraacetate. Since there were no reports of the acetate labeling pattern in daunomycin, single labeled acetate studies were of special interest. Using a method analogous to that described for islandicin, *S. peucetius* was cultured separately in the presence of [1-^{13}C]- and [2-^{13}C]acetate, acetylated, and observed under established ^{13}C NMR conditions. A molar ratio of 0.55 $Cr(acac)_3$ to daunomycinone tetraacetate was found to optimize the peak height of the carbonyl and quarternary carbons, although some line broadening of the methoxyl carbon occurred at the high absolute $Cr(acac)_3$ concentration used in studying the unlabeled sample. (To shorten observation times, a high concentration of both sample and $Cr(acac)_3$ was used in this case.)

The results of the [1-^{13}C]- and [2-^{13}C]acetate experiments are presented in Table V. Appreciable incorporation of [1-^{13}C]acetate was observed at carbons 2, 4, 5, 6, 7, 15, 18, and 19, while [2-^{13}C]acetate incorporation was observed at carbons 1, 3, 8, 10, 11, 12, 16, 17, and 20. Signals of enriched carbons generally appear two or three times more intense than the natural abundance level, with incorporations of the [2-^{13}C]-acetate being slightly greater than that of the [1-^{13}C]acetate. However, because the peak intensities are relative to the methoxyl carbon, which showed appreciable line broadening

Table VI. ^{13}C–^{13}C Coupling Constants for [1,2-^{13}C]Acetate-Enriched Islandicin Triacetate

Coupled carbons	J, Hz	Coupled carbons	J, Hz
C(2)–C(15)	44.3	C(7)–C(8)	56.4
C(15)–C(2)	44.4	C(8)–C(7)	55.5
C(4)–C(12)	71.2	C(14)–C(9)	53.7
C(12)–C(4)	72.1	C(9)–C(14)	54.6
C(10)–C(13)	56.4	C(11)–C(1)	73.1
C(13)–C(10)	56.4	C(1)–C(11)	74.0
C(5)–C(6)	70.2		
C(6)–C(5)	70.3		

Table VII. ^{13}C–^{13}C Coupling Constants for [1,2-^{13}C]Acetate-Enriched Daunomycinone Tetraacetate

Coupled carbons	J, Hz	Coupled carbons	J, Hz
C(1)–C(15)	53.1	C(6)–C(20)	~70
C(15)–C(1)	a	C(20)–C(6)	a
C(2)–C(3)	a	C(7)–C(8)	37.8
C(3)–C(2)	62.4	C(8)–C(7)	37.2
C(4)–C(16)	70.1	C(11)–C(19)	~63
C(16)–C(4)	70.3	C(19)–C(11)	a
C(5)–C(17)	54.3	C(12)–C(18)	~55
C(17)–C(5)	55.3	C(18)–C(12)	a

[a] In these cases either overlap was too extensive or signal intensity too weak to measure the values.

only in the high concentration unlabeled sample, a comparison of the peak intensities for the two labeled samples (run at approximately the same concentrations) proved, in most cases, the best measure of incorporation.

Peak intensity variation due to insufficient computer points and/or other factors (see islandicin discussion) was again a problem,[14] particularly in regard to C-13 and C-14, which together with C-9 are key to the "propionate starter" interpretation. While C-14 shows some [2-^{13}C]acetate incorporation, a careful comparison of spectra indicated that incorporation was probably not as extensive as observed for the other [2-^{13}C]acetate derived carbons, a result which is more evident in relation to C-9. (This result is expected if C-14 is derived from propionate which has, in turn, been derived from labeled acetate.) Unfortunately, C-13 showed an abnormally undefined peak height in the spectrum of the [1-^{13}C]acetate derived sample. Expansion of the C-13 signal in this particular spectrum showed that no computer point had fallen near the top of the peak, which is particularly narrow, so that its true peak height was not established. Conclusions, therefore, are based on other key carbons.

To summarize the results, it is clear that the biosynthesis of daunomycin does not conform to a "classical" acetate–polymalonate pathway (Figure 1, pathway c or d). In particular the carboxyl and methyl derived carbons are opposite from those predicted by these two routes; the three-carbon-fragment, carbons 9, 13, 14, is apparently not directly acetate derived at all, and there is one more methyl-derived carbon than there are carboxyl-derived carbons.

Incorporation of Doubly Labeled Acetate. We have previously described the expected appearance of ^{13}C NMR spectra when a low level (1–2% on a per acetate unit) of exogenous [1,2-^{13}C]acetate has been incorporated into the compound under observation.[3] At sites where incorporation has occurred, carbons appear either as singlets, if a decarboxylation or skeletal rearrangement has occurred resulting in the loss of one carbon from the double labeled acetate molecule, or as triplets. Triplets occur because the doublet due to ^{13}C–^{13}C coupling (arising, or course, when doubly labeled acetate is incorporated), is approximately the same height as the central singlet due to unlabeled material.

Islandicin Triacetate. In the 67-MHz spectrum of islandicin triacetate grown from [1,2-^{13}C]acetate (Figure 4b), 14 of the 15 nucleus carbon atoms appear as distinct triplets (although three overlap at ~125.3 ppm), while C-3 is a distinct singlet. Therefore, the islandicin skeleton is derived from eight acetate units with one decarboxylation occurring during the biosynthesis. The observed coupling pattern (reported in Table VI), together with the distinct singlet for C-3, unequivocally establish the folding pattern a in Figure 2 as the biosynthetic pathway. Obviously pattern b would predict a different coupling pattern and a singlet for C-1 rather than C-3. Numerous other possibilities are also ruled out.

Close inspection of the enriched sample spectrum (Figure 4b) shows that most peaks, in particular C-3, have associated with them small symmetrically placed satellite pairs. These are of the expected intensity for multiply labeled species and apparently represent neither incursion of alternate biosynthetic pathways nor machine anomalies. We conclude this for the following reasons: (a) the C-1 resonance shows this effect and should not were configuration b operable, since the configuration would result in a singlet for this resonance; (b) the acetate carbonyl and methyl resonances do not show this effect (they probably would if the cause were machine anomalies); and (c) the C-2 methyl does not show this effect because it can have only one enriched neighboring carbon.

While it is thus unlikely that these satellite peaks arise from an alternative biosynthetic pathway, there are several ways in which multiple labeled species can arise. (1) The inherent probability of incorporation of labeled acetate at adjacent positions at this level of incorporation. In this case, the calculated satellite peak height at the observed incorporation level is ~3–4% of the main peak, while the observed satellites are larger than this (~8% of main peak), seemingly too large to be accounted for by this alternative. (2) The presence of a substantial preformed pool of islandicin at the time of initiation of feeding. This would give rise to an enhanced intensity of the satellite peaks over that expected on the basis of average acetate incorporation. No perceptible islandicin is formed at the time of initial pulsing, but any committed biosynthetic precursor (e.g., a polyketide) would give the same effect. (3) A lag period after initial pulsing during which exogenous acetate is not incorporated but islandicin is formed.

All of these are possible, but in the absence of accurate integration, possibilities 2 and 3 above, which predict an enhanced intensity of the satellite peaks, can only be suggested.

To summarize, these results demonstrate unequivocally that of the two pathways a and b (Figure 2), the former is correct.

Daunomycinone Tetraacetate. Analysis of the ^{13}C NMR spectrum of daunomycinone tetraacetate derived from [1,2-^{13}C]acetate (Figure 5b) in conjunction with the singly labeled acetate results permitted the following strong conclusions. First, the carbon fragment (C-9, C-13, C-14) is either not acetate derived or is derived by a process that entails both dilution of the isotope (relative to the other carbons in the molecule) and loss of the integrity of the added two-carbon acetate fragments. Second, while the coupling of all carbon pairs was not evident (Table VII), enough couplings could be measured (and enough chemical-shift assignments firmly established) to specifically implicate pathway a in Figure 1. Specific considerations were the following. (a) Because C-7 and C-8 appear to be derived from the same acetate unit, while C-10 is a singlet, pathway a is uniquely required. Although this conclusion could be reversed (pathway b) if the assignments of C-8 and C-10 were reversed, while C-19 and C-10 as well as C-7 and C-20 were coupled, this seems unlikely on the basis of single-frequency decoupling experiments (C-8 vs. C-10) and other pairings which implicate pathway a (see below). (b) The

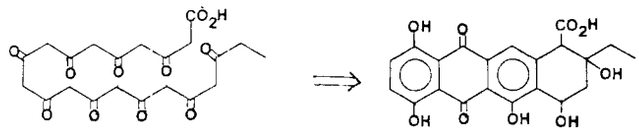


Figure 6. Proposed biosynthetic pathway for rutilantinone.

coupling of C-5 to C-17 and of C-4 to C-16 was apparent, firmly establishing that each of these pairs is derived from the same acetate unit. (c) A distinct 55-Hz coupling can be measured for C-12, presumably due to coupling to C-18; only one satellite peak is visible for C-18. However C-18 is not coupled to C-11, and postulating coupling to C-17 would require a most esoteric connectivity pattern. The elimination process confirms that C-12 and C-18 are derived from the same acetate unit. (d) C-3 appears with a satellite doublet (J = 68 Hz) and since it cannot be coupled to C-4 (whose coupling to C-16 is established), it must be coupled to C-2, although the splitting of C-2 is immersed in the 135-ppm complex and could not be determined. Even if the assignment of C-1 and C-3 were reversed, C-3 still cannot be coupled to C-4; it must be coupled to C-2. (The assignment reversal was considered because the carbon shifts are very close; however, the corresponding protons are separated by 0.44 ppm, enough to use single-frequency decoupling to establish that C-3 is coupled to the higher field aromatic proton.)

The small symmetrically placed satellite pairs (3–4% of mean peak in the islandicin triacetate spectrum, Figure 4b) were not observed in the daunomycin tetraacetate spectrum. However, the signal/noise level was much less favorable in the latter case which may have precluded their observation.

Taken together, the above results are uniquely consistent with pathway a (Figure 1) and show an acetate connectivity pattern both strikingly different from that determined for islandicin and strikingly similar to that proposed some time ago for metabolite rutilantinone (Figure 6),[15] wherein the three-carbon fragment corresponding to carbons 19, 13, and 14 was shown to be propionate derived. In the latter compound, sodium [1-^{14}C]propionate was used to demonstrate the involvement of a propionate "starter" in the polyketide synthesis.

Experimental Section

Carbon spectra for islandicin triacetate were obtained on a Bruker WH 270 spectrometer at 67.92 MHz. The computer associated with this instrument yielded 16K data points in the real transformed spectrum. Carbon spectra for the substituted anthraquinones and daunomycinone tetraacetate were obtained on a Varian XL-100-15 spectrometer operating at 25.16 MHz. Its computer yielded 4K data points in the real transformed spectrum. Chemical shifts are in parts per million relative to internal tetramethylsilane and $Cr(acac)_3$ was used as a relaxation agent in all experiments. Standard techniques were used for single frequency, broad band noise, and off-resonance decoupling experiments.

Islandicin Experimental. Organism. *Penicillium islandicum* Sopp was obtained from the American Type Culture Collection (strain no. 10127) and maintained on malt extract agar (Balkeslee).[16] This medium produced abundant sporulation.

Culture Conditions. Medium Preparation. Czapek–Dox–2% agar was prepared in the following manner. To 1 L of distilled water were added the following: 50 g of glucose (Mallinckrodt), 2.0 g of $NaNO_3$ (MCB), 1.0 g of KH_2PO_4 (Mallinckrodt), 0.5 g of KCl (Mallinckrodt), 0.5 g of $MgSO_4 \cdot 7H_2O$ (Baker), 0.01 g of $FeSO_4 \cdot 7H_2O$ (Allied), and 20 g of Bacto agar (Difco). This molten solution was sterilized and then poured into sterile petri dishes. Each petri dish received approximately 30–40 mL of the medium.

Inoculation and Growth Conditions. *P. islandicum* spores from a culture which was at least 3 weeks old were scraped from the agar (Blakeslee malt extract) surface and spread over the fresh Czapek–Dox medium. The new cultures were allowed to grow 22–23 days at 20–24 °C. At this point the agar plates were removed from the petri dishes and dried in an oven.

Isolation of Islandicin. The dried agar mats were ground to a powder and exhaustively extracted with Skellysolve A to remove lipid material. This process also extracted some islandicin and other pigments, but the islandicin could be isolated from this extract by basic extraction and chromatography. The mycelial powder was then extracted with chloroform, a process which removed a substantial amount of colored pigments. This extract was chromatographed (silica gel/benzene) to yield islandicin and its dimer, iridoskyrin; the islandicin could be purified by sublimation at temperatures not exceeding 180 °C. The iridoskyrin could be recovered unchanged from the sublimation vessel. The islandicin is recrystallized from chloroform.

Preparation of Sodium Acetate Solutions. Sodium [2-^{14}C]acetate (2.0 mCi/mmol, 2.1 mg) was dissolved in 100 mL of water and cold sodium acetate was added to bring the concentration up to the desired level (4.0, 5.0, 6.0, 8.0 mg/mL). The solutions were sterilized and the radioactivity was determined on a Packard Tri-Carb Model 3375 counter in Aquasol (New England Nuclear) solution. Sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-^{13}C]acetate solutions were prepared analogously from 90% [^{13}C]acetates (Merck) with added [2-^{14}C]acetate as tracer.

Addition of Sodium Acetate. Solutions (0.5 mL) of known sodium acetate concentration were added daily to the cultures starting at day 7 and continuing through day 16 of the growth period. The yield of islandicin and the percent incorporation of [^{14}C]acetate as a function of acetate concentration are as shown.

Acetate added per 12 cultures, mmol	Islandicin produced per 12 cultures, mmol	% incorporation of added acetate
2.93	0.186	1.76
3.66	0.181	2.32
4.19	0.209	2.75
5.63	0.208	3.72

Islandicin Triacetate. Recrystallized islandicin (51.8 mg; 0.192 mmol) was placed in a small test tube along with 1.5 mL (17.3 mmol) of pyridine and the mixture heated to 110 °C. Acetic anhydride (1.5 mL; 15.9 mmol) was then added and the mixture was sitrred and warmed intermittently over a 90-min period. The yellow solution was then quenched in ice water and stirred for 15 min, and the resultant precipitate was filtered, washed, and dried in vacuo. The crude product (86.6 mg) was sublimed [175 °C (0.1 Torr)] to yield 83.2 mg (0.185 mmol; 96%) of the purified islandicin triacetate, mp 207.5–208 °C (lit. 208 °C). The material could be recrystallized from methanol to give pale yellow needles: NMR (100 MHz; $CDCl_3$) δ 2.30 (3 H, s) (this methyl group shows a reduced peak height, although the 1.5-Hz coupling to the proton at 7.30 ppm was not observable), 2.42 (6 H, s), 2.49 (3 H, s), 7.30 (1 H, q, J = 1.5 Hz), 7.38 (1 H, dd, J = 1.5, 7.5 Hz), 7.75 (1 H, t, J = 7.5 Hz), 8.13 (1 H, dd, J = 1.5, 7.5 Hz); IR (KBr) ν 1791, 1779, 1685, 1602, 1385, 1342, 1271, 1212, 1191, 1036, 921, 910, 822 cm^{-1}; UV–vis $\lambda_{max}(CH_3OH)$ 342 (ϵ 6470), 270 (ϵ 14 200 sh), 250 nm (ϵ 36 800); MS m/e 354 (3), 312 (31.5), 270 (100).

Counting Protocol. Radioactivity of sodium [^{14}C]acetate (New England Nuclear) was determined by counting in Aquasol. Radioactive islandicin (100 mg) was dissolved in 50 mL of chloroform. A 50 μL aliquot was added to 5 mL of toluene-based scintillation fluid (5 g of PPO, 0.066 g of POPOP/L of toluene) and counted. Self-quenching was corrected for by means of a concentration–count plot.

Daunomycin Experimental. Organism. *Streptomyces peucetius* was obtained from the American Type Culture Collection (strain no. 21354) and was maintained on a malt extract agar at 20–24 °C (Hesseltire[17]).

Culture Conditons. Vegetative Medium Preparation. To 1 L of distilled water were added: 3.0 g of yeast extract (Difco), 3.0 g of malt extract (Difco), 5.0 g of Bacto peptone (Difco), and 10.0 g of dextrose (Mallinckrodt). To each of several small shake flasks was added 40 mL of this medium, and the flasks were subsequently sterilized.

Productive Medium Preparation. To 790 mL of tap water was added 10 mL of an ion solution containing Zn^{2+} (10^{-4} M), Cu^{2+}, Mn^{2+}, Co^{2+} (all 10^{-5} M), and Mo^{2+} (10^{-6} M). To this solution were added the following: 15.0 g of dry yeast (Red Star), 2.0 g of NaCl (Merck), 1.0 g of KH_2PO_4, 1.0 g of $CaCO_3$ (Allied), 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.01 g of $FeSO_4 \cdot 7H_2O$. This solution was divided equally among four 2-L *creased* flasks and sterilized. Dextrose solutions were prepared separately by dissolving 10-g portions of dextrose in 50-mL aliquots of distilled water and sterilizing prior to use.

Inoculation and Growth Conditions. *S. peucetius* mycelia were

scraped from an agar surface and added to the vegetative media. Mycelia between 9 and 12 days old proved most viable for production of the metabolite. The vegetative cultures were grown on a rotary shaker at 28 °C for 45 h. The vegetative mycelia thus prepared served as inocula for the productive media prepared above. Dextrose solutions were added separately. Cultures were grown on a rotary shaker for 120 h.

Isolation of Daunomycinone. The culture broth from the shake flasks was centrifuged with Celite at 0 °C and the solids were extracted several times with a 3:1 mixture of acetone/0.5 N HCl. The aqueous acetone solution was neutralized and the acetone removed in vacuo. The aqueous suspension remaining was immediately hydrolyzed with 0.5 N HCl (1 h; 90 °C) and the resultant suspension extracted with chloroform.

The chloroform extracts were pooled, concentrated, and chromatographed (silica gel; 1% methanol/chloroform) to yield 25–30 mg of daunomycinone from 1 L of broth. The daunomycinone thus obtained was not purified further.

Preparation of Sodium [^{13}C]Acetate Solutions and Pulsing Conditions. In 18 mL of distilled water was dissolved 1.8 g of sodium [^{13}C]acetate (1-^{13}C, 2-^{13}C, and 1,2-^{13}C for the three experiments). The solutions were sterilized and added daily to growing cultures of *S. peucetius*. Additions commenced 45 h after inoculation and continued at 12-h intervals through the 108th hour of the growth period. Each pulse consisted of a 0.5-mL aliquot of the solution and this ensured that the concentration of acetate would be <200 mg/L.

Daunomycinone Tetraacetate. The chromatographed daunomycinone (32 mg; 0.08 mmol) was dissolved in 1.0 mL (12.4 mmol) of pyridine. Acetic anhydride (1.5 mL; 15.0 mmol) was then added and the mixture was stirred at 60 °C for 3 h. The solution was quenched in ice water and extracted with chloroform. The crude product, a mixture of tri- and tetraacetates, was chromatographed on silica gel (5% methanol/benzene) to yield 31.1 mg (0.055 mmol; 68%) of the tetraacetate. This was used for the ^{13}C NMR experiments without additional purification; NMR (100 MHz; $CDCl_3$) δ 2.01 (6 H, s), 2.22 (3 H, s), 2.42 (3 H, s), 2.48 (3 H, s), 3.10 (1 H, AB quartet, J = 18 Hz), 3.96 (3 H, s), 6.34 (1 H, bd), 7.24 (1 H, dd, J = 7.5, 2 Hz), 7.59 (1 H, t, J = 7.5 Hz), 7.70 (1 H, dd, J = 7.5, 2 Hz) (The multiplet expected for H-8 is obscured by the resonances in the 2.0–2.6-ppm range, but shows in the integration.); IR (KBr) 1781, 1746, 1681, 1590, 1375, 1242, 1192, 1077, 1019 cm^{-1}; UV-vis λ_{max}(MeOH) 375 (ϵ 5960), 252 (ϵ 30 200); MS *m/e* 488 (2), 362 (50), 60 (61), 44 (42).

Registry No.—**1a,** 20830-81-3; **2a,** 476-56-2; **2b,** 18713-46-7; daunomycinone, 21794-55-8; daunomycinone tetraacetate, 32384-96-6.

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Synthesis and Resolution of 3-Fluoro-D,L-alanine-*2-d:* A Selective Deuteration via Reductive Amination with Sodium Borodeuteride

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3-Fluoro-D,L-alanine-2-*d* (**11**) is synthesized in aqueous (protio) ammonia from lithium fluoropyruvate (**3**) via reductive amination with sodium borodeuteride with complete retention of isotopic purity. Fluoropyruvate salts (**5**) equilibrate in 13, 6.5, and 4 M aqueous ammonia to 95:5, 85:15, and 80:20 mixtures of 3-fluoro-2,2-diaminopropionate (**8**) and 3-fluoro-2-amino-2-hydroxypropionate (**6**), respectively. The reduction of these mixtures with sodium borodeuteride to **11** and 3-fluoro-2-hydroxypropionic-2-*d* acid (**10**), a side product, is studied in detail. A mechanistic scheme is proposed in which the rate-limiting step for the formation of **10** is the reequilibration of **8** to **5,** and for the formation of **11** it is the reduction of 3-fluoro-2-iminopropionate (**7**) with sodium borodeuteride. The yield of **11** is maximized with respect to an efficient use of sodium borodeuteride. Racemic **11** is resolved via the *N*-carbobenzoxy derivative with quinine and by a continuous resolution via preferential crystallization of the benzenesulfonate salt.

3-Fluoro-D-alanine-2-*d* in combination with the 2,4-pentanedione enamine of cycloserine, sodium salt, constitutes a novel, uniquely synergistic, bactericidal antimicrobial with an unusually broad spectrum.[1] The first synthesis of 3-fluoro-D,L-alanine by fluorination of 2-phenyl-4-chloromethylene-5-azlactone, followed by hydrolysis, hydrogenation, and saponification was reported by Yuan et al.[2] Later, Lettré and Wölcke obtained the racemic amino acid by α-bromination and subsequent ammonolysis of 3-fluoropropionic acid.[3] However, neither approach can introduce deuterium selectively into the α position. Photofluorination of D-alanine-2-*d* with CF_3OF in liquid HF at −78 °C produced the first 3-flu-

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