MACROLIDE BIOSYNTHESIS: STEREOCHEMISTRY OF THE HYDROXYLATION OF BREFELDIN C

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Brefeldin C (**2**) is the penultimate intermediate in the biosynthetic pathway to brefeldin A (**1**). Using stereospecifically ^{2}H-labeled forms of **2**, the hydroxylation of **2** to **1** is shown to involve stereospecific removal of the 7 *pro-S* hydrogen from **2**.

The biosynthesis of brefeldin A (**1**), a macrolide antibiotic produced by *Eupenicillium brefeldianum* and some other fungi, has beeen studied in several laboratories[1~6]. Brefeldin C (**2**) is the penultimate intermediate of the brefeldin A pathway[7,8] and its hydroxylation involves molecular oxygen since that is the source of the oxygen attached to C-7[6]. These observations suggest that the conversion of **2** to **1** is catalyzed by a monooxygenase; if true, then stereospecific removal of one of the two diastereotopic hydrogens at C-7 should occur during the hydroxylation of **2** *in vivo*. We have confirmed this assumption by the results of the isotopic labeling experiments described here.

Results

Three samples of deuterium labeled **2** were prepared from unlabeled **1** by the chemical transformations shown in Fig. 1. Assuming that reduction of 7-*O*-mesyl-**2** with sodium cyanoborodeuteride occurred with inversion of the C-7 configuration, which is precedented in other cases[10,11] and supported by the data given below, (7*R*)-[7-^{2}H]-**2** (**4**) was thus obtained from **5** and its 7 *pro-R* ^{2}H characterized by a signal at 1.54 ppm in the ^{2}H NMR spectrum, and at 25 ppm (t, J_{CD}=19 Hz) in the ^{13}C NMR spectrum. Similarly, (7*S*)-[7-^{2}H]-**2** (**7**) was obtained from **6** and its 7 *pro-S* ^{2}H observed to have a signal at 1.63 ppm in the ^{2}H NMR spectrum and at 26.1 ppm (t, J_{CD}=20 Hz) in the ^{13}C NMR spectrum. A sample of (7*RS*)-[7-^{2}H]-**2** (**8**) was prepared from **6** and found to be an equal mixture of **4** and **7** by ^{2}H NMR spectroscopy. The deuterium content of all of these labeled compounds was 92% d_1 or d_2 (or greater), as determined by mass spectral analysis of their *tert*-butyldimethylsilyl (TBDMS) ethers.

These three compounds were fed separately in about equal concentrations to cultures of *E. brefeldianum*. A trace amount of (7*RS*)-[7-^{3}H]-**2** was fed along with **7** and **8** for an internal control, since we anticipated that one of these compounds would loose most of its deuterium label during biological hydroxylation; thus its incorporation into **1** would have to be verified by a radioactivity measurement.

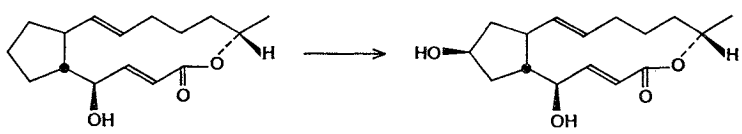

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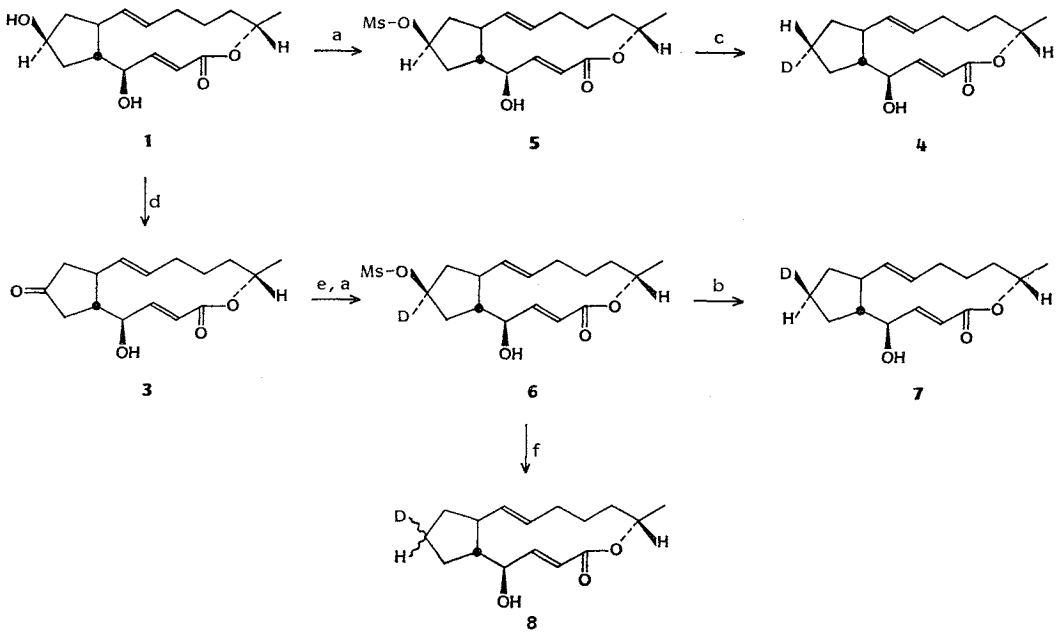
Fig. 1. Synthetic scheme for the preparation of deuterium labeled brefeldin C.

a) Mesyl chloride - pyridine; triethylamine; −13°C; 49% yield. b) $NaBH_3CN$ - toluene; hexadecyltributyl phosphonium bromide ($HDTBP^+Br^-$); 25°C; 72% yield for **2** and 59% for **7**. c) $NaBD_3CN$ - toluene; $HDTBP^+Br^-$; 25°C; 80% yield. d) Ag_2CO_3 on Celite - benzene; 70°C; 68.7% yield. e) $NaBD_4$ - MeOH; −78°C; 89.6% yield. f) $NaBH_3CN$ - toluene; $HDTBP^+Br^-$ (catalyst); 25°C; 50% yield.

MS: Mesyl.



The ^2H NMR spectrum of the samples of **1** isolated from each of the three feeding experiments showed a signal at 4.5 ppm, which was assigned to the deuterium atom attached to C-7[12], but the intensity of this signal was significantly different in each case. For an accurate determination of isotopic enrichment, the *bis*-TBDMS ethers of these samples were prepared and their deuterium enrichments determined by mass spectral analysis. The specific incorporations of **4**, **7** and **8** into **1** calculated from these data are listed in Table 1.

Table 1. Specific incorporations of labeled precursors into **1**.

Precursor	Specific incorporation (%)[a]	
	^2H	^3H
4	45.7	
7	6.7	18.9
8	21.9	16.5

[a] Calculated by the formula, specific incorporation = $f_aA \times 100/f_bB$, where f_a is the percent isotopic enrichment (or specific radioactivity) of the isolated **1**, f_b is the corresponding enrichment (or specific radioactivity) of **4**, **7** or **8**, A is the mmol of **4**, **7** or **8**, and B is the mmol of isolated **1**.

The data show that the incorporation of ^2H into **1** from **4** was approximately twice that from **8**. This indicates that the 7 *pro-S* hydrogen of **4** was stereospecifically removed during the biological hydroxylation of **2**. The incorporation of ^3H into **1** was about the same in the feeding experiments with **7** and **8**, which shows that these two precursors were similarly taken up and metabolized by the fungal cells. We therefore expected that the incorporation of **7** would not result in deuterium labeling of **1** since the result with **4** indicated that the 7 *pro-S* ^2H label in **7** should be removed during its hydroxylation. The fact that **1** contained some deuterium

enrichment at C-7 from **7** is most likely due to a slight lack of stereospecificity during the reduction of **6**. The resolution of the signals for the 7 *pro-R* and 7 *pro-S* deuterium atoms in the 30.6 MHz ^{2}H NMR spectra of **4** and **7**, respectively, was not large enough to permit detection of a small amount of contamination of the (7*R*)-[7-^{2}H] isomer in the sample of **7** used in the feeding experiment. We therefore conclude that the hydroxylation of C-7 of **2** was stereospecific and occurred with retention of configuration.

Discussion

Our finding that **2** is converted to **1** by stereospecific hydroxylation of the diastereotopic C-7 position with retention of configuration is consistent with many other examples showing that biological hydroxylations mediated by monooxygenases involve the stereospecific replacement of a C-H bond by a C-O bond[13]. This suggests that the transformation of **2** to **1** is catalyzed by a cytochrome P-450 type of hydroxylase.

Experimental

General

The procedures used for culture maintenance and fermentation, standard chemical methods, and isolation and spectral analyses of **1** are described in refs 6, 8 and 9. Chemical shifts (δ) were determined at ambient probe temperatures in $CHCl_3$ or $CDCl_3$ for synthetic compounds and in pyridine for **1** that was isolated from the feeding experiments, and are reported relative to TMS as the external standard. Coupling constants (J) are given in Hz.

7-*O*-Mesylbrefeldin A (5)

Mesyl chloride (184 μl, 2.38 mmol, 1.3 eq) was added dropwise to a solution of **1** (500 mg, 1.79 mmol) and triethylamine (184 μl, 2.38 mmol, 1.3 eq) in pyridine (6 ml) at -13°C over a period of 10 minutes. The reaction mixture was stirred at -13°C for 35 minutes, then diluted with ethyl acetate, washed with 5% HCl, 10% $NaHCO_3$ and brine; and dried and concentrated to obtain a mixture of 4,7-*bis*-*O*-mesylbrefeldin A, **5** and starting material [Rf 0.54, 0.4 and 0.45, respectively, in *n*-hexane - ethyl acetate (2 : 3)]. This mixture was separated by preparative TLC, eluted with *n*-hexane - ethyl acetate (2 : 3), to yield **5** as a white precipitate (311 mg, 0.87 mmol, 49%): Rf 0.4 in *n*-hexane - ethyl acetate (2 : 3); ^{1}H NMR (200 MHz) δ 7.27 (1H, dd, J_1=15.7 and J_2=3.1), 5.86 (1H, dd, J_1=15.7 and J_2=1.8), 5.72 (1H, ddd, J_1=15.1, J_2=104 and J_3=5.0), 5.21 (1H, dd, J_1=15.1 and J_2=8.8), 5.15~5.05 (1H, m), 4.84 (1H, m), 4.1 (1H, br d), 3.0 (3H, s), 1.24 (3H, d, J=6.2); MS *m/z* (relative intensity, %) 262 (15, $M^+ - CH_3 - SO_3H$), 202 (18), 151 (40), 79 (100).

(7*R*)-[7-^{2}H]Brefeldin C (4)

7-*O*-Mesylbrefeldin A (155 mg, 0.43 mmol) and hexadecyltributyl phosphonium bromide ($HDTBP^+Br^-$)[11] (305 mg, 0.60 mmol) in dry toluene (5 ml) was added *via* syringe to a suspension of $NaBD_3CN$ (98 atom % D, 259 mg, 3.9 mmol) in dry toluene (1 ml), under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 15 hours then diluted with ethyl acetate and poured into a separatory funnel containing brine (20 ml). The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The organic extracts were combined, washed with 10% HCl, 10% $NaHCO_3$ and brine; and dried to yield a white crystalline solid (90.1 mg, 0.34 mmol, 80%): MP 152~153°C (recrystallized from hexane - acetone); ^{1}H NMR (500 MHz) δ 1.56~1.46 (2H, m); ^{2}H NMR δ 1.54; ^{13}C NMR δ 25.0 (t, J_{CD}=19); MS *m/z* (relative intensity, %) 265 (5, M^+), 247 (7), 205 (28), 122 (100).

The TBDMS ether was prepared by the literature method[6] from **4** (7.4 mg, 0.03 mmol), imidazole (58 mg, 0.85 mmol), TBDMS chloride (105 mg, 0.7 mmol) in DMF (1 ml) to yield a white solid (6.1 mg, 0.016 mmol, 36%): Rf 0.36 in *n*-hexane - ethyl acetate (95 : 5); MS *m/z* (relative intensity, %) 322 (12.9), 321 (1, $M^+ - 57$, d_0=7.3%, d_1=92.7%).

7-Oxobrefeldin A (3)

Fetizon reagent[14] was prepared as follows. Purified Celite was added to a mechanically stirred solution of silver nitrate (34 g, 200 mmol) in distilled H_2O (200 ml). A solution of $KHCO_3$ (21 g, 210 mmol) in distilled H_2O (300 ml) was added slowly to the homogeneous suspension. When the $KHCO_3$ addition was complete, stirring was continued for a further 10 minutes and the yellow green precipitate which had formed was filtered off and dried in a rotary evaporator over a period of several hours (ratio of Ag_2CO_3 - Celite 1 mmol/0.57 g). Fetizon reagent (32.3 g, 56.7 mmol) was added to a suspension of **1** (1.02 g, 3.64 mmol) in dry benzene. The reaction mixture was heated to reflux for 8 hours, cooled, filtered through a pad of Celite, and washed with ethyl acetate. The combined filtrate and washings were concentrated to give an oily residue consisting of a mixture of **3**, small amounts of 4,7-dioxobrefeldin A and starting material (Rf 0.38, 0.33 and 0.17, respectively). This mixture was separated by preparative TLC on silica gel, eluted with *n*-hexane - ethyl acetate (1 : 1), to yield **3** as a crystalline white solid (696 mg, 2.5 mmol, 68.7%), which was recrystallized from *n*-hexane - acetone, mp 120~122°C (literature[6] 122~123.5°C): Rf 0.52 in chloroform - methanol (9 : 1); ^{1}H NMR (200 MHz) δ 7.34 (1H, dd, J_1=15.7 and J_2=3.4), 5.90 (1H, dd, J_1=15.7 and J_2=1.8), 5.76 (1H, ddd, J_1=15.1, J_2=9.5 and J_3=4.7), 5.16 (1H, dd, J_1=15.1 and J_2=9.0), 4.85 (1H, m), 4.18 (1H, br d), 1.22 (3H, d, J=6.2); ^{13}C NMR δ 215.5, 166.4, 150.9, 135.3, 132.4, 118.5, 76.7, 71.9, 50.1, 46.8, 45.1, 42.6, 34.5, 31.7, 26.5, 20.8; MS *m/z* (relative intensity, %) 278 (2, M^+).

[7-^{2}H]-7-*O*-Mesylbrefeldin A (6)

Solid $NaBD_4$ (218 mg, 5.19 mmol) was added to a solution of **3** (629 mg, 2.26 mmol) in dry methanol (10 ml), magnetically stirred, at −78°C under a nitrogen atmosphere. The reaction mixture was maintained at the same conditions for 1 hour, the quenched with acetic acid - chloroform (1 : 1, 2 ml), diluted with ethyl acetate (50 ml), and poured into a separatory funnel containing 5% HCl (20 ml). The organic layer was separated, washed with 10% $NaHCO_3$, dried and concentrated to obtain a solid residue which was column chromatographed on silica gel in ethyl acetate to yield [7-^{2}H]brefeldin A as a crystalline white solid (567 mg, 2.02 mmol, 89.6%) which was recrystallized from ethyl acetate, mp 195~197°C (literature[15] 204~205°C): Rf 0.41 in chloroform - methanol (9 : 1); ^{1}H NMR (200 MHz, pyridine-d_5) δ no signal at 4.55; ^{13}C NMR (pyridine-d_5) δ very weak signal at 71.9.

The mesylate of [7-^{2}H]brefeldin A was prepared as described for **5**, using [7-^{2}H]-**1** (518 mg, 1.84 mmol), triethylamine (0.26 ml, 2.41 mmol), mesyl chloride (0.188 ml, 2.43 mmol) in pyridine (8 ml) to yield **6** as a white precipitate (430 mg, 1.20 mmol, 65%): ^{1}H NMR (200 MHz) δ no signal at 5.15~5.05 (1H, m).

(7*S*)-[7-^{2}H]Brefeldin C (7)

Solid $NaBH_3CN$ (200 mg, 3.18 mmol) was added to a solution of **6** (79 mg, 0.22 mmol) and $HDTBP^+Br^-$ (335 mg, 0.66 mmol) in dry toluene (5 ml). The reaction mixture was stirred at room temperature, and after 24 hours, only a small amount of 7-bromobrefeldin C and starting material were detected by TLC. The reaction was stirred 48 hours more at the same conditions, and worked up as described above to obtain a mixture of **7** and some 7-bromobrefeldin C which was separated by preparative TLC on silica gel, eluted with *n*-hexane - ethyl acetate (3 : 1), to yield **7** as a crystalline white solid (34.6 mg, 0.13 mmol, 59%): MP 151~153°C (recrystallized from *n*-hexane - ethyl acetate); Rf 0.3 in *n*-hexane - ethyl acetate (4 : 1); ^{1}H NMR (500 MHz) δ 1.64~1.57 (1H, m); ^{13}C NMR δ 26.1 (t, J_{CD}=20); ^{2}H NMR δ 1.63.

The TBDMS ether of this brefeldin C was prepared as described above from **7** (0.8 mg, 0.003 mmol), imidazole (10 mg, 0.15 mmol), TBDMS chloride (50 mg, 0.3 mmol) in DMF (0.4 ml) to yield the desired product: Rf 0.36 in *n*-heptane - ethyl acetate (95 : 5); MS *m/z* (relative intensity, %) 322 (19.74), 321 (1.74, M^+ −57, d_0=8.2%, d_1=91.8%).

(7*RS*)-[7-^{2}H]Brefeldin C (8)

Solid $NaBH_3CN$ (440 mg, 6.98 mmol) was added to a solution of **6** (178 mg, 0.49 mmol) and $HDTBP^+Br^-$ (48.6 mg, 0.1 mmol) in dry toluene (4 ml). The reaction mixture was stirred at room temperature for 5 days, then worked up as described before to yield **8** (65 mg, 0.25 mmol, 50%) as a

white crystalline solid: MP 151~152°C; the ^{1}H NMR spectrum was essentially identical to the spectrum of **2**; the ^{13}C NMR spectrum was identical to the spectra of the other deuterium labeled isomers; ^{2}H NMR (55.3 MHz) δ 1.65 and 1.57 (1:1).

The TBDMS ether of this brefeldin C was prepared as described above from **8** (1.8 mg, 0.007 mmol), imidazole (10 mg, 0.15 mmol), TBDMS chloride (50 mg, 0.3 mmol) in DMF (0.5 ml) to yield the desired product (2 mg, 0.005 mmol, 77%): Rf 0.36 in *n*-heptane - ethyl acetate (95:5); MS *m/z* (relative intensity, %) 322 (28.14), 321 (1.18, $M^+ -57$, d_0=4.1%, d_1=95.9%).

(7*RS*)-[7-^{3}H]Brefeldin C

This compound was prepared as described in ref 9.

TBDMS Ethers of **1** Isolated from the Feeding Experiments

These were prepared and analyzed by mass spectrometry as described in ref 6.

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