BIOSYNTHESIS OF BREFELDIN A

INTRODUCTION OF OXYGEN AT THE C-7 POSITION

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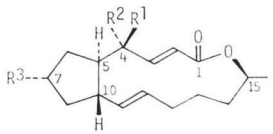
The biosynthetic introduction of oxygen in position 7 of brefeldin A, a structurally unique macrolide with an alicyclic ring, was studied. [4-^{2}H]Brefeldin C was prepared efficiently from brefeldin A. A high incorporation ratio of the labeled brefeldin C into brefeldin A by *Eupenicillium brefeldianum* clearly indicates that the oxygen in position 7 of brefeldin A does not contribute to the cyclopentane ring formation but is introduced during the last step of brefeldin A biosynthesis.

Recent progress in the spectroscopic analysis of stable isotopes, *e.g.* ^{13}C, ^{2}H or ^{18}O, has enabled more detailed biosynthetic study of multifunctional polyketides, such as macrolide and polyether antibiotics, to be done. Especially, the stereochemical consideration in those biogeneses has become possible with the utilization of ^{13}C–^{2}H or ^{13}C–^{18}O double labeling experiments.[1~3] The problem, however, may be that in most cases a single step in the biosynthesis of those antibiotics would not be studied unless an enzyme(s) which contributes to their biogenesis or to that of an appropriate intermediate can be obtained.

Antibiotic brefeldin A (**1**)[4] isolated from several Ascomycetes is a structurally unique macrolide in that it has an alicyclic ring and its cyclopentanol ring formation has been of interest in relation to that of prostanoid fatty acids.[5] Although the contribution of two oxygens at C-4 and C-7 in brefeldin A to the carbon ring closure has not been clarified yet, HUTCHINSON (see MABUNI *et al.*[5]) has proved that, in contrast to the case of prostanoids, two oxygen atoms in brefeldin A originate from two different molecules of oxygen, which was clearly shown by the study using ^{16}O and ^{18}O.

We have been interested in the biosynthetic ring formation in brefeldin A and have isolated brefeldin C (**2**)[6] from *Eupenicillium brefeldianum* in the course of searching for a biosynthetic intermediate or an analog of brefeldin A (**1**). The fact that brefeldin C (**2**) has no hydroxyl at C-7 indicates two possibilities. One is that **2** is a deoxygenated metabolite and the other is that brefeldin C is a precursor of brefeldin A. If the latter were true, C-7 hydroxyl of brefeldin A (**1**) should have been introduced after the cyclopentane ring formation. This suggests that synthetically labeled brefeldin C may be transformed into brefeldin A at a high incorporation ratio in the fungi. Thus we have synthesized brefeldin C, labeled stereospecifically with deuterium at C-4, and administered it to *E. brefeldianum*. The following is the synthesis of [4-^{2}H]brefeldin C (**3**) and the biosynthetic consideration of its incorporation into [4-^{2}H]brefeldin A (**4**).

Fig. 1.

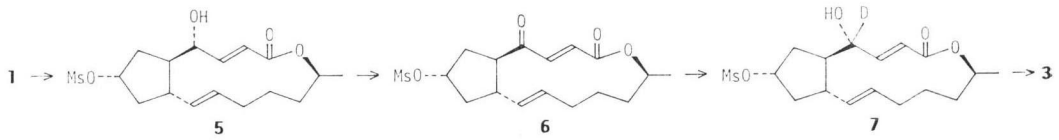


1 R^1 = H, R^2 = R^3 = OH
2 R^1 = R^3 = H, R^2 = OH
3 R^1 = D, R^2 = OH, R^3 = H
4 R^1 = D, R^2 = R^3 = OH
8 R^1 = D, R^2 = R^3 = OAc

Results and Discussion

[4-^{2}H]Brefeldin C (**3**) was prepared from brefeldin A (**1**) in 4 steps (Scheme 1). **1** was first mesyl-

Scheme 1.



ated (position-selectively) to obtain 7-mesyloxy brefeldin A (**5**) followed by Collins oxidation. 4-Dehydro derivative **6** was then reduced with $NaBD_4$ in MeOH at $-78°C$ to give [4-^{2}H]-7-mesyloxy brefeldin A (**7**)[7]. Deuterium was introduced stereospecifically into C-4β and no epimer was detected. Demesylation of **7** with NaI and Zn in 1,2-dimethoxy ethane gave [4-^{2}H]brefeldin C (**3**) in an overall yield of 41.6% from brefeldin A (**1**). Deuterium enrichment was 85.6%. The stereostructure of prepared **3** was confirmed by comparing its ^{13}C NMR with natural abundance spectrum of brefeldin C (**2**) (Table 1). ^{13}C resonances of **2** were assigned based on the comparison of spectra of natural abundance- and ^{13}C incorporated brefeldin A.[8] The latter was prepared from [1-^{13}C]-, [2-^{13}C]- or [1,2-^{13}C]acetate by incorporating those into *E. brefeldianum*. Labeled brefeldin A was isolated by AcOEt extraction from each fermentation broth followed by silica gel chromatography and by recrystallization from MeOH-$CHCl_3$. ^{13}C NMR of brefeldin A derived from [1,2-^{13}C]acetate showed eight pairs of doublets in which $^1J_{C-C}$ of two pairs due to C-3/C-4 and C-11/C-12 were so close, as shown in Table 1, 44.2 and 44.1 Hz respectively, that chemical shifts of the four carbons were confirmed by single frequency proton decoupling experiments.* Assignments for the rest of signals were made by comparing carbon resonances of brefeldin A and brefeldin C, and by the mode of ^{13}C enrichment in the ^{13}C labeled brefeldin A. C-4 signal observed at δ 75.6 for brefeldin C (**2**) shifts to δ 75.0, triplet ($^1J_{C-D}$=20.6 Hz), for [4-^{2}H]brefeldin C (**3**) and no ^{13}C{^{1}H} signal was observed.

Synthesized [4-^{2}H]brefeldin C was subjected to the following incorporation experiment using *E. brefeldianum* (IFO 8945) which shows high brefeldin A production efficiency. The fungus was fermented in shake flasks at 27° for 5 days and then [4-^{2}H]brefeldin C (**3**) was added to the broth. After three more days of fermentation, the culture broth was extracted with AcOEt and the extract was chromatographed on silica gel to obtain ^{2}H labeled brefeldin A (**4**).

Another experiment was performed in the presence of cerulenin[9] which is known to be an inhibitor of β-ketoacyl-acyl carrier protein synthetase in biosyntheses of fatty acids and other polyketides.[10] Since the preliminary experiment showed that cerulenin regulates the production of brefeldin A, we had expected higher enrichment of deuterium in [4-^{2}H]brefeldin A (**4**) with the controlled addition of cerulenin. Thus 15 μg/ml of cerulenin was added daily from the 3rd to the 7th day in the second experiment and [4-^{2}H]brefeldin A was isolated from the culture broth on the 8th day. Regardless of the presence of cerulenin, [4-^{2}H]brefeldin C (**3**) was transformed into [4-^{2}H]brefeldin A (**4**) efficiently in 99.5 or 75% yields depending on the respective condition (Table 2). In the EIMS analysis of brefeldin A, we have used 30 ionization voltage (eV) to reduce fragmentation of the molecular ion (M^+) since M^+ was too weak to measure at 70 eV. The effect of cerulenin appeared as the increased deuterium enrichment of *ca.* 47% (Table 2).

Deuterium incorporation into brefeldin A was confirmed by ^{13}C NMR of [4-^{2}H]brefeldin A (**4**) and ^{2}H NMR of its diacetate.[1] In the proton noise-off-resonance decoupled ^{13}C NMR spectrum of **4**

* Assignments for C-3 and C-11 are inconsistent with those reported.[8]

Table 1. ^{13}C NMR data[a] for brefeldins **1**, **2** and **3**.

Carbon	**1**[b]		**2**	**3**
	δ	$^1J_{C-C}$(Hz)	δ	δ
1	166.6	75.1	166.6	166.5
2	117.4	75.1	117.5	117.5
3	154.8	44.2	154.9	154.8
4	57.7	44.2	75.6	75.0(t[c])
5	52.9	33.8	54.6	54.4
6	42.2	33.8	32.7	32.6
7	71.8	35.3	25.5	25.5
8	44.0	35.3	35.5	35.5
9	44.6	45.6	47.0	47.0
10	137.8	45.6	137.0	136.9
11	129.8	44.1	130.2	130.1
12	32.2	44.1	32.3	32.2
13	27.0	35.3	27.1	27.0
14	34.1	35.3	34.2	34.2
15	71.3	38.2	71.3	71.2
16	21.0	38.2	21.0	21.0

[a] Recorded on a JEOL FX-100 spectrometer at 25.05 MHz in C_5D_5N; δ_c's were referred to internal TMS; temperature 25°C, aquisition time 0.68 seconds, spectral width 6,250 Hz, data points 8192, flip angle 36° or 45°, 10,000~20,000 scans.

[b] Labeled by the incorporation of [1,2-^{13}C]acetate.

[c] Multiplicity by proton noise decoupling.

Table 2. EIMS data for [4-^2H]brefeldin A[a].

		Isotopic labeling ratios[b] (normalized isotopic distribution)	
		Experiment 1[c] (mole%)	Experiment 2[d] (mole%)
m/z	281[e]	20.1 (0.25)	29.5 (0.42)
	280	79.9 (1.00)	70.5 (1.00)
	263	19.4 (0.24)	31.1 (0.45)
	262	80.6 (1.00)	68.9 (1.00)
	245	18.2 (0.22)	30.9 (0.45)
	244	81.8 (1.00)	69.1 (1.00)
Incorporation ratio[f]		99.5%	75.0%

[a] Mass spectra were recorded on a JEOL O1SG-2; ionization voltage 30 eV.

[b] Average of three determinations corrected for the natural isotopic abundances.

[c] [4-^2H]Brefeldin C incorporation without cerulenin.

[d] [4-^2H]Brefeldin C incorporation in the presence of cerulenin.

[e] Found 281.1711, Calcd. 281.1736 for $C_{16}H_{23}DO_4$.

[f] Brefeldin A (g)×mole%/brefeldin C (g)/0.856/281×265.

which is 29.5% deuterium enriched, the signal due to C-4 carbon bearing deuterium was observed at δ 75.2 as a triplet ($^1J_{C-D}$=19.1 Hz) which was not found in the spectrum by proton noise decoupling. Although the observation of natural abundunce ^{13}C coupled with incorporated deuterium has not been utilized in ^{13}C NMR analysis for any biosynthetic study, our results indicate that this technique could be useful in the incorporation study using a biosynthetic intermediate. Incorporated deuterium was also found to show a signal at δ 5.17 in the ^2H NMR spectrum of 4,7-di-*O*-acetyl-[4-^2H]brefeldin A (**8**) and its chemical shift was compatible with that observed in ^1H NMR of brefeldin A diacetate.

Conclusions

We have synthesized [4-^2H]brefeldin C stereospecifically and have shown that isotopically labeled brefeldin C was incorporated into brefeldin A at a high conversion ratio*. The latter fact clearly indicates that the oxygen in position 7 of brefeldin A does not contribute to the cyclopentane ring closure but is introduced in the last step of brefeldin A biosynthesis. The contribution of C-4 oxygen to the ring formation is under investigation.

Observation of the natural abundance ^{13}C signal which couples with incorporated deuterium may be the first successful application to the incorporation study of a biogenetic intermediate. This technique could be utilized as a support of ^2H NMR analysis, especially when an assignment(s) of ^2H shift(s) is troublesome.

* Similar results have been obtained by Dr. C. R. HUTCHINSON; personal communication.

Experimental

Melting points were taken on a Yanagimoto micro melting point apparatus and are uncorrected. Mass spectra were recorded on a Hitachi M-52-G mass spectrometer or a JEOL JMS-O1SG-2 instrument interfaced with a JMA-2000 data system. Gas chromatography-mass spectrometric conditions in the latter instrument were as follows; column, 1 m×3 mm id. glass column packed with 1% OV-17 on Uniport-HP; carrier gas, helium (0.8 kg/cm²); injection temperature, 250°C; column temperature, 196°C. IR spectra were recorded on a Hitachi IR-27G spectrophotometer; ^{1}H NMR on a Hitachi R-20 or a JEOL FX-100 spectrometer. Chemical shifts (δ) are referred to internal tetramethylsilane (δ 0). ^{2}H-{^{1}H} NMR spectrum was taken on a Bruker CXP-300 spectrometer at 46.06 MHz in which the chemical shift is reported in δ units with $CDCl_3$ as an internal standard; Spectral width 1000 Hz, acquisition time 2.05 seconds, 128 scans.

7-*O*-Mesyl Brefeldin A (**5**)

To brefeldin A (**1**, 400 mg, 1.43 mmole) and Et_3N (204 μliter, 1.46 mmole) in pyridine (5 ml) was added mesyl chloride (146 μliter, 1.89 mmole) dropwise at −13°C over a period of 11 minutes; the mixture was stirred at −13°C for 35 minutes. The reaction mixture was diluted with AcOEt, washed with 5% HCl, 10% $NaHCO_3$ aqueous and saturated NaCl aqueous, dried over anhydrous Na_2SO_4 and evaporated. The residue was chromatographed on silica gel; elution with *n*-hexane - AcOEt (3: 2) gave dimesylate (181 mg), **5** (365.5 mg, 86.4%) and the starting material (69.8 mg). **5**: mp 121~122°C, MS *m/z* 358 (M^+), IR ($CHCl_3$) ν 3410, 1710, 1645, 1260 cm^{-1}, ^{1}H NMR ($CDCl_3$, 100 MHz) δ 1.22 (3H, d, J=6.5 Hz), 3.23 (3H, s), 4.32 (1H, m), 4.94 (2H, m), 5.28 (1H, m), 5.71 (1H, m), 6.44 (1H, dd, J=1.5, 16 Hz), 7.55 (1H, dd, J=3, 16 Hz).

4-Dehydro-7-*O*-mesyl Brefeldin A (**6**)

To dipyridinium chromate (2.96 g, 11.4 mmole) in dry CH_2Cl_2 (8 ml) was added mesylate **5** (282 mg, 0.78 mmole) in dry CH_2Cl_2 (2 ml) dropwise at 0°C; the mixture was stirred at room temperature for 10 minutes. Silica gel chromatography of crude product on elution with 20% AcOEt in *n*-hexane gave ketone **6** which was crystallized from the same solvent. **6**: mp 110~111°C, MS *m/z* 260 ($M^+ - CH_3 \cdot SO_3H$), IR (KBr) δ 1728,1690, 1280, 1168 cm^{-1}, ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.34 (3H, d, J=6 Hz), 3.02 (3H, s), 4.75 (1H, m), 5.10 (1H, m), 5.72 (1H, m), 6.48 (1H, d, J=16 Hz), 7.78 (1H, d, J=16 Hz).

[4-^{2}H]-7-*O*-Mesyl Brefeldin A (**7**)

4-Dehydro derivative **6** (145.5 mg, 0.41 mmole) in MeOH (10 ml) was stirred with 98 atom % $NaBD_4$ (46 mg, 1.1 mmole) at −78°C for 2.1 hours. The reaction mixture was diluted with AcOEt (50 ml) and washed with cold 5% HCl, cold 10% $NaHCO_3$ aqueous and saturated NaCl aqueous, dried over anhydrous Na_2SO_4 and evaporated. The residue was chromatographed on silica gel [elution with *n*-hexane - AcOEt (3: 2)] to give **7** (72.2 mg, 68%) and the substrate **6** (40.2 mg). **7**: IR (KBr) ν 3400, 1700, 1340, 1265, 1170 cm^{-1}, ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.26 (3H, d, J=6.5 Hz), 3.00 (3H, s), 4.7~5.8 (4H, m), 5.92 (1H, d, J=15.5 Hz), 7.34 (1H, d, J=15.5 Hz).

[4-^{2}H]Brefeldin C (**3**)

7 (98 mg, 0.27 mmole) in 1,2-dimethoxy ethane (12 ml) was refluxed with NaI (212 mg, 1.41 mmole) and Zn powder (183 mg, 2.80 mmole) for 3.1 hours. Filtration and evaporation gave a crude product which was purified by silica gel chromatography (*n*-hexane - AcOEt, 2: 1) to give **3** (53.3 mg, 79%) and **6** (6.6 mg). **3**: mp 161~162°C, MS *m/z* (rel. intensity) 265, M^+ (Found 265.1793, Calcd. 265.1787 for $C_{16}H_{23}DO_3$) (1.00), 264, M^+ ($C_{16}H_{24}O_3$) (0.17), IR (KBr) ν 3460, 1690, 1640, 1267 cm^{-1}, ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.24 (3H, d, J=6.5 Hz), 4.86 (1H, m), 5.1~5.8 (2H, m), 5.90 (1H, d, J=16 Hz), 7.38 (1H, d, J=16 Hz).

Incorporation of [4-^{2}H]Brefeldin C (**3**)

a) *E. brefeldianum* (IFO 8945) was grown in three shake flasks each containing 100 ml of medium at 27°C for 4 days. The composition of the medium was glucose - malt extract - yeast extract - peptone - KH_2PO_4 - $MgSO_4 \cdot 7H_2O$ (20–2–2–2–2–2 g/liter of medium). [4-^{2}H]Brefeldin C (**3**, 15 mg, 5 mg/100 ml) was added to the culture which was cultured for three more days. Mycelia were filtered off and the broth was extracted with AcOEt. Silica gel chromatography of AcOEt extract and elution with AcOEt

gave a crystalline mass which was recrystallized from $CHCl_3$-MeOH to give [4-^{2}H]brefeldin A (**4**, 67.4 mg): mp 212~213°, IR (KBr) ν 3370, 1711, 1641, 1260 cm^{-1}, ^{1}H NMR (C_5D_5N, 60 MHz) δ 1.26 (3H, d, J=6.5 Hz), 4.4 (*ca.* 1.8H, m), 4.9 (1H, m), 5.6 (2H, m), 6.48 (1H, dd, J=1.5, 15.5 Hz), 7.74 (1H, dd, J=2, 15.5 Hz). b) The experimental procedure was essentially the same except that cerulenin (6.73 μmole/100 ml) was added to the culture from the 3rd to the 7th day. Isolated [4-^{2}H]brefeldin A (**4**) was 34.6 mg.

[4-^{2}H]Brefeldin A 4,7-Diacetate (**8**)

4 (22 mg) in pyridine (1 ml) and Ac_2O (0.5 ml) were left standing at room temperature overnight. Et_2O extract was chromatographed over silica gel to give **8** (28.9 mg, 99.5%): IR (KBr) ν 1726, 1710, 1255, 1235 cm^{-1}, ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.24 (3H, d, J=7 Hz), 2.00 (3H, s), 2.09 (3H, s), 4.9 (1H, m), 5.17 (*ca.* 0.8H, m), 5.2~5.8 (2H, m), 5.71 (1H, dd, J=1.5, 15.5 Hz), 7.27 (1H, dd, J=2, 15,5 Hz).

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References

1) HUTCHINSON, C. R.; I. KUROBANE, D. E. CANE, H. HASLER & A. G. MCINNES: Biosynthesis of macrolide antibiotics. 4. Stereochemistry of hydrogen labeling of brefeldin A by [2-$^2H_3$]acetate. J. Am. Chem. Soc. 103: 2477~2480, 1981
2) HUTCHINSON, C. R.; M. M. SHERMAN, A. G. MCINNES, J. A. WALTER & J. C. VEDERAS: Biosynthesis of macrolides. 6. Mechanism of stereocontrol during the formation of lasalocid A. J. Am. Chem. Soc. 103: 5956~5959, 1981
3) CANE, D. E.; T.-C. LIANG & H. HASLER: Polyether biosynthesis. Origin of the oxygen atoms of monensin A. J. Am. Chem. Soc. 103: 5962~5965, 1981
4) WEBER, H. P.; D. HAUSER & H. P. SIGG: Structure of brefeldin A. Helv. Chim. Acta 54: 2763~2766, 1971, and references cited therein.
5) MABUNI, C. T.; L. GARLASCHELLI, R. A. ELLISON & C. R. HUTCHINSON: Biosynthesis of the C_{15}- macrolide antibiotics. 1. Biochemical origin of the four oxygen atoms in brefeldin A. J. Am. Chem. Soc. 101: 707~714, 1979, and references cited therein.
6) SUNAGAWA, M.; T. OHTA & S. NOZOE: Isolation and structure of brefeldin C. Heterocycles 13: 267~270, 1979
7) COREY, E. J. & R. H. WOLLENBERG: Useful stereoselective and position-selective transformations of brefeldin A and derivatives at carbons 4, 7 and 15. Tetrahedron Lett. 1976: 4701~4704, 1976
8) HUTCHINSON, C. R.; I. KUROBANE, C. T. MABUNI, R. W. KUMOLA, A. G. MCINNES & J. A. WALTER: Biosynthesis of macrolide antibiotics. 3. Regiochemistry of isotopic hydrogen labeling of brefeldin A by acetate. J. Am. Chem. Soc. 103: 2474~2477, 1981
9) HATA, T.; Y. SANO, A. MATSUMAE, Y. KAINO, S. NOMURA & R. SUGAWARA: Study of new antifungal antibiotic. Jpn. J. Bacteriol. 15: 1075~1077, 1960
10) ŌMURA, S.: The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. 40: 681~697, 1976