Biosynthesis of the C_{15} Macrolide Antibiotics. 1. Biochemical Origin of the Four Oxygen Atoms in Brefeldin A[1]

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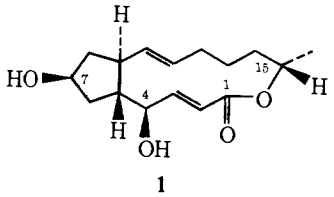
Abstract: The biochemical origin of the four oxygen atoms present in brefeldin A (**1**), a 16-membered macrolide antibiotic, is determined by feeding experiments with sodium [$^{18}O_2$,2-^{3}H]acetate and $^{36}O_2$ in which the labeling regiochemistry of **1** is shown by EI mass spectral analysis. Brefeldin A is labeled only at C-1 and C-15 by [$^{18}O_2$]acetate. These two positions are proven to be equally ^{18}O enriched by analysis of the EI mass spectral fragmentation pattern of a derivative of **1**. The resulting equal C-1 and C-15 labeling is interpreted as evidence for a one-step, intramolecular mechanism of macrolide ring formation in vivo. The incorporation of [$^{18}O_2$]acetate into **1** is accompanied by loss of 46% of its ^{18}O enrichment as determined from the dilution of the [2-^{3}H]acetate reference label. This is interpreted to imply that the carbonyls of the intermediate polyketide may exist as ketone hydrates in vivo. Since the antibiotic was labeled at C-4 and C-7 by $^{36}O_2$, but by two different molecules of oxygen as proven by the results of a feeding experiment in a $^{32}O_2$–$^{36}O_2$ gas mixture, it is concluded that the bioorganic mechanism for formation of the cyclopentanol ring of **1** does not closely parallel prostaglandin biosynthesis. A new hypothesis for this ring formation is made, based on the oxidosqualene to lanosterol cyclization as precedent. The lack of C-15 labeling of **1** by $^{36}O_2$ is further evidence against a C-16 fatty acid biosynthetic origin for **1**.

The biosynthesis of macrolide antibiotics has been studied for many years, in part owing to the considerable interest in the chemistry and industrial production of the important chemotherapeutic members of this class of antibiotics.[3] Since the advent of rapid spectroscopic techniques for localization of isotopic labels introduced by ^{13}C- and ^2H-labeled precursors, knowledge about the early stages of macrolide biosynthesis, i.e., identification of the biochemically simple C_2–C_4 carbon precursors, has been uncovered rapidly.[4] The results of such studies can also define certain boundary conditions for the biochemical elaboration of the penultimate C_{12}–C_{38} carbon skeletons, although the most fruitful use of this approach has been in biosynthetic studies of aromatic polyketide antibiotics via the use of doubly ^{13}C-labeled[5] and ^2H-labeled[6] acetate. In the case of macrolide antibiotics such technology has not yet been applied toward a deeper understanding of the biosynthetic transformations subsequent to the chain-forming condensation reaction of the C_2–C_4 thiol esters. For the most studied of macrolide antibiotics, the erythromycins, the limited amount of information available about the biochemistry of the dehydrations, reductions, and oxidations that must occur during their biosynthesis has come from the use of blocked mutants and the isolation of relevant enzymes (glycosyl transferases, methylases, oxidases).[7]

We are intrigued by the stereochemical homology among the 14- and 16-membered macrolide antibiotic aglycones,[8] specifically its underlying biochemical basis. Thus we have initiated biosynthetic studies with stable and radioactive isotopes of certain macrolide antibiotics, with the goal of elucidating stereochemical details of their biogenesis such as are associated with chain-lengthening condensations, hydrogen-atom introduction, and ring-forming cyclizations. The results from one biosynthetic study are described in detail here.

Brefeldin A (**1**), a 16-membered macrolide antibiotic produced by several genera of Ascomycetes,[9] has an obvious structural resemblance to the prostaglandins. This has led to the speculation that **1** might also be biosynthesized from a fatty acid in a manner paralleling prostaglandin biosynthesis, because it was reported in 1969 that [9-^{14}C]palmitic acid was specifically incorporated into **1** to a limited extent in *Penicillium cyaneum*.[10] However, since the intact incorporation of [^{14}C]palmitate into **1** could not be confirmed in *P. lilacinum*,[11] nor in *P. brefeldianum*,[12] this biosynthetic hypothesis was withdrawn subsequently.[12]

We commenced our study with the knowledge from earlier biosynthetic investigations that **1** was a polyketide secondary metabolite based on the reported specific incorporations of [^{14}C]acetate[13] and [^{14}C]malonate[13c] into **1**, in which an acetate "starter effect" had been noted for carbons 15 and 16 during the incorporation of [2-^{14}C]malonate. Our principal interest was in the bioorganic mechanism of cyclopentanol ring formation. Two hypothetical mechanisms for this had been advanced by previous investigators: one involving a [2 + 2 + 2] cycloaddition between O_2 and C_{16} diene[13a] and the other invoking a dihydro-1,2-dioxin intermediate in analogy to the 1,2-dioxolane (PGG_1) involved in prostaglandin biosynthesis.[10] Since the validity of these biogenetic proposals depends on the biochemical origin of the oxygens at C-4 and C-7 of **1**, we sought the answer to this question via [$^{18}O_2$]acetate and [$^{16}O_2$,$^{18}O_2$]O_2 feeding experiments.



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Results and Discussion

Since we planned to determine the ^{18}O labeling regiochemistry of **1** obtained from feeding experiments by low-resolution electron-impact mass spectrometric (EIMS) analysis, it was necessary to determine the mass spectral fragmentation pattern of **1** and suitable derivatives of it. Both **1** and its 4,7-diacetate[9a] fragment under EIMS conditions by loss of 2 mol of water or acetic acid giving an ion at *m/e* 244.1453, whose elemental formula is consistent with the molecular structure **3**. EIMS analysis of bis(4-[$^2H_3$]acetyl-7-[H_3]acetyl)-**1** (Experimental Section) showed that the loss of acetic acid occurs stepwise, first by heterolysis of the allylic C^4–O bond (→ **2**, *m/e* 304), then of the aliphatic C^7–O bond (→ **3**). For complementary data, a sample of [7-^{18}O]-**1** was prepared as follows. Acid-catalyzed exchange of 4-*O*-acetyl-7-oxo-**1**[14]

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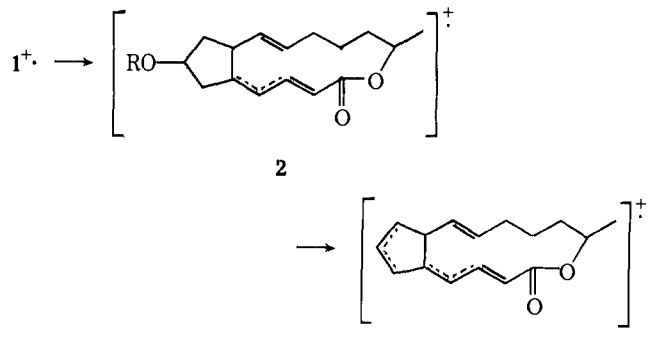

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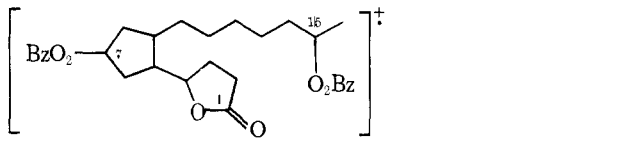
with $[^{18}O]H_2O$ gave 4-*O*-acetyl-[7-^{18}O]-7-oxo-**1**. Low-temperature reduction of this ketone with $NaBH_4$ gave a major product identical with 4-*O*-acetyl-**1** and a minor product, not fully characterized but which appeared to be 4-*O*-acetyl-7-*epi*-**1** by NMR and chromatographic analysis. Deacetylation of this mixture with methanolic base gave the desired [7-^{18}O]-**1** and presumably its C-7 epimer, which were separated and purified chromatographically. EIMS analysis of [7-^{18}O]-**1** (Experimental Section) showed that the loss of water from $\mathbf{1}^{+\cdot}$ also occurs stepwise in a sequence identical with that of its diacetate: $\mathbf{1}^{+\cdot} \rightarrow \mathbf{2}$ (*m/e* 264) $\rightarrow$ **3**.

Since it was anticipated that **1** would become ^{18}O labeled at the oxygens attached to carbons 1 and 15, the tetrahydro-γ-lactone (**4**), obtained from **1** by catalytic hydrogenation,



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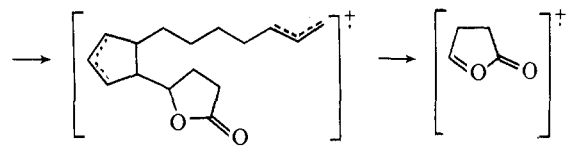

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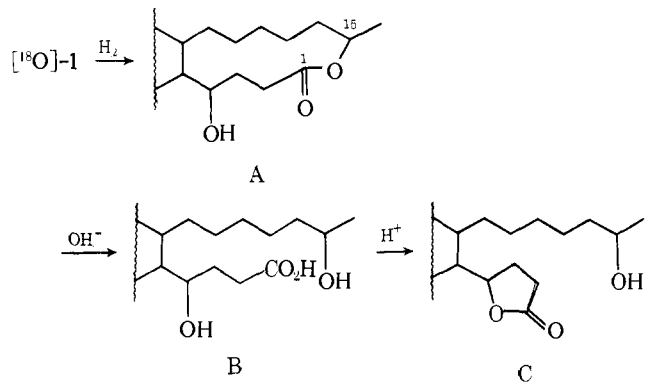
saponification, acid-catalyzed relactonization,[9b] and benzoylation, was chosen as a suitable derivative for EIMS analysis.[15] The EIMS spectrum of a sample of [7-^{18}O]-**4**, prepared from [7-^{18}O]-**1** (vide supra), indicated that a random loss of 2 equiv of $C_7H_6O_2$ from the weak molecular ion occurs, resulting in a fragment ion at *m/e* 248. We assign the arbitrary structure **5** to this fragment ion since it did not contain any ^{18}O enrichment (Experimental Section). The oxygen of the C-1 carbonyl of **4** is contained in the fragment ion (**6**) at *m/e* 85.0292, which was proven as follows. Tetrahydro-**1**[9b] was saponified in methanolic potassium methoxide containing $[^{18}O]H_2O$ (95 mol % ^{18}O) and [1-^{18}O]-**4** obtained from this reaction mixture by acidification, benzoylation, and chromatographic purification. EIMS analysis of a sample of this ^{18}O-labeled lactone showed that 30 ± 4 mol % of the molecules contained one ^{18}O atom. Since trial runs had showed that the experimental manipulations resulted in a small dilution of the ^{18}O content of the labeled water used in the synthesis (to 82 ± 5 mol % ^{18}O), the theoretical ^{18}O enrichment of [1-^{18}O]-**4** is 41 ± 5 mol % excess ^{18}O. The lower value found experimentally reflects the expected acid-catalyzed exchange of carbonyl-bound ^{18}O during lactonization of the intermediate [1-^{18}O]tetrahydro-**1**-ω-2-hydroxy acid. This appears to have resulted in a $\leq$27% loss of ^{18}O enrichment. Another consideration is the weak intensity of the molecular ion in EIMS analysis of **4**, which can lead to a rather poor experimental accuracy. Nevertheless, it was clear that the fragment ion (**5**) contained all the ^{18}O present in [1-^{18}O]-**4**, and that this ^{18}O appeared to reside solely in **6** (Experimental Section), which represents carbons 1-4 and the C-1 and C-4 oxygens of **1**.[9b]

[^{18}O]Acetate Incorporation. *Penicillium brefeldianum* Dodge (NRRL 2083), the best of several reported producers of **1**[9] that we screened, was grown in shake culture on a defined medium (Czapek-Dox) until a bright yellow pigment was just appearing.[16] This succeeded the appearance of **1** in the fermentation medium by ca. 1 day and coincided with the maximum in the pH of the cultures (8.2). Sodium $[^{18}O_2]$acetate[17] admixed with sodium [2-^{3}H]acetate was fed all at once[18] to one culture and, after 3 days of further incubation, labeled **1** was isolated and purified by chromatography and recrystallization to a constant specific radioactivity. Separate aliquots of this [^{18}O,^{3}H]-**1** were converted to its 4,7-diacetate and **4**, the latter by conditions identical with those used in the preparation of synthetic [1-^{18}O]-**4**.

The results of the EIMS analysis of these two derivatives of [^{18}O,^{3}H]-**1** are shown in Table I. It is immediately apparent from the fragmentation pattern of 4,7-diacetyl-[^{18}O,^{3}H]-**1** that all of the ^{18}O enrichment of the $M^{+\cdot}$ is present in the *m/e* 244 fragment ion **3** within experimental error. Therefore, either the oxygen at C-7 of **1** is not labeled by [^{18}O]acetate even though it is at a carbonyl position of some putative polyketide biosynthetic intermediate, or the ^{18}O label was lost by exchange from carbonyl-bound oxygen during the biosynthesis of **1**.[19] The lack of ^{18}O labeling by [^{18}O]acetate of the oxygen at C-4 of **1** is to be expected since this is not at a carbonyl position of any intermediate polyketide.

Additional information that is of general relevance to macrolide antibiotic biosynthesis is available if the data in Table I are analyzed further.

The processes used to make **4** from biosynthetically ^{18}O-labeled **1** can be depicted as A $\rightarrow$ B $\rightarrow$ C. If [^{18}O]-**1** consists

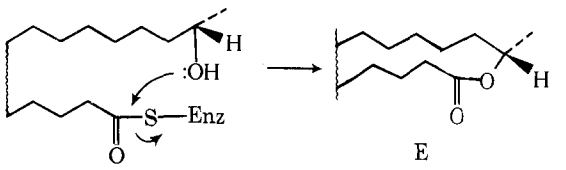


A B C

of a statistically equal mixture of molecules labeled equally at C-1 and C-15 with ^{18}O, so would A, but B would have its ^{18}O enrichment at C-1 diluted by 50%. This is a result of saponification of the macrolide ester bond, since it was carried out in methanolic KOH. Then during the relactonization to give C, a further dilution of ca. 27% of the ^{18}O enrichment at C-1 of B would occur as proven above. Therefore, the ratio of the relative ^{18}O enrichment of the oxygen bound to C-1 vs. C-15 of **1** is calculated to be A, 1:1; B, 1:2; C, 1:2.7. Since C is **4** when benzoylated it then should be found that the isotopically labeled ions giving rise to the M· + 2 peak of **4** that is obtained from [^{18}O]-**1** consist of a statistical mixture of molecules, 27% of which are labeled at C-1 and 73% of which are labeled at C-15. Of course, this cannot be proven if only the molecular ions' isotopic abundance is considered, but it can if the EIMS fragmentation pattern is analyzed for the relative isotopic abundances of the fragment ions. The normalized isotopic distribution values shown for **4** in Table I indicate that 26% of the normalized ^{18}O enrichment of the M· + 2 peak of **4** at *m/e* 494 is contained in the M· + 2 ions of **5** at *m/e* 250. Since no further loss of ^{18}O occurs on fragmentation of **5** to **6**, which

contains the oxygen originally attached to C-1 of **4**, the excellent agreement between the theoretical and the experimental ^{18}O enrichment of C-1 of **4** is proof that **1** was equally ^{18}O labeled at C-1 and C-15.

The equal ^{18}O labeling of C-1 and C-15 of **1** is evidence that the macrolide ring closure step takes place in a single, intramolecular event in vivo. This cannot involve a free 15-hydroxy carboxylic acid intermediate; otherwise, the ^{18}O enrichment of C-1 of **1** would have been one-half that of C-15.[20] Consequently, it is sensible to conclude that the precursor of **1** is enzyme bound as a thioester and is released from the enzyme directly as a macrolide (D → E). If this can be proven experimentally in future studies using cell-free or pure enzyme systems, it would be the biological analogy to the recently developed synthetic methodologies that employ thioesters for macrolide ring closure.[7]



D E

The dilution of ^{18}O label relative to the dilution of ^{3}H label during the incorporation of [$^{18}O_2$,2-^{3}H]acetate into **1** indicates if any ^{18}O was lost by exchange of carbonyl-bound ^{18}O with environmental [^{16}O]water. (a) One can calculate the dilution of ^{18}O label as follows. The acetate fed contained 82.1 mol % $^{18}O^{18}O$, 14.3 mol % $^{16}O^{18}O$, and 3.6 mol % $^{16}O^{16}O$. Since one of its two oxygen atoms must be lost during its incorporation into **1**, it follows that the doubly labeled molecules contribute 82.1% and the singly labeled molecules contribute 7.1% (one-half of 14.3, assuming that the acetate is labeled equally at both oxygen sites) of the molar ^{18}O content of the acetate fed. Consequently, 89.2% of this acetate contained a potentially exchangeable ^{18}O label. The isolated [^{18}O]-**1** contained 8.2 mol % $^{18}O^{18}O$, 35.4 mol % $^{16}O^{18}O$, and 53.8 mol % $^{16}O^{16}O$ as shown in Table I for the isotopic distribution of the molecular ions of 4,7-diacetyl-**1**. Since each doubly labeled molecule contributes 2 molar equiv of ^{18}O, the total ^{18}O content of this [^{18}O]-**1** is the sum of 35.4 and twice 8.2 = 51.8 mol % ^{18}O. There are only two sites labeled by [^{18}O]acetate in **1**; therefore, if all the molecules were ^{18}O labeled, it would contain 200 mol % ^{18}O. Since it contains 51.8 mol % ^{18}O, only 25.9% of the molecules are ^{18}O labeled. Hence, the [^{18}O]acetate was diluted 89.2 divided by 25.9 = 3.44-fold upon incorporation into **1**.[21]

(b) The dilution of the ^{3}H label is calculated by dividing the specific radioactivity of the [^{3}H]acetate fed by one-eighth[22b] of the specific radioactivity of the isolated [^{3}H]-**1**, since eight C_2 units are assembled in vivo to make **1**. However, the specific radioactivity of the [^{3}H]-**1** isolated must be corrected for the fact that acetate loses 71.9% of its ^{3}H content on incorporation into **1**.[22a] When this is done (Experimental Section), the dilution of ^{3}H label per C_2 unit is found to be 1.84-fold during the incorporation of the [2-^{3}H]acetate into **1**. If no ^{18}O were lost by exchange processes during the incorporation of [^{18}O]acetate into **1**, the dilution of ^{18}O and ^{3}H labels would be identical. Since the ^{18}O label is diluted 1.89 times more than the ^{3}H label, it calculates that about 46% of the molar ^{18}O content of the [$^{18}O_2$]acetate fed is lost by exchange with water during its incorporation into **1**. This result is contrary to that published originally,[1] wherein the interpretation was based on an erroneous method of calculating the ^{18}O dilution value.

The exchange of ^{18}O with the environment during the metabolism of acetate in vivo is to be expected based on the known exchange rates of carbonyl-bound oxygen as a function of pH.[23] It is impossible to determine when this occurs from the available data, although it could occur most easily before acetate is converted to the thioester intermediates of the polyketide biosynthetic pathway. The latter should have a much slower exchange rate with water than a free carboxylic acid.[23] Nevertheless, it invites speculation in the finding that only about one-half the ^{18}O label exchanges between the stage of acetate and **1**. Since the same amount of ^{18}O loss relative to a radioactive reference label has been observed in two other studies of the incorporation of [^{18}O]acetate into polyketide secondary metabolites,[20] it seems too coincidental for this loss to occur at the level of acetate. An alternative explanation of these data is that in the intermediate polyketide chain on the enzyme governing the assembly of the C_2 units, the carbonyls exist as ketone hydrates.[20] This might help stabilize the growing polyketide chain, and could result in the loss of one-half of the ^{18}O from carbonyl positions during the incorporation of [^{18}O]acetate. It is of considerable interest to confirm or invalidate this hypothesis in vitro since there is yet no satisfactory explanation of how the chemically reactive polycarbonyl intermediates of polyketide biosynthesis are stabilized in vivo.

Table I. EIMS Analysis of **1** Labeled by Sodium [$^{18}O_2$,^{3}H]-Acetate[a]

isotopic distribution[b] (normalized isotopic distribution)			
4,7-diacetyl-**1**		**4**	
m/e	mol %	*m/e*	mol %
368	8.2 ± 0.7 (0.15)	496	2.1 ± 2.8
367	*c*	495	2.7 ± 3.6
366	35.4 ± 0.5 (0.66)	494	28.8 ± 2.8 (0.47)
365	2.5 ± 2.1	493	5.1 ± 3.6
364[d]	53.8 ± 3.1 (1.00)	492[d]	61.3 ± 4.0 (1.00)
309	1.7 ± 0.8	374	1.0 ± 0.4
308	6.5 ± 1.4 (0.13)	373	*c*
307	4.1 ± 3.5	372	19.6 ± 0.5 (0.25)
306	31.1 ± 1.6 (0.60)	371	*c*
305	4.7 ± 1.6	370	79.4 ± 0.9 (1.00)
304	51.9 ± 0.4 (1.00)		
		251	0.5 ± 0.1
248	8.4 ± 1.5 (0.15)	250	9.7 ± 0.2 (0.12)
247	1.1 ± 0.8	249	6.6 ± 0.5
246	31.6 ± 0.9 (0.55)	248	83.1 ± 0.3 (1.00)
245	1.1 ± 0.6		
244	57.2 ± 1.6 (1.00)	88	0.5 ± 0.4
		87	10.4 ± 0.3 (0.12)
		86	2.0 ± 0.2
		85	87.0 ± 0.5 (1.00)

[a] Sodium [$^{18}O_2$]acetate, 82 mol % $^{18}O_2$, 2.38 g admixed with [2-^{3}H]acetate (25.8 μCi) was fed to one 500-mL shake culture. [b] Average of three determinations; corrected for the natural isotopic abundances[33]. [c] No ion observed owing to weak intensity. [d] M^+.

$^{36}O_2$ Incorporation. *P. brefeldianum* was grown in shake culture as before but in a system that could be made air-tight. After the pigment's appearance, the system was flushed free of $^{32}O_2$ by repeated evacuation–refilling cycles with N_2 and filled with $^{36}O_2$ (Table II, expt 1), or with a $^{32}O_2/^{36}O_2$ mixture (Table II, expt 2). A [^{14}C]acetate reference label also was included in these experiments and one flask was left unsealed for a control. After 3 days further metabolism in the sealed system, [^{18}O,^{14}C]-**1** was isolated and purified as before. EIMS analysis of the [^{18}O,^{14}C]-**1** from expt 1 showed a negligible intensity for the M· + 2 peak at *m/e* 282, which reflects the small amount of $^{32}O_2$ present during the experiment. The fragment ions at *m/e* 262 (**2**) showed a negligible intensity for their P· + 4 peak, but the same mol % ^{18}O content in their P· + 2 peak as found in the M· + 4 ions of [^{18}O,^{14}C]-**1**. This shows that ^{18}O label was located at C-4 of **1** as proven above. Finally,

Table II. EIMS Analysis of **1** Labeled by $^{36}O_2$

isotopic distribution[a] (normalized isotopic distribution)			
1, expt 1[b]		**7**, expt 2[c]	
m/e	mol %	*m/e*	mol %
284	40.9 ± 0.7 (0.71)	341	2.3 ± 0.2 (0.025)
282	*d*	340	*d*
280[e]	57.7 ± 1.6 (1.00)	339	5.5 ± 0.03 (0.060)
		338	*d*
264	39.5 ± 1.9 (0.68)	337[f]	92.2 ± 0.2 (1.00)
262	58.4 ± 0.9 (1.00)		
247	3.0 ± 0.4 (0.04)		
246	10.5 ± 3.5 (0.12)		
245	*d*		
244	86.5 ± 1.6 (1.00)		

[a] Average of three determinations corrected for the natural isotopic abundances.[33] [b] The mole percent $^{36}O_2$ in the system was 94.1 at 0 h; $^{32}O_2$, 3.9; and no oxygen could be detected at 72 h. [c] The mole percent $^{36}O_2$ of all oxygen in the system was 46 at 0 h, 44 at 11 h, and 40 at 79 h. No $^{34}O_2$ could be detected, although the purchased gas analyzed (Mound Laboratory, Miamisburg, Ohio) for 3% $^{34}O_2$ and no $^{16}O^{18}O$. [d] Peak intensity too weak to measure. [e] $M^{+\cdot}$. [f] $M^{+\cdot} - 57$.

almost all the remaining ^{18}O was lost on further fragmentation to the *m/e* 244 ions (**3**), indicating that **1** had not been labeled at C-1 or C-15 by $^{36}O_2$, but only at C-4 and C-7. The small apparent ^{18}O enrichment of **3** may be real, reflecting a minor pathway for $^{36}O_2$ incorporation into carbonyl-derived positions of **1** via water.[24] We cannot comment further about this since additional EIMS analysis of the [^{18}O,^{14}C]-**1** was not done.

EIMS analysis of the [^{18}O,^{14}C]-**1** from expt 2 indicated that only a small ^{18}O enrichment of **1** had been achieved. This probably was the result of a low rate of brefeldin A biosynthesis during the experiment, since the control had a specific ^{14}C incorporation of acetate into **1** ca. four times greater than that of the sealed system. The sealed system also gave a specific ^{14}C incorporation of acetate into **1** ca. one-sixth that of the **1** produced in expt 1. Since the intensity of the $M^{+\cdot}$ of **1** or its diacetate was weak under the EIMS analyses conditions, the mono-C^7-*tert*-butyldimethylsilyl derivative of [^{18}O,^{14}C]-**1** (**7**) was prepared and used for mass spectral analysis. This derivative gives an intense $M^{+\cdot} - 57$ fragment ion, due to the loss of isobutene,[25] that permitted precise EIMS analysis of this ion's isotopic enrichment despite the low ^{18}O content. As shown in Table II, expt 2, the brefeldin produced when the sealed system's atmosphere contained an approximately equal mixture of $^{32}O_2$ and $^{36}O_2$ consisted of both singly and doubly ^{18}O-labeled **1**.

If the relative abundance of the M· + 2 and M· + 4 ions of [^{18}O,^{14}C]-**1** is analyzed, it can be determined if the oxygens located at C-4 and C-7 come from the same molecule of oxygen or from two separate oxygen molecules.[26] The rationale for the experiment is as follows. If the metabolite, which contains two positions known to be labeled by O_2, is produced in a system containing a mixture of $^{32}O_2$ and $^{36}O_2$ (but no $^{16}O^{18}O$ or $^{34}O_2$), the probability that the positions will be labeled by 2 ^{16}O, ^{16}O + ^{18}O, or 2 ^{18}O is a function of the relative amount of $^{32}O_2$ and $^{36}O_2$ in the system. This must be considered in two ways. (a) If both positions are labeled by the same molecule of oxygen, only $M^{+\cdot}$ and M· + 4 ions will be observable in the mass spectrum. Thus the ratio of the relative abundance of the M· + 2 and M· + 4 ions will be ca. zero. Of course, when the atmosphere contains a significant quantity of $^{16}O^{18}O$ or $^{34}O_2$, this ratio may be greater than zero. In expt 2, the $^{34}O_2$ content of the purchased oxygen was 3 mol %; thus, the theoretical ratio of M· + 2 to M· + 4 in [^{18}O,^{14}C]-**1** is ≤0.07. (b) If both positions are labeled by two different molecules of O_2, M· + 2 and M· + 4 ions will be observable in the mass spectrum. Their relative abundance ratio is calculated by the equation

$$(X + Y + Z)^2 = X^2 + Y^2 + Z^2 + 2(XY + XZ + YZ) \quad (1)$$

where X equals the mol % $^{32}O_2$, Y equals the mol % $^{36}O_2$, and Z equals the mol % $^{34}O_2$ in the system. Thus, the M· + 2 ions' abundance is represented by $2XY + Z^2$, and the M· + 4 ions' by Y^2, from which their ratio, $(M\cdot + 2)/(M\cdot + 4) = Z^2/Y^2 + 2XY/Y^2$. In expt 2, the mol % of $^{36}O_2$ in the system decreased slowly during the metabolism period.[27] Using an average value of 43 mol % $^{36}O_2$, the theoretical intensity ratio of M· + 2 to M· + 4 is 2.66 for [^{18}O,^{14}C]-**1**. Since the ratio found from the *m/e* 339 and 341 ions' relative abundance (Table II) is 2.39, it appears that C-4 and C-7 of brefeldin A were labeled by two different molecules of O_2, not one.

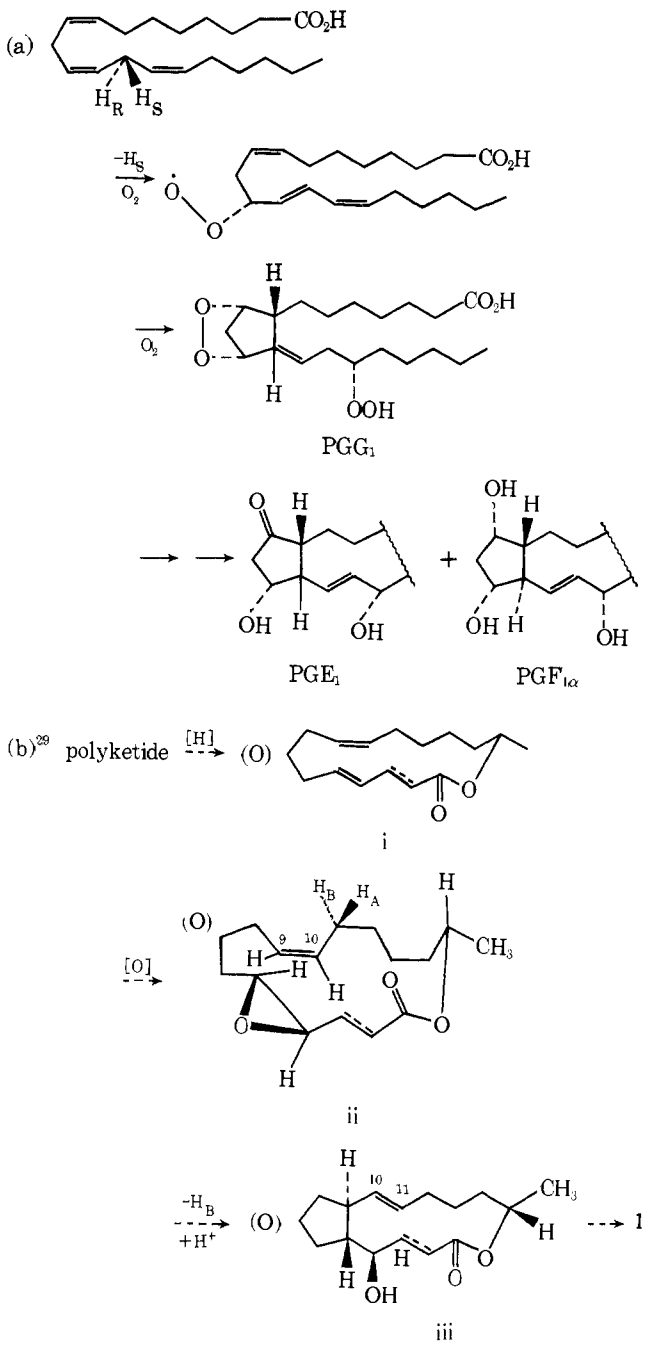
Conclusions

We have shown that the four oxygen atoms present in brefeldin A (**1**) are derived from the carbonyl position of some polyketide intermediate (at C-1 and C-15), and from two separate molecules of atmospheric O_2 (at C-4 and C-7). The former result does not support a fatty acid origin for **1**, since C-15 oxygenation of a saturated (or unsaturated) fatty acid precursor would be expected to occur via an $\omega 2$ mixed-function monooxygenase.[28] This necessarily would involve molecular oxygen.[28] The latter result is convincing evidence that the bioorganic mechanism by which the cyclopentanol ring of **1** is formed does not closely parallel prostaglandin biosynthesis, since one molecule of O_2 is known to give rise to the cyclopentanol oxygens in this case[26] via PGG_1 (Scheme Ia). The latter result does not rule out the possibility that two molecules of O_2 could be involved in the ring cyclization mechanism, perhaps by a slight modification of the Bu'Lock and Clay hypothesis.[10] We favor an alternate cyclization mechanism (Scheme Ib) that is modeled after the squalene → lanosterol cyclization involving 2-oxidosqualene.[30] This hypothesis implies that the C-4 hydroxyl of **1** would be a necessary result; hence, we plan to ascertain if the hydroxyls at C-4 or C-7 of **1** are a prerequisite for ring formation, or simply "cosmetic afterevents" via 4,7-dideoxybrefeldin A. Finally, the apparently equal ^{18}O enrichment of C-1 and C-15 by [^{18}O]acetate is the first evidence to have been obtained about the mechanism of macrolide ring closure during the biosynthesis of such antibiotics. The conclusion we have drawn about this indicates that a separate "macrolide cyclase" enzyme may not be involved in the biosynthesis of **1**. In fact, we feel that the single-step, intramolecular mechanism of lactonization would be the logical outcome of macrolide biosynthesis by a multienzyme complex, i.e., the final step leading to release of the macrolide from the enzymatic complex, after which additional, separate enzymes could further modify the macrolide by oxidation, glycosidation, etc. Since very little information is presently available about the enzymology of macrolide antibiotic biosynthesis,[31] our belief must remain conjectural. Nevertheless, it is a testable mechanistic concept whose generality will be an important fact to validate for other members of this antibiotic class.

Experimental Section

General. Melting points were determined on a Kofler micro hot stage melting point apparatus and are uncorrected. UV absorption spectra were recorded on a Cary Model 14 spectrophotometer; IR spectra on a Perkin-Elmer Model 257 grating spectrophotometer; ^1H NMR spectra on a Varian EM-390 or Bruker HX-90E spectrometer at 90 MHz in which chemical shifts are reported relative to Me_4Si (0 ppm) or $CHCl_3$ (7.26 ppm); mass spectra on either a Finnigan Model 1016 quadrupole mass spectrometer interfaced with a Finnegan M-6000 computer or an AEI MS-9 high-resolution mass spectrometer interfaced with a Nova 2 data system. Elemental analyses were done

Scheme I



at Galbraith Laboratory, Knoxville, Tenn. Radioactivity measurements were done by liquid scintillation in a Packard Model 2002 or 3255 spectrometer using a toluene (5 g of PPO and 0.3 g of POPOP in 1 L) or a dioxane (100 g of naphthalene, 7.0 g of PPO, and 0.5 g of POPOP in 1 L) scintillation solution. Counting efficiencies were determined using a [^{3}H]hexadecane or [^{14}C]hexadecane internal standard. High-pressure liquid chromatography (LC) was done on a Waters ALC 202 liquid chromatograph with UV absorbance monitoring at 254 nm of the column effluent. Thin layer (TLC) and preparative (PLC) chromatography was done using Brinkmann analytical grade silica gel 60 TLC plates or preparative grade ($SGPF_{254}$) adsorbent. All reagents were analytical grade and all solvents were glass distilled before use. Radio-labeled compounds were purchased from Amersham-Searle or New England Nuclear and used without further purification. ^{18}O-Labeled compounds were purchased from Monsanto Laboratories, Miamisburg, Ohio. Evaporation in vacuo refers to solvent removal on a rotary evaporator at $\leq$40°C.

Maintenance and Fermentation of Fungi. *Penicillium brefeldianum* (NRRL 2083) was maintained on malt extract agar slants (5 g of Difco malt extract, 5 g of glucose, 9.25 g of Difco peptone, 6.25 g of Difco agar made up to 250 mL), sterile soil, or sterile water suspension at 4 °C. Aseptic transfers were carried out in a laminar flow hood.

Inoculum for metabolite production was prepared by using a spore-mycelium suspension from one slant culture of the mold to inoculate a 50-mL volume of a sterile nitrate-sucrose fermentation medium [0.3 g of $NaNO_3$, 0.1 g of KH_2PO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, 1 mg of $FeSO_4 \cdot 7H_2O$, 3.0 g of sucrose, plus 0.3 mL of a solution containing 3×10^{-3}% $ZnSO_4 \cdot 7H_2O$, 2×10^{-9}% $(NH_4)MoO_4$, and 2×10^{-9}% $MnSO_4$, in a total volume of 100 mL] contained in a 250-mL Erlenmeyer flask, which was incubated at 27 °C in a NBS Model G-25 gyrorotary shaker (200–300 rpm). After 2–4 days the 50-mL inoculum was transferred to 450 mL of the fermentation medium in a 2-L Erlenmeyer flask and incubation continued for the fermentation period.

Isolation of Brefeldin A (1). The shake cultures were filtered through two layers of cheesecloth to remove the mycelia, which was discarded since very little **1** was found to be present in mycelial extracts. The clear yellow filtrate was extracted with $CHCl_3$ (4 × ½ to ¾ vol) and the aqueous fraction discarded. The combined $CHCl_3$ extracts were washed with brine, dried (Na_2SO_4), and evaporated to a viscous oil. In large-scale incubations this residue was partitioned between Skelly B and MeOH, and the solid, light yellow residue obtained by evaporation of the methanol layer was recrystallized from MeOH–EtOAc or MeOH–H_2O. The colorless, crystalline **1** so obtained was identified by melting point, mixture melting point, ^{1}H NMR, and mass spectral comparison to an authentic reference standard. In small-scale incubations, the $CHCl_3$ solubles were first purified by PLC on silica gel in $CHCl_3$–MeOH (9:1), R_f 0.25, giving a violet coloration on spraying the plate with 5% concentrated H_2SO_4 in EtOH and heating at ca. 120 °C. The band of **1** was eluted from the adsorbent with $CHCl_3$–MeOH (8:2), the solvent removed in vacuo, and the resulting solid residue recrystallized as above. These procedures were found to be the best way to remove the yellow pigment impurities from small samples of **1**.

7-Acetylbrefeldin A. A suspension of brefeldin A (200 mg, 0.71 mmol), anhydrous sodium acetate (400 mg, 4.87 mmol), and acetic anhydride (2.4 mL) was stirred at room temperature for 20–48 h. The reaction was quenched by careful addition of MeOH and the reagents were removed in vacuo to give an oily residue. This was purified by PLC in $CHCl_3$–MeOH (98:2) to give unreacted brefeldin A (**1**, 0.31 mmol), 7-acetyl-**1** (85 mg, 66%), 4-acetyl-**1** (4 mg, 3%), and 4,7-diacetyl-**1** (50 mg, 34%). The 7-acetyl-**1** had a lower R_f than the 4-acetyl-**1** and gave the following physical and spectral data: mp 96.5–98.5 °C ($EtOH-H_2O$); IR ($CHCl_3$) ν 3610 w, 3460 br, 3020 m, 2980 m, 2940 m, 1730 sh, 1712 s, 1645 w, 1450 m, 1375 m, 1355 m, 1269 s, 1115 m, 1070 m, 980 m, and 970 m cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.65–2.37 (m, 11 H), 1.26 (d, J = 6 Hz, H-16), 2.03 (s, 3 H), 4.08 (m, H-4), 4.86 (m, H-15), 5.24 (m, H-10,11), 5.91 (dd, J = 2.2, 14 Hz, H-2), and 7.35 (dd, $J \simeq 3$, 14 Hz, H-3); MS *m/e* (rel intensity) 322, M^+ (0.6) ($C_{18}H_{26}O_5$, calcd 322.1781, found 322.1793), 262 (0.8), 244 (7), 43 (100).

7-Acetyl-**1** was distinguished from 4-acetyl-**1**[14] by double irradiation experiments: ^{1}H{H-4} of 7-acetyl-**1** caused the signals for H-2 and H-3 to collapse to an AB quartet; ^{1}H{H-7} of 4-acetyl-**1** (δ 4.32) did not affect the H-2 and H-3 resonances.

Bis(4-[^{2}H$_3$]acetyl-7-[H$_3$]acetyl)-1. 7-Acetyl-**1** (1.4 mg) was heated for 3 h at 70 °C with acetic anhydride-d_6 (0 μL) and dry pyridine (50 μL). The reagents were removed in vacuo and the resulting residue was purified by TLC in Skelly B–EtOAc (4:1) to give bis(4-[^{2}H$_3$]-acetyl-7-[H$_3$]acetyl)-**1** (0.9 mg). This had an R_f identical with that of 4,7-diacetyl-**1** and exhibited the expected absence of the one acetyl methyl singlet at δ 2.11 in its ^{1}H NMR spectrum, relative to the latter compound (CH_3CO- at δ_H 2.03, 2.11). A sample of the deuterated diacetate was purified further by LC (⅛ in. × 4 ft C_{18} Corasil, 55% H_2O in MeOH, 0.5 mL/min) before EIMS analysis (Table III).

4-Acetylbrefeldin A. Brefeldin A (200 mg, 0.71 mmol) was stirred for 20 h at room temperature with acetic anhydride (ca. 160 mg, 1.57 mmol) in dry pyridine (10 mL). The reaction mixture was treated as for 7-acetyl-**1** to give unreacted **1** (0.18 mmol), 7-acetyl-**1** (17%), 4-acetyl-**1** (84 mg, 49%), and 4,7-diacetyl-**1** (26%) after chromatographic separation. The 4-acetylbrefeldin A had the following physical and spectral characteristics: mp 131.5–132.5 °C (lit.[14] 128–129 °C); IR ($CHCl_3$) ν 3600 w, 3490 br w, 3010 m, 2978 m, 2930 m, 1732 s, 1710 s, 1650 w, 1450 m, 1375 m, 1245 s, 1260 s, 1125 w, 1070 m, 1010 m, 975 m cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.99–2.51 (m, 11 H), 1.25 (d, J = 6 Hz, H-16), 2.11 (s, 3 H), 4.32 (m, H-7), 4.85 (m, H-15), 5.31 (m, H-10,11), 5.55–5.79 (m, 1 H), 5.69 (dd, J = 1.2, 16 Hz, H-2), and

Table III

fragment ion	4,7-diacetyl-**1** *m/e* (rel intensity)	bis(4-[$^2H_3$]acetyl-7-[H_3]acetyl)-**1** *m/e* (rel intensity)
$M^{+\cdot}$	364 (5.2)	367 (11.3)
$M^{+\cdot} - H_2O$	346 (0.1)	349 (0.1)
$M^{+\cdot} - CH_2CO$	322 (0.8)	323 (1.3)
$M^{+\cdot} - CH_3CO_2H$	304 (4.8)	304 (4.8)
$M^{+\cdot} - 2CH_3CO_2H$	244 (28.8)	244 (42.4)

Table IV

fragment ion	*m/e*	isotope distribution, mol %[a]
$M^{+\cdot}$	282	28.0 ± 3.2[b]
	281	3.9 ± 2.6
	280	68.2 ± 5.9
$M^{+\cdot} - H_2O$	264	26.6 ± 7.0
	263	1.9 ± 0.6
	262	71.6 ± 6.4
$M^{+\cdot} - 2H_2O$	246	1.4 ± 0.9
	245	5.2 ± 3.5
	244	91.8 ± 2.3

[a] Corrected for the natural isotopic abundances according to ref 33. [b] Average of three mass spectra.

7.24 (dd, J = 2.3, 16 Hz, H-3); MS *m/e* (rel intensity) 322, $M^{+\cdot}$ (0.6), 263 (8), 245 (7), 43 (100).

4-Acetyl-7-oxobrefeldin A. 4-Acetylbrefeldin A (41.4 mg, 0.13 mmol) dissolved in CH_2Cl_2 (1.5 mL) was added all at once to a suspension of pyridinium chlorochromate[32] (50 mg) in CH_2Cl_2 (1 mL). The mixture was stirred magnetically for 3 h at room temperature, then filtered through a silica gel plug (0.5 g). The effluent was taken to dryness in vacuo and the resulting residue purified by PLC in $CHCl_3$–MeOH (98:2) to give pure 4-acetyl-7-oxo-**1**: 39 mg, 92%; mp 122–123.5 °C (lit.[14] 123–124 °C); ^1H NMR ($CDCl_3$) δ 1.12–2.35 (m, 11 H), 1.25 (d, J = 6.3 Hz, H-16), 2.11 (s, 3 H), 2.35–2.87 (m, 3 H), 4.91 (m, H-4), 5.33 (m, H-10,11), 5.74 (dd, J = 1.3, 15 Hz, H-2), and 7.27 (dd, J = 3.9, 15 Hz, H-3); MS *m/e* (rel intensity) 329, $M^{+\cdot}$ (3), 260 (12), 218 (21), 43 (100).

[7-^{18}O]-4-Acetyl-7-oxobrefeldin A. A solution of 4-acetyl-7-oxobrefeldin A (13.5 mg, 0.05 mmol) in dry THF (2 mL) was stirred with $H_2{}^{18}O$ (222 mg, 11.1 mmol, 96.6 mol % ^{18}O) and ca. 14 mg of acetic acid for 4 h at room temperature. After evaporation of the solution to dryness, TLC analysis showed that no degradation of the starting material had occurred. Mass spectral analysis indicated that a ca. 33% ^{18}O enrichment of the ketone had been achieved.

[7-^{18}O]Brefeldin A. The above sample of [7-^{18}O]-4-acetyl-7-oxobrefeldin A was dissolved in dry MeOH (1.5 mL), the solution cooled to −78 ° C in a dry ice-2-propanol bath, and $NaBH_4$ (33 mg, 0.87 mmol) added. After the reaction mixture was magnetically stirred for 1 h, the excess hydride was decomposed by the dropwise addition of a 50% acetic acid in $CHCl_3$ solution. The reaction mixture was concentrated to a syrup in vacuo and the residue partitioned between saturated aqueous $NaHCO_3$ and $CHCl_3$ (3 × equal volume). The combined $CHCl_3$ extracts were dried (Na_2SO_4), the solvent was removed in vacuo, and the resulting residue was purified by TLC in $CHCl_3$–MeOH (98:2). Ca. 10 mg of a mixture of two compounds was obtained.

Part of this mixture (ca. 5 mg) was dissolved in MeOH (0.4 mL) and stirred with anhydrous K_2CO_3 (40 mg) for 8 min at room temperature. Excess base was neutralized with glacial acetic acid and the reaction mixture was partitioned between water–$CHCl_3$. The $CHCl_3$ layer was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated to dryness in vacuo. TLC purification [$CHCl_3$–MeOH (95:5), fourfold elution] of the resulting residue gave [7-^{18}O]-**1** free of its C-7 epimer. This material was subjected to EIMS analysis (Table IV).

Identical results were obtained from the EIMS analysis of [7-^{18}O]-4,7-diacetyl-**1**.

7,15-Dibenzoyl-2,3,10,11-tetrahydrobrefeldin A γ-Lactone (4). Brefeldin A was converted to its 2,3,10,11-tetrahydro-γ-lactone by the literature procedures.[9b] A sample of this lactone (13.2 mg, 0.046 mmol) was dissolved in dry pyridine (0.2 mL) and benzoyl chloride (0.26 mmol, freshly distilled from P_2O_5) was added. The reaction mixture was stirred for 20 h at room temperature and quenched with EtOH, and the resulting solution was partitioned between 1 N aqueous HCl and $CHCl_3$. The $CHCl_3$ layer was washed with 1 N aqueous NaOH and brine and then dried (Na_2SO_4). PLC of the residue, resulting from solvent removal in vacuo, in Skelly B–EtOAc (4:1) gave the dibenzoate derivative (**4**) as a glass: 24 mg, 100%; ^1H NMR ($CDCl_3$) δ 1.10–2.65 (m, 22 H), 4.51 (m, 1 H), 5.13 (m, 1 H), 5.35 (m, 1 H), 7.44 (m, 7 H), and 8.00 (m, 4 H); MS *m/e* (rel intensity) 492, $M^{+\cdot}$ (0.5) ($C_{30}H_{36}O_6$, calcd 492.2513, found 492.2502), 370, $M^{+\cdot} - C_7H_6O_2$ (12.5) ($C_{23}H_{30}O_4$, calcd 370.2145, found 370.2112), 248, $M^{+\cdot} - 2C_7H_6O_2$ (14) ($C_{16}H_{24}O_2$, calcd 248.1777, found 248.1770), 105 (100), and 85 ($C_4H_5O_2$, calcd 85.0290, found 85.0292).

[7-^{18}O]-4. A sample of [7-^{18}O]brefeldin A (4.5 mg), prepared as above from 4-acetyl-7-oxo-**1** and [^{18}O]H_2O (95.1 mol % ^{18}O), was reduced catalytically (10% Pd/C, 2 mg) in an alcohol solution (0.7 mL). The catalyst was removed by filtration through Celite and the filtrate evaporated in vacuo to a solid, crystalline residue (ca. 4 mg). The latter was shown to be 2,3,10,11-tetrahydro-**1** by TLC in $CHCl_3$–MeOH (90:10) against an authentic reference standard.

The [7-^{18}O]tetrahydro-**1** was saponified by mixing with MeOH (0.7 mL) and 20% KOH in MeOH (0.7 mL) and stirring the mixture for 8 h at room temperature. The reaction mixture was neutralized with 2 N aqueous H_2SO_4 and evaporated to a crystalline residue, and the latter was filtered through a silica gel plug (0.5 g) as a solution in 20% MeOH in $CHCl_3$. The residue from evaporation of the filtrate in vacuo was purified by PLC in $CHCl_3$–MeOH (92:8) to give the 2,3,10,11-tetrahydro-γ-lactone derivative (ca. 4 mg) of [7-^{18}O]-**1**, by TLC comparison with an authentic reference standard.

The [7-^{18}O]tetrahydro-γ-lactone was converted to [7-^{18}O]-**4** by the procedure described above, giving ca. 3 mg of this dibenzoate after purification by PLC. A sample of this was purified further by LC (⅜ in. × 3 ft C_{18} Corasil, 20% water in MeOH, 2.5 mL/min) for EIMS analysis (Table V).

Table V

fragment ion	*m/e*	isotope distribution, mol %[a]
$M^{+\cdot}$	494	56 ± 9[b]
	493	7 ± 3
	492	11 ± 0.7
$M^{+\cdot} - C_7H_6O_2$	372	46 ± 0.3
	371	19 ± 0.3
	370	28 ± 0.3
$M^{+\cdot} - 2C_7H_6O_2$	250	~1
	249	~1
	248	94 ± 0.3
	247	4 ± 0.3

[a] Corrected for the natural isotopic abundances according to ref 33. [b] Average of three mass spectra.

[1-^{18}O]-4. Potassium methoxide was prepared by reacting a 22.7% mineral oil dispersion of potassium hydride (0.9 mL, washed three times with dry Skelly A under N_2) as a suspension in dry THF (0.1 mL) with anhydrous MeOH (ca. 400 μL, distilled from $Mg(OMe)_2$) dissolved in dry THF (0.5 mL) at room temperature under N_2. The KOMe that was generated was freed of solvent by a N_2 stream and dissolved in anhydrous MeOH (1 mL). An aliquot (0.08 mL) of this solution was added to a solution of 2,3,10,11-tetrahydro-**1** (6.8 mg) in anhydrous MeOH (0.1 mL) and [^{18}O]H_2O (ca. 8 mg, 95 mol % ^{18}O). The resulting solution was stirred for 8 h at room temperature, weakly acidified with 2 N aqueous H_2SO_4, and concentrated in vacuo to a wet, crystalline mass. This was triturated with $CHCl_3$–MeOH, the resulting soluble material was filtered through a silica gel plug (0.5 g), and the filtrate was evaporated to dryness. The residue obtained was purified by TLC in $CHCl_3$–MeOH (90:10), then in $CHCl_3$–MeOH (92:8), twice developed, to give the [1-^{18}O]tetrahydro-γ-lactone derivative of **1** (3 mg).

This [1-^{18}O]lactone was converted to [1-^{18}O]-**4** (2.4 mg) by the procedure described above for **4**. A final purification of [1-^{18}O]-**4** by TLC in Skelly B–EtOAc (4:1), twice developed, was done prior to EIMS analysis (Table VI).

Sodium [$^{18}O_2$,2-^{3}H]Acetate Feeding. Sodium [$^{18}O_2$]acetate was prepared from [^{18}O]H_2O (567 mg, 95.1 mol % ^{18}O) as described in ref 17, yielding 2.39 g of anhydrous solid. A portion was converted to its *p*-phenylphenacyl ester derivative[17] and the ^{18}O content of this determined by EIMS analysis: *m/e* 258 ($M^{+\cdot}$ + 4), 82.1 ± 1.6 mol % $^{18}O_2$; *m/e* 256 ($M^{+\cdot}$ + 2), 14.3 ± 1.1 mol % $^{16}O^{18}O$. A second portion was shown to be ≥91% pure sodium acetate by radioisotope dilution analysis.[17] The remaining sodium [$^{18}O_2$]acetate (2.38 g) was admixed with sodium [2-^{3}H]acetate (25.8 μCi), yielding sodium [$^{18}O_2$,2-^{3}H]acetate of 1.95 × 10^6 dpm/mmol. This was fed to one 500-mL culture of *P. brefeldianum* and the culture incubated 3 days longer. Isolation of [^{18}O,^{3}H]-**1** was carried out as described above, yielding 15 mg of crude **1** and, then, 8 mg of crystalline **1** (MeOH-H_2O and MeOH-EtOAc).

A sample of [^{18}O,^{3}H]-**1** was purified by LC (⅜ in. × 3 ft, C_{18} Corasil, 40% MeOH in H_2O, 2.5 mL/min) to give 1.57 mg of crystalline material. This was diluted with radioinactive **1** (9.44 mg) and recrystallized to a constant specific radioactivity from MeOH-EtOAc: 2.40 × 10^6 dpm/mmol. The specific radioactivity, corrected for the loss of ^{3}H during the incorporation of [2-^{3}H]acetate,[21a] is 8.57 × 10^6 dpm/mmol.

A second sample of [^{18}O,^{3}H]-**1** (1.5 mg) was converted to its **4**,7-diacetate,[9b] which was purified by TLC in $CHCl_3$-MeOH (90:10) and LC (as before) prior to EIMS analysis.

A third sample of [^{18}O,^{3}H]-**1** (2.5 mg) was converted to [^{18}O,^{3}H]-**4** by the procedures described above for synthetic [7-^{18}O]-**4**. This gave 2.7 mg of dibenzoate after LC purification, which was used for EIMS analysis.

$^{36}O_2$ Feeding. Two 2-L Erlenmeyer side-arm suction flasks, each containing ca. 200-mL cultures of *P. brefeldianum* to which sodium [1-^{14}C]acetate (11–17 μCi per flask) had been added, were sealed with sterile rubber stoppers containing 5-mL gas sampling bulbs that could be isolated from the system by vacuum stopcocks. The two flasks were connected jointly to a gas handling manifold via a sterile glass wool filled air filter.

A tank of N_2 and of $^{36}O_2$ (98.6 mol % $^{36}O_2$, ca. 80 mL volume containing 0.96 L of $^{36}O_2$) were connected to the manifold, which could be evacuated by a water aspirator and refilled to atmospheric pressure under a small, constant N_2 pressure via a Hg bubbler valve. The system was evacuated and refilled three times with N_2 while the cultures were magnetically stirred to facilitate degassing of the medium. The $^{36}O_2$ was then permitted to enter the system after which the pressure inside was adjusted to ambient by the addition of N_2. The two flasks were clamped off from the manifold, but still left connected together by a common air line, and the incubation was continued for 72 h in the rotary shaker. Samples of the system's atmosphere were taken at time zero, +43 h, and +72 h for EIMS analysis. A third culture was incubated in a cotton-plugged flask to serve as a control, although it was not subjected to the evacuation-refill procedure. The [$^{18}O_2$,^{14}C]-**1** was isolated and purified as before.

flask	crude **1**, mg	specific incorporation of [1-^{14}C]acetate, %
1	5.5	n.d.
2	7.4	0.023
control	11.5	0.004

EIMS analysis of [^{18}O,^{14}C]-**1** and its 4,7-diacetate from flask 1 gave essentially identical results for their ^{18}O enrichment.

$^{32}O_2$–$^{36}O_2$ Feeding. The general procedures were as described above. The $^{32}O_2$–$^{36}O_2$ gas mixture was prepared for us by Mound Laboratories; it contained 49.9 mol % $^{32}O_2$ and 48.5 mol % $^{36}O_2$. The three cultures were incubated 79 h after addition of the oxygen and sodium [1-^{14}C]acetate.

flask	crude **1**, mg	specific incorporation of [1-^{14}C]acetate, %
1	9.1	0.0035
2	10.2	n.d.
control	n.d.	0.011

Samples of [^{18}O,^{14}C]-**1** and its 4,7-diacetate from flasks 1 and 2 were subjected to EIMS analysis. Although the data were in agreement with that shown in expt 2, Table II, the analyses' precision was poor because of the weak molecular ion intensity and low ^{18}O enrichment. Consequently, a sample of [^{18}O,^{14}C]-**1** was converted to its C^7-*tert*-butyldimethylsilyl ether by a literature method[25] as described below for EIMS analysis.

Table VI

fragment ion	*m/e*	isotopic mol %[a]	distribution normalized
$M^{+\cdot}$	494	30 ± 4[b]	0.47
	493	*c*	*c*
	492	64 ± 3	1.00
	491	5 ± 3	0.08
$M^{+\cdot} - C_7H_6O_2$	371	18 ± 5	0.28
	370	64 ± 5	1.00
	369	9 ± 3	0.14
$M^{+\cdot} - 2C_7H_6O_2$	250	28 ± 4	0.44
	249	2 ± 1	0.03
	248	63 ± 2	1.00
	247	6 ± 1	0.10
6	87	22 ± 15	0.29
	86	3 ± 1	0.04
	85	75 ± 14	1.00

[a] Corrected for the natural isotopic abundance according to ref 33. [b] Average of three mass spectra. [c] Peak intensity too weak for accurate measurement.

***tert*-Butyldimethylsilyl Ether of 1 (7).** [^{18}O,^{14}C]-**1** (2.56 mg) was heated for 1 h at ca. 100 °C in a sealed tube containing *tert*-butyldimethylsilyl chloride (25 μL of a 1 M solution in dry DMF) and imidazole (50 μL of a 1 M solution in dry DMF). The tube was cooled and opened, and its contents were evaporated in vacuo to a residue. This residue was purified by passage through a Sephadex LH 20 column in 25% EtOAc in Skelly C. Evaporation of the effluent gave 1.1 mg of a residue that was further purified by TLC in $CHCl_3$-EtOAc (95:5) to give **7** (0.9 mg) and 4,7-di-*tert*-butyldimethylsilyl-[^{18}O,^{14}C]-**1** (**8**, 0.7 mg).

These two silyl ethers also were prepared from unlabeled **1** in 63 (**7**) and 13% (**8**) yield. The spectral data for **7**: IR ($CHCl_3$) ν 3600 w, 3450 m, 2940 s, 2845 s, 1708 s, 1640 m, 1350 s, 1255 s, 1110 s, 1070 s, 835 s cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 9 H), 1.1–2.6 (m, 12 H), 1.24 (d, *J* = 6 Hz, H-16), 4.08 (m, H-4), 4.25 (m, H-7), 4.84 (m, H-15), 5.10–5.40 (dd, 1 H), 5.59 (m, 1 H), 5.84 (dd, *J* = 2.2, 16 Hz, H-2), 7.36 (dd, *J* = 3.3, 16 Hz, H-3); MS *m/e* (rel intensity) 394, $M^{+\cdot}$ ($C_{22}H_{38}O_4Si$, calcd 394.2541, found 394.2563), 337, $M^{+\cdot}$ − 57 ($C_{18}H_{29}O_4Si$, calcd 337.1836, found 337.1841) (35), 192 (26), 75 (100).

The key spectral parameters for structures **7** and **8** are that (a) H-4 in **7** is a dd, *J* = 2, 7 Hz at δ 4.08 and 4.13, which compares to H-4 in **1** at δ 4.11, m; (b) H-4 in **8** is a dt, *J* = 2, 8 Hz, at δ 3.96 and 4.06, and H-7 is a triplet, *J* = 4 Hz, at δ 4.19; (c) H-7 in **7** is a br t, *J* ≃ 5 Hz, at δ 4.25, and in **1**, a m at δ 4.31; and (d) **8** shows an $M^{+\cdot}$ at *m/e* 508.3418 ($C_{28}H_{52}O_4Si_2$) and an $M^{+\cdot}$ − 57 at *m/e* 451.2719 ($C_{24}H_{43}O_4Si_2$).

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Conformational Analysis by Nuclear Magnetic Resonance Spectroscopy: ^{15}N NMR of a Cyclic Pentapeptide

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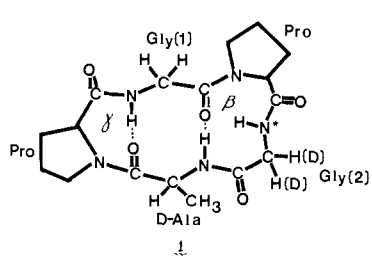
Abstract: The cyclic pentapeptide *cyclo*-(Gly(1)-Pro-Gly(2)-D-Ala-Pro), which has been shown by ^{1}H and ^{13}C NMR to incorporate both β and γ turns, has been used as a model system to explore the use of ^{15}N NMR to analyze the conformations of peptides. Assignments of ^{15}N resonances to specific amino acids have been made by analogy with similar peptides and confirmed by ^{15}N labeling. Nitrogen chemical shifts of the peptide, which is soluble in a wide variety of solvents, are sensitive to solvent changes. In water, two conformations corresponding to different cis–trans configurations of the peptide bonds are present. By means of ^{15}N labeling and an analysis of ^{15}N chemical shifts the involvement of Gly(1) in this cis–trans isomerism has been established. These results indicate substantial utility for ^{15}N NMR in the conformational analysis of peptides.

The cyclic pentapeptide *cyclo*-(glycyl-L-prolyl-glycyl-D-alanyl-L-prolyl), **1** [*cyclo*(Gly(1)-Pro-Gly(2)-D-Ala-Pro)],



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has been proposed on the basis of ^{1}H and ^{13}C NMR data[2] to adopt a rigid conformation containing two distinct types of intramolecular hydrogen bonds, one forming a seven-membered ring γ turn[3] and one forming a ten-membered ring β turn.[4]

The primary purpose of the present work was to explore the use of ^{15}N NMR in determining conformations of polypeptides, and the cyclic pentapeptide **1** provides a model system with favorable solubility characteristics and well-defined conformational populations. The procedure was to obtain spectra in a variety of solvents and to assign the ^{15}N resonances to specific amino acid residues. Solvent shifts were interpreted in terms of the relative strengths of hydrogen bonds and the results correlated with the structure of the peptide as indicated by ^{1}H and ^{13}C NMR, circular dichroism,[2] and X-ray crystallography.[5]

Experimental Section

The cyclic peptide[2b] is soluble in acetonitrile, water, chloroform,

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