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(15) The following abbreviations are used: HF, Hartree-Fock method; UHF, unrestricted Hartree-Fock method; RHF, restricted Hartree-Fock method; MC-SCF, multiconfiguration self-consistent field method; CI, configuration interaction; CEPA, coupled electron pair approximation.

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Biosynthetic Origin of the Oxygen Atoms in the C_{15} Macrolide Antibiotic Brefeldin A[1]

Sir:

Brefeldin A (**1**), a C_{15} macrolide antibiotic produced by several genera of *Ascomycetes*,[2] has an obvious structural resemblance to the prostaglandins, which has led to the speculation that **1** might also be biosynthesized from a fatty acid in a manner paralleling prostaglandin biosynthesis. Although earlier evidence obtained from labeled acetate[3,4] and malonate[4b] feeding experiments was consistent with a polyketide biosynthetic route for **1**,[5] Bu'Lock and Clay reported in 1969 that [9-^{14}C]palmitic acid apparently was specifically incorporated into **1** to a limited extent in *Penicillium cyaneum*.[6] These authors proposed a mechanism (Figure 1a) for the biosynthetic formation of the cyclopentanol ring of **1** analogous to the biosynthesis of PGG$_1$, PGE$_1$, and PGF$_{1\alpha}$ (Figure 1b). However, since the intact incorporation of palmitate into **1** could not be confirmed in *P. lilacinum* subsequently by Cross and Hendley,[7] nor in *P. brefeldianum* at Manchester,[8] this biosynthetic hypothesis was withdrawn.[7] Since the validity of biogenetic proposals like that of Bu'Lock and Clay depends on the origin of the oxygens of C-4 and C-7 of **1**—if both of these oxygens originate from the same oxygen molecule, their hypothesis could be valid mechanistically although in a slightly modified format—we sought the answer to this question via [$^{18}O_2$]acetate and $^{16,18}O_2$ feeding experiments. The results of our experiments reported herein indicate that the bioorganic parallel implicit in Figure 1 is not valid and rule out a fatty acid biosynthetic origin for **1**.

P. brefeldianum Dodge (NRRL 2083) growing in shake culture in defined media was fed sodium [$^{18}O_2$,2-^{3}H]acetate at a time when a bright yellow (unknown) pigment was just appearing. After 3 days' further incubation, labeled **1** was isolated and purified, and separate aliquots of it were converted to the 4,7-diacetate[2a] and tetrahydro-γ-lactone[2b] dibenzoate (**2**) derivatives. Using bis(4-[$^2H_3$]acetyl-7-[$^1H_3$]acetyl)-**1** and

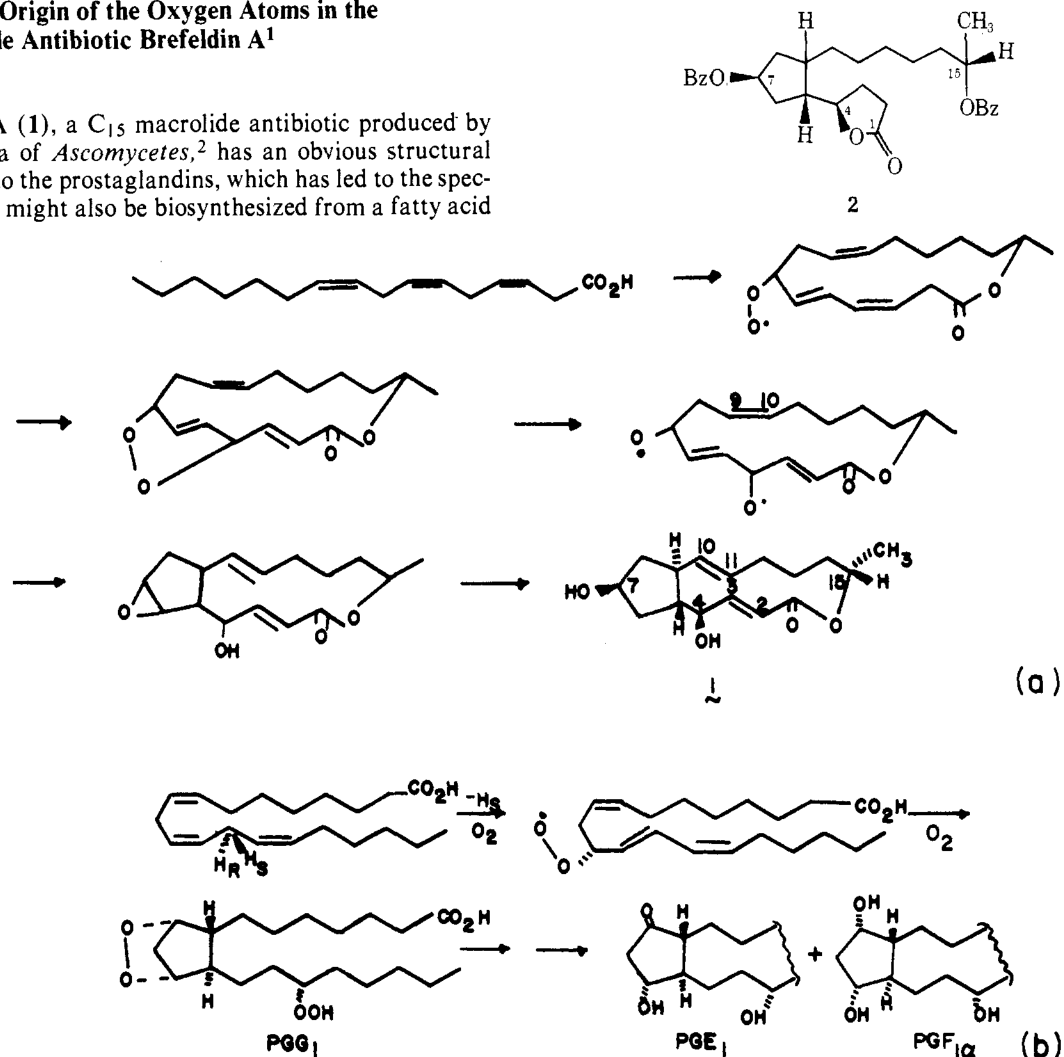

Figure 1. (a) Bu'Lock and Clay biosynthetic hypothesis for brefeldin; (b) established biosynthetic pathway of prostaglandins.

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		2	
m/e	Mole %	*m/e*	Mole %
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	61.3 ± 4.0 (1.00)
309	1.7 ± 0.8	3.74	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 ± 1.4 (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 mole % $^{18}O_2$, 2.38 g, prepared from $H_2{}^{18}O$ and ethyl orthoacetate (C. T. Mabuni and C. R. Hutchinson, *J. Labelled Compd. Radiopharm.*, in press) admixed with [2-^{3}H]-acetate (25.8 μCi) was fed to one 500-ml shake culture. [b] Mole percent, average of three determinations. [c] No ion observed owing to weak intensity. [d] M^+.

synthetic 7-[^{18}O]diacetyl-**1**,[9] it was established that diacetyl-**1** fragmented under 70-eV electron impact mass spectral (EIMS) conditions by stepwise loss of the C-4 acetoxyl ($M^{+\cdot}$ − 63) and then the C-7 acetoxyl [$M^{+\cdot}$ − 123 (^{2}H) or − 122 (^{18}O)] to a fragment at *m/e* 244.1453, which contained only the C-1 and C-15 oxygens. Similarly, EIMS analysis of [7-^{18}O]-**2** indicated that a random loss of $C_7H_6O_2$ from C-7 and/or C-15 occurred to give an *m/e* 248 fragment ion and of synthetic [1-^{18}O]-**2**[11] showed that the *m/e* 248 fragment ion contained only one ^{18}O atom, all of which was located in the *m/e* 85.0291 fragment ion comprising carbons 1–4 and the C-1 and C-4 oxygens of **1**.[2b] EIMS analysis of **1** biosynthetically labeled by [$^{18}O_2$]acetate showed that ^{18}O was present only at C-1 and C-15 (Table I). Surprisingly the C-7 oxygen of **1** was not ^{18}O labeled by acetate, even though C-7 is a carbonyl position of the unknown polyketide intermediate. Also no significant exchange of ^{18}O from the C-1[12] or C-15 carbonyl of polyketide biosynthetic intermediates apparently occurred in vivo since the dilution of the [$^{18}O_2$]acetate fed calculated from the dilution of the [^{3}H]acetate reference (4.12-fold) agrees well with the assumed even distribution of ^{18}O between C-1 and C-15[12] observed in EIMS analysis of **1** (19.9 vs. 21.8%).

P. brefeldianum was grown in shake culture as before, but, after the pigment's appearance, the system was N_2 flushed, filled with $^{18}O_2$ (Table II, expt 1) or with $^{16,18}O_2$ (Table II, expt 2), and left sealed for a further 3 days' metabolism. EIMS analysis of the ^{18}O-labeled **1** from expt 1 indicated that ^{18}O was present at both C-4 and C-7,[13] but not at C-1 or C-15. Finally, EIMS analysis of the ^{18}O-labeled **1**, diacetyl-**1**, and particularly the mono-C^7-*tert*-butyldimethylsilyl ether of **1** (**3**)[14] obtained from expt 2 was clear evidence for the biosynthetic origin of the C-4 and C-7 oxygens from *two* oxygen molecules, not one.[15]

The bioorganic mechanism by which the cyclopentanol ring of brefeldin A is formed, therefore, does not closely parallel prostaglandin biosynthesis. In future feeding experiments we will attempt to ascertain if the C-4 and C-7 hydroxyls of **1** are a necessary result of ring formation, or simply cosmetic "after events".[16] The fact that the C-15 oxygen does not come from O_2 further supports a non-fatty-acid, polyketide 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, which necessarily would involve molecular oxygen.[17]

Table II. EIMS Analysis of **1** Labeled by $^{18}O_2$

Isotopic distribution[a] (normalized isotopic distribution)			
1, expt 1[b]		**3**, expt 2[c]	
m/e	Mole %	*m/e*	Mole %
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] Mole percent, average of three determinations. [b] The mole percent $^{18}O_2$ of all oxygen in the system was 94.1 at 0 h and no oxygen could be detected at 73 h. [c] The mole percent $^{18}O_2$ of all oxygen in the system was 46 at 0 h, 44 at 11 h, and 40 at 79 h. No $^{17}O_2$ could be detected although the purchased gas analyzed (Mound Laboratory, Miamisburg, Ohio) for 3% $^{17}O_2$ and no $^{16}O^{18}O$. [d] Peak intensity too weak to measure. [e] $M^{+\cdot}$. [f] $M^{+\cdot}$ − 57.

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(5) Namely, that **1** was uniformly labeled by [1-^{14}C]acetate as eight C_2 units,[3] in which an acetate starter effect was noted for carbons 15 and 16 in [2-^{14}C]malonate's incorporation.[4b]

(6) J. D. Bu'Lock and P. T. Clay, *Chem. Commun.*, 237–238 (1969).

(7) B. E. Cross and P. Hendley, *J. Chem. Soc., Chem. Commun.*, 124–125 (1975). [16-^{14}C]Palmitate was catabolized to [2-^{14}C]acetate before label entered **1**.

(8) Footnote in ref 7 and personal communication from Professor J. D. Bu'Lock, Jan 1977.

(9) By the sequence 4-*O*-acetyl-7-oxo-**1**[10] → 4-*O*-acetyl-[7-^{18}O]-7-oxo-**1** → 4-*O*-acetyl-[7-^{18}O]-**1** → [7-^{18}O]diacetyl-**1**.

(10) H. P. Weber, D. Hauser, and H. P. Sigg, *Helv. Chim. Acta*, **54**, 2763–2767 (1971).

(11) By the sequence 2,3,10,11-tetrahydro-**1**[2b] → [1-^{18}O]-2,3,10,11-tetrahydro-**1**-ω-hydroxy acid → [1-^{18}O]-2,3,10,11-tetrahydro-**1**-γ-lactone → [1-^{18}O]-**2**.

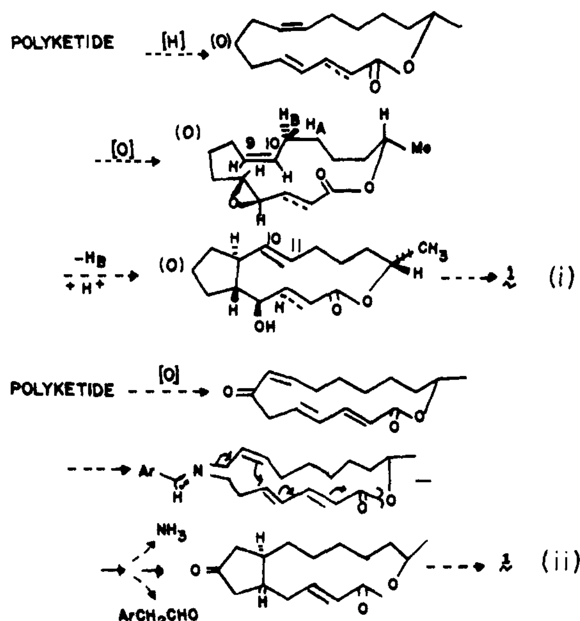
(12) The apparently smaller amount of ^{18}O at C-1 than at C-15 of **2** (Table I) is due to some loss of ^{18}O during preparation of **2**, as shown by an independent synthetic labeling experiment.[11]

(13) EIMS of synthetic [7-^{18}O]-**1**[9] showed that the oxygen at C-4 (M^+ − 18) was lost first, then the C-7 oxygen (M^+ − 38) to give the *m/e* 244 fragment ion.

(14) Prepared from **1** according to R. W. Kelley and P. L. Taylor, *Anal. Chem.*, **48**, 465–467 (1976): 62%; mass spectrum *m/e* (rel intensity), 337 (M^+ − 57, 35%), 192 (26%), 75 (100%); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 9 H), 4.08 (M, 1 H, C-4), 4.25 (m, 1 H, C-7) ppm. The bis(4,7-*t*-BDMS) ether of **1** also was obtained (13%).

(15) The calculated intensity ratio of (M + 2)/(M + 4) of **3** if these oxygens came from two O_2 molecules is 2.63 (found 2.39), whereas, if from one O_2 molecule, it is ≤0.07.

(16) For example, in our working hypothesis i for ring formation, the C-4 hydroxyl would be a necessary result, but not the C-7 hydroxyl; in the alternative hypothesis ii, the C-7 hydroxyl could be a necessary result, but not the C-4



hydroxyl; in the proton-initiated analogue of hypothesis i, neither the C-4 nor C-7 hydroxyl would be a necessary result.

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A Nonbleachable Rhodopsin Analogue Formed from 11,12-Dihydroretinal

Sir:

It is now commonly accepted[1] that the chromophore of the visual pigment rhodopsin consists of the protonated Schiff base[2] of 11-*cis*-retinal (**1**) bound to an ε-amino group of lysine[3] of opsin, and that light triggers a series of reactions the terminal products of which are *all-trans*-retinal and opsin. Although the detection of the initial photolysis product, bathorhodopsin,[4] and the room temperature[5] and low temperature measurements[6] of bleaching have been achieved, the nature of complex transformations and spectral changes are largely unsolved and remain to be the central problems in understanding the visual process on a chemical basis. A major factor which renders studies of rhodopsin difficult is its great lability toward light and heat. In conjunction with our studies on model retinals and rhodopsins formed therefrom,[7] we report the preparation of the first nonbleaching rhodopsin and some spectral data which have direct bearing in clarifying the chemistry of rhodopsin.

The synthesized chromophore was *all-trans*-11,12-dihydroretinal (**2**), which, in view of the flexibility around the C_{11}–C_{12} bond, could conceivably bind to opsin; this was found to be the case. This chromophore is pertinent since (i) there is no cis/trans isomerism around the 11–12 bond and hence the rhodopsin analogue would be "nonbleachable"; (ii) owing to the separation of the chromophore into triene and enal moieties, spectroscopic properties of the pigment would contribute to clarifying the cause of the enigmatic red shift accompanying its formation.

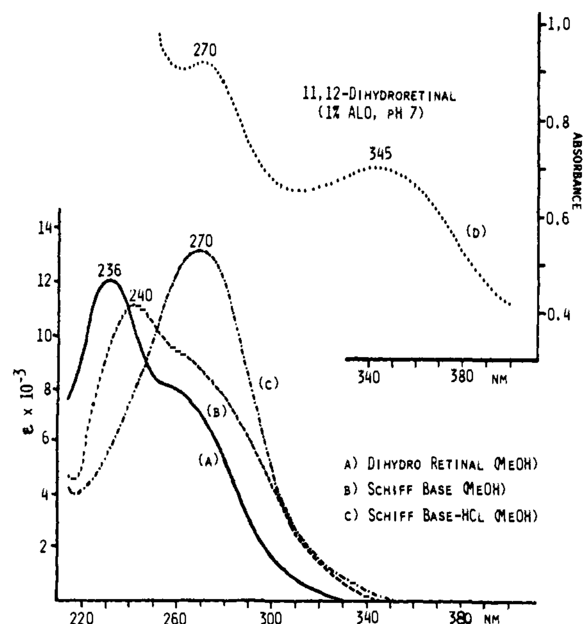

Figure 1. Absorption spectra: Curve a: aldehyde **2** in methanol; curve b, Schiff base **6** in methanol; curve c, protonated Schiff base **7** in methanol; curve d, pigment from 11,12-dihydroretinal **2** and bovine opsin in ALO, pH 7.0.

Table I. Absorption Spectral Data of 11-*cis*-Retinal and Dihydroretinal

	Dihydroretinal, in MeOH	11-*cis*-Retinal, in EtOH
Aldehyde	*236 (12 000)*,[a] 255 (8 000)	375 (20 000)
Schiff base with $BuNH_2$	*240 (11 000)*,[b] 255 (8 000)	350 (29 000)[b]
Schiff base-HCl	*270 (13 000)*[c]	440 (34 000)[c]
Pigment	345 (in ALO, pH 7.4)	500 (in ALO, pH 7.4)[d]

[a] Maxima due to the enal chromophore are italicized. [b] Prepared by keeping the solution of aldehyde in neat amine over molecular sieves, −20 °C, 12 h, under nitrogen in the dark, blowing off excess amine, and dissolving residue in MeOH—*strictly anhydrous conditions.* [c] Prepared by bubbling dry HCl gas into methanol solution of the Schiff base at −78 °C, under argon in the dark. [d] Data for bovine opsin.

11,12-Dihydroretinal (**2**) was prepared by the Emmons reaction between the ethyl phosphonate reagent derived from the chloro ketal **3** and β-ionone. The ketal was hydrolyzed to the dihydro C_{18} ketone **4** with 10% HCl/THF, and this was submitted to a second Emmons reaction with ethyl (2-carbethoxy)phosphonate to give the ethyl ester **5**, which was converted to the aldehyde by reduction with diisobutylaluminum