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# COMPARATIVE BIOCHEMISTRY OF FATTY ACID AND MACROLIDE ANTIBIOTIC (BREFELDIN A). FORMATION IN *PENICILLIUM BREFELDIANUM*†

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**Abstract**—The stereochemistry of the D labeling of mycelial steric (**3**) and oleic acids (**4**) by $^{13}C^{2}H_3CO_2H$ *in vivo* in *Penicillium brefeldianum* is the same at their even numbered, non-starting group positions as found for saturated fatty acid biosynthesis in two other eukaryotes. This conclusion was reached by comparing the D labeling at C-10 of methyl stearate with that of methyl oleate as determined by $^{13}C$ NMR spectroscopy. Since brefeldin A (**1**) is labeled by $CD_3CO_2H$ at C-6 and C-8 with the enantiotopic configurational result, the stereochemistry of antibiotic formation either differs at two points from fatty acid formation; or the C-6 and C-8 configuration of **1** is determined by some process unique to cyclopentane ring formation, but not by reduction of enolylthioester intermediates of its carbon chain assembly process.

The comparison of biochemical reaction mechanisms in primary and secondary metabolic pathways frequently is useful to construct hypotheses about the biosynthesis of natural products. The mechanism of C chain assembly for the biosynthesis of macrolide and polyether antibiotics is a case of this type. Since the principal C skeleton of these natural products is made from simple fatty acids (e.g. acetate, butyrate and propionate), the well-known biochemistry of long-chain fatty acid formation has been the model for C-chain assembly in the antibiotics' biosynthetic pathway. This analogy, however, is too simple: it does not clarify how the secondary biochemical pathway controls the sequence of $C_2$ to $C_4$ subunit assembly, or the absolute configuration of the $R^1{*}CH(R^2)R^3$ chiral centers that frequently occur at alternate positions in the main C chain of the antibiotics.

We have been studying the biosynthesis of brefeldin A (**1**) as one model of C chain assembly in macrolide antibiotics. The structural features of **1** allow us to view the types of C–H and C–O stereochemical determinants that are seen in the intermediates of saturated fatty acid biosynthesis (Fig. 1). Positions 2,3 and 10,11 of **1** may correspond to the enolylthioester intermediate (**C**); positions 4, 6, 8 and 12 to the *alpha*-C of saturated thioester intermediate (**D**); positions 5, 7, 9 and 13 to the *beta*-C of the same intermediate; position 14 to the *alpha*-C of the *beta*-ketothioester intermediate (**A**); and position 15 to the *beta*-hydroxythioester intermediate (**B**). Since **1** is assembled by *Penicillium brefeldianum* from one acetate (the C-15,16 $C_2$ starter unit) and seven malonates,[1-3] it is possible that its C chain is formed by a biochemical process similar to the formation of saturated fatty acids. Therefore, we can compare the mechanisms of the reactions in the two biochemical pathways by determining the stereochemistry of brefeldin A biosynthesis, then comparing this with the well-understood stereochemistry of saturated fatty acid formation (Fig. 1).

Prior to the work described in this paper, we had investigated the stereochemistry of brefeldin A biosynthesis by determining the regiochemistry and stereochemistry of its isotopic labeling by $CH_3{}^{13}CO_2H$, $^{13}CH_3{}^{13}CO_2H$, $CH_3C^{18}O_2H$[4], $^{13}CD_3CO_2H$[5] and $CD_3CO_2H$[6]. The results of these studies established the isotopic labeling relationships shown in Fig. 2. We also have determined recently that brefeldin C (7-deoxybrefeldin A)[7] is very efficiently biotransformed to **1** by the fungal cells,[8] which may be the last step in the biosynthetic pathway to **1**.

The results of our previous isotope labeling experiments are consistent with the formation of **1** by a biochemical process that is similar, but not identical, to the biosynthesis of saturated fatty acids. Both processes result in the regiospecific alternate labeling of the carbon positions of **1** and palmitic acid (**2**), for example, by $CH_3{}^{13}CO_2H$ (Fig. 2). These processes also result in retention of the D atoms of $CD_3CO_2H$ at the acetate starter unit, and some retention of the $^{18}O$ isotope of $CH_3C^{18}O_2H$ at the chain terminating carboxyl group.‡ These data represent the biochemical similarities of the secondary (macrolide) and primary (fatty acid) metabolism. On the other hand,

†NRCC No. 20467.

‡We are not certain if this has been proven directly for fatty acid biosynthesis, but it is a logical conclusion.

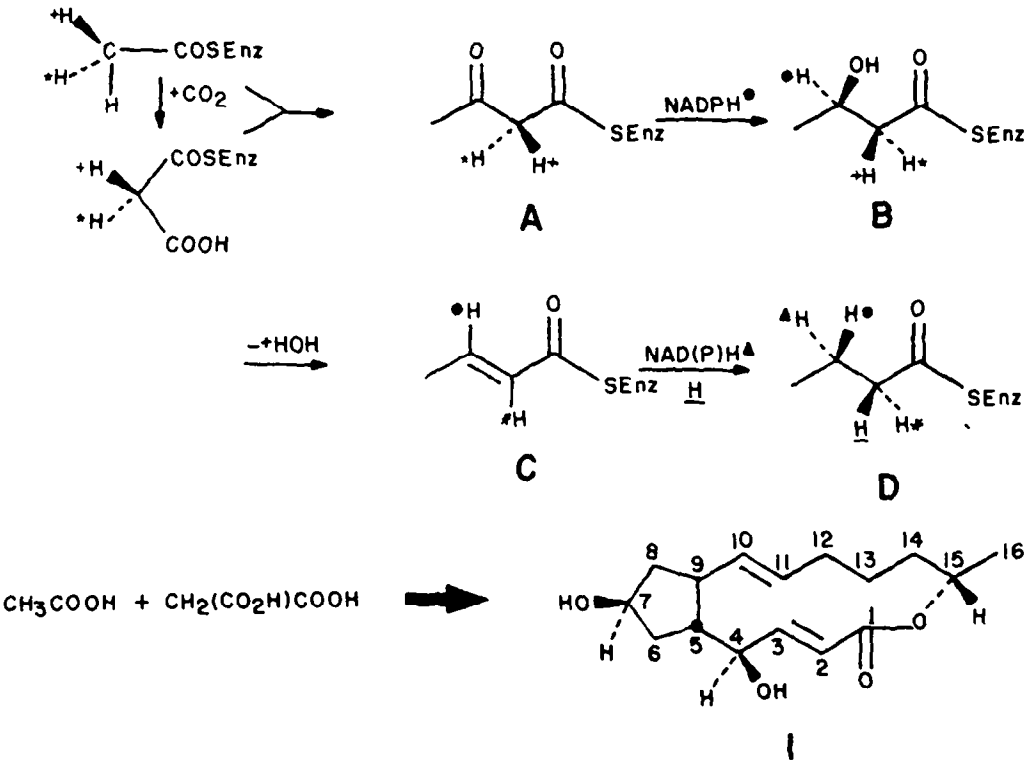



Fig. 1. The mechanism of fatty acid biosynthesis in *E. coli*, which uses for four intermediates A–D for carbon chain building; and the biosynthesis of brefeldin A (1), which uses acetate and malonate in a unknown way to form the carbon skeleton.

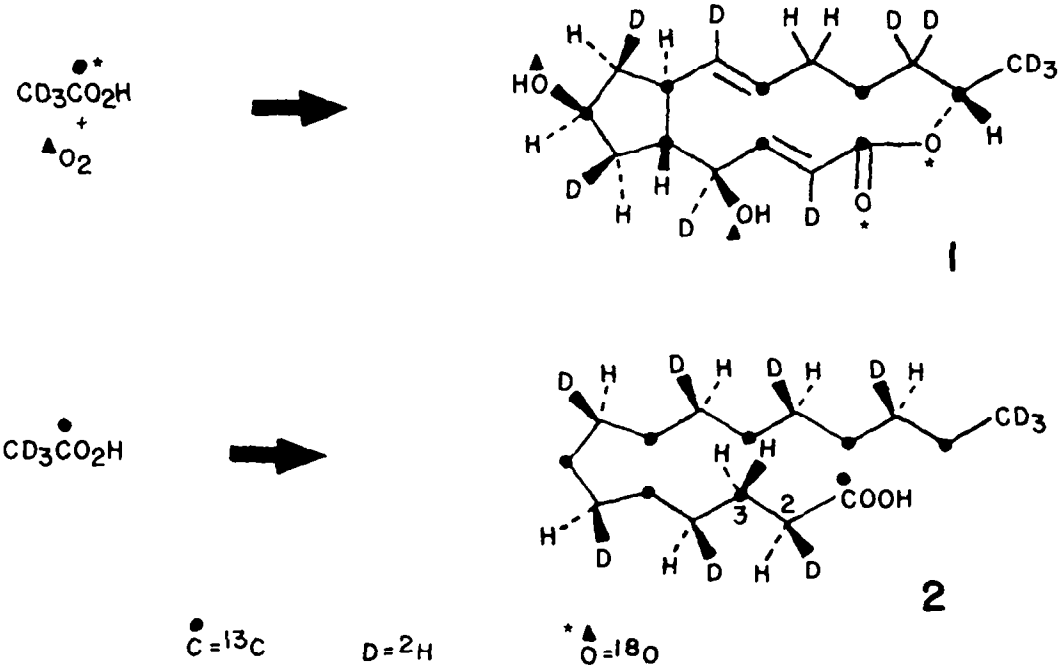



Fig. 2. The regiochemistry and stereochemistry of precursor incorporation into brefeldin A (1) by the fungus and palmitic acid (2) by prokaryotes.

$CD_3CO_2H$ labels the non-starter unit even-numbered positions of the $C_{16}$ fatty acid with one D atom,[9,10] but labels the corresponding position of **1** with none (C-12), one (C-2, 4, 5, 8 and 10) or two (C-14) D atoms[5]. The latter result has no analogy in saturated fatty acid biosynthesis since their formation necessarily involves the loss of two of the three hydrogens from the Me group of acetate at all even-numbered positions (other than the starter unit). Finally, the secondary alcohol at position 15 of **1** acquires an $^{18}O$ label from $CH_3C^{18}O_2H$, which does not come from the C-1 carboxyl group of an intermediate in the biosynthetic pathway,[4] and has an absolute configuration (*L*) that is enantiotopic to the configuration (*D*) of the *beta*-C in the corresponding intermediate of fatty acid biosynthesis. These data, then, reveal the biochemical dissimilarities between the two metabolic systems, and give us some insight about the mechanism of macrocyclic ring formation in the biosynthesis of **1**.[4,5]

Formation of the cyclopentane ring of **1** is a more intriguing event than closure of its macrolide ring.[4-6] The biosynthesis of the type D, E, F, G and H prostaglandins[11] is only analogy, but one that cannot be directly applied to the formation **1**.[4,5] Since there are no closely analogous cases in other secondary metabolic pathways, we proposed[4] that the cyclopentane ring is formed by closure of an epoxy-olefin intermediate, itself a 16-membered macrolactone (**F**→**G** in Fig. 3). This process would be similar to the formation of lanosterol from 2,3-oxidiosqualene.[12] We have yet to test this hypothesis directly, but have argued that the absolute configuration of positions 4, 6 and 8 of **1** labeled by $CD_3CO_2H$ supports this mechanism.[6] This argument was based, in part, on the presumption that positions 6 and 8 of **1** correspond to the *alpha*-C of the saturated intermediate of fatty acid chain elongation (**D** in Fig. 1). Their absolute configuration, when chiral due to labeling by $CD_3CO_2H$, would have been set during reduction of an enolylthioester intermediate like **C**, and thus would correspond to the absolute configuration of the even-numbered positions in saturated fatty acids like **2** that also had been labeled by $CD_3CO_2H$. Since the stereochemistry of this labeling varies among different species (*vide infra*), we felt it important to test our hypothesis by comparing the D labeling stereochemistry of fatty acids made by *P. brefeldianum* with positions 6 and 8 of **1**.

## RESULTS

We used the method of isotope-induced shift of $^{13}C$ NMR resonances by directly attached $^2H$ atoms[5,9,16,17] to measure the $^2H$, $^{13}C$-labeling regiochemistry of the fatty acids produced by *P. brefeldianum*. Samples of [$^2H$, $^{13}C$]-steric (**3**) and -oleic (**4**) acids were obtained by saponification of the crude mycelial lipids from *P. brefeldianum* that had been grown in shake cultures for 24 hr in the presence of 5 $\mu$Ci [1-$^{14}C$] acetate and 5*mM* [2-$^2H_3$, 2-$^{13}C$] acetate (98% $^2H_3$, 93% $^{13}C$). Conversion of these samples to their methyl esters and purification by chromatography on $AgNO_3$ impregnated thick layer silica gel plates gave the samples of the methyl[$^2H$, $^{13}C$]-stearate and methyl[$^2H$, $^{13}C$]-oleate used for spectral analysis.

$^{13}C$ NMR spectroscopic analysis of these two samples, using the triple resonance method of McInnes *et al.*[9,18] gave the data shown in Table 1. As expected, the $^{13}C$ enrichment of the even-numbered positions in the

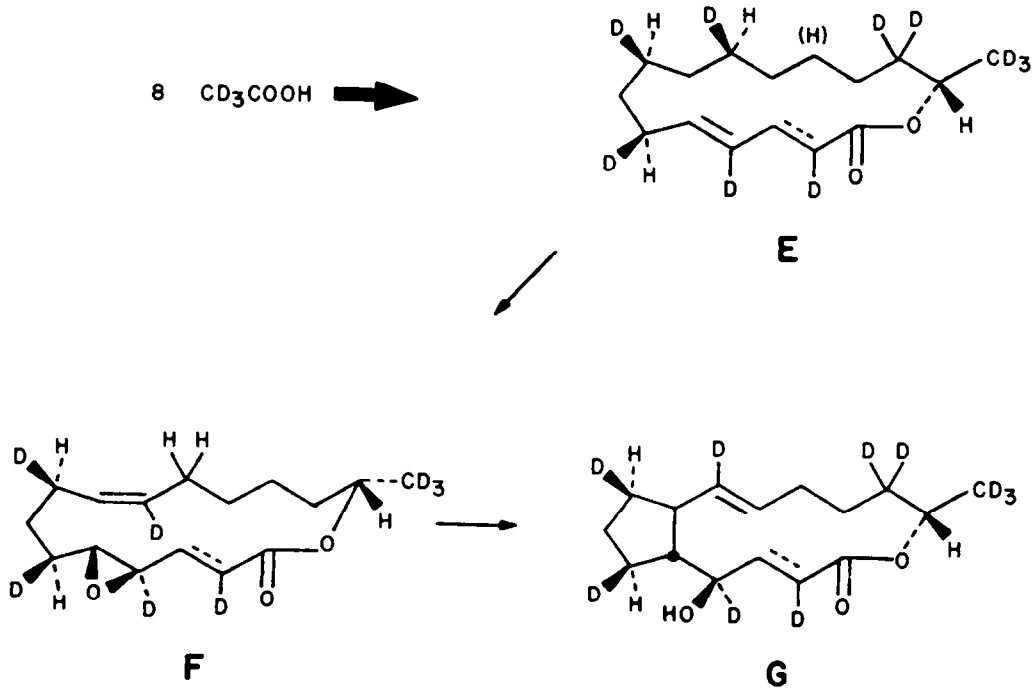



Fig. 3. A hypothesis for formation of the cyclopentane ring of brefeldin A. The labeling stereochemistry of the final product resulting from the incorporation of $CD_3CO_2H$ is shown in the intermediate structures, **F**–**G**.

Table 1. Isotopic labeling pattern of methyl esters of **3** and **4** from the biosynthetic incorporation of $^{13}CD_3CO_2H$[a]

| Carbon | $\delta_C$[b] | $\Delta\delta_C$ | Incorporation of $^{13}C$ (%)[c] | Percent $C^1H^2H$ from $^{13}C_2H_3CO_2H$[d] | $F_D$[d] (%) |
|---|---|---|---|---|---|
| | | **3** | | | |
| 2 | 34.16 | 0.30 | 2.9 | 43 | 43 |
| 4 | 29.36 | 0.48[e] | 2.5 | 51 | 51 |
| 6 | 29.68 | 0.48[e] | 2.5 | 51 | 51 |
| 8,10,12,14 | 29.9 | 0.47[e] | 2.5 | 51 | 51 |
| 16 | 32.14 | 0.50[e] | 2.6 | 48 | 48 |
| 18 | 13.98 | $CH_3$, 0.00<br>$CH_2{}^2H$, 0.31<br>$CH^2H_2$, 0.60<br>$C^2H_3$, 0.91 | 3.1 | $CH_3$, 11<br>$CH_2{}^2H$, 10<br>$CH^2H_2$, 24<br>$C^2H_3$, 55 | 47 ± 7 |
| Error | ± 0.03 | ± 0.06 | ± 0.04 | ± 0.05 | ± 5[f] |
| | | **4** | | | |
| 2 | 34.81 | 0.29 | 4.2 | 40 | 40 |
| 4,6 | 30.08 | 0.43[e] | 4.8[g] | 49[g] | 49[g] |
| 8 | 28.01 | 0.37 | 4.2 | 48 | 48 |
| 10 | 130.72 | h | 3.8 | h | h |
| 12 | 30.66 | 0.38[g] | 4.8[g] | 49[g] | 49[g] |
| 14 | 30.46 | 0.38[g] | 4.8[g] | 49[g] | 49[g] |
| 16 | 32.88 | 0.49 | 4.0 | 51 | 51 |
| 18 | 14.42 | $CH_3$, 0.00<br>$CH_2{}^2H$, 0.30<br>$CH^2H_2$, 0.59<br>$C^2H_3$, 0.89 | 3.9 | $CH_3$, 0<br>$CH_2{}^2H$, 11<br>$CH^2H_2$, 17<br>$C^2H_3$, 72 | 87 |
| Error | ± 0.03 | ± 0.06 | ± 0.6 | ± 0.07 | ± 7 |

[a] See Experimental Section for details of feeding experiments and refs 9 and 18 for details of the spectral analyses.
[b] Solvent was $CDCl_3$:$C_6F_6$ (2:1) containing 8 mg/mL $Cr(acac)_3$.
[c] Based on $^{13}C$ content of precursor (93% $^{13}C$).
[d] Calculated as described in refs. 9 and 18.
[e] Not accurately measurable from the spectra.
[f] Except for C-18
[g] Aver. of C-4, 6, 12 and 14.
[h] No detectable $C^2H$ signal and no change in signal for C-9 $C^1H$ with $^1H$ decoupling only.

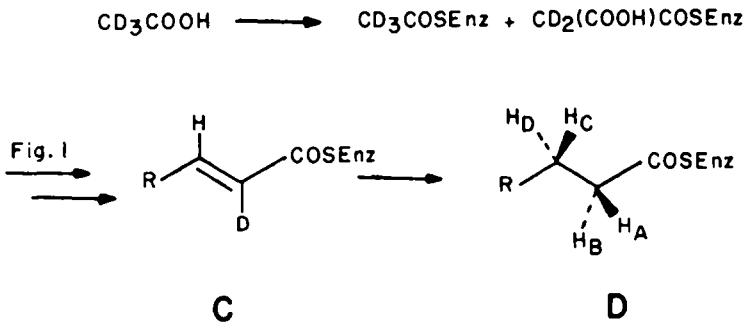
isotopically labeled **3** and **4** was the same within the limits of experimental error. This was also true for the $^2H$ labeling, except for the C-18 Me groups which retain more $^2H$ relative to $^{13}C$ than the other even-numbered positions, as had been found in other studies.[5,9,10] The $F_D$ values for **3** and **4**, which represent the amount of $^2H$ retained per $^{13}C$ atom relative to the precursor $^{13}CD_3CO_2H$ (an FD of 100% would mean complete retention of the $^2H$)[18], have the same range (40–51%) as found for **1** (44–57%)[5]. Of most significance, however, was the complete loss of $^2H$ at C-10 of **4**, yet 51% retention of $^2H$ at this position in **3**.

## DISCUSSION

The stereochemistry of hydrogen flow through the fatty acid biosynthetic pathway is different for the prokaryotes and eukaryotes. Isotopically labeled $C^*H_3CO_2H$ introduces an H label at the 2 *pro S* position during reduction of the enolylSEnz intermediate in three prokaryotes. Two eukaryotes (*C. pyrenoidosa*, *P. tricornutum*) exhibit this same labeling stereochemistry, but two others show the opposite stereochemistry (Table 2). Saito *et al.* have recently shown that at some point during fatty acid biosynthesis (when [2-$^2H_2$]malonate is the labeling source) the configuration at C-2 of **D** is partially racemized.[20] For example, the purified yeast fatty acid synthetase gave palmitate and stearate that were each labeled at their 2 *pro S* (18%), and 2 *pro R* (82%) positions†.[20] The partial loss of $^2H$ in fatty acids labeled by [$^*H_3$]-acetate or [$^*H_2$]-malonate also occurs and has been called

†This result had been obtained earlier by G. R. Drysdale (unpublished results), who observed that crotonyl CoA reductase from yeast gave butyryl CoA with 86% 2 *pro S* and 14% 2 *pro R* T labeling when the reaction occured in the presence of $^3H_2O$ *in vitro*.

Table 2. Stereochemistry of isotopic labeling of fatty acids by $CD_3CO_2H$




C D

| Organism | Position of $^2H$ label in D | Stereospeicificy of α-carbon protonation in C |
|---|---|---|
| E. coli[15,19] | $H_A$[a] | Re |
| B. ammoniagenes[20] | $H_A$ | Re |
| A. nidulans[9] | $H_A$ | Re |
| C. pyrenoidosa[9] | $H_A$ | Re |
| P. tricornutum[9] | $H_A$ | Re |
| P. brefeldianum | $H_B$[b] | Si |
| Yeast[9,21] | $H_B$ | Si |
| Rat liver[22] | $H_B$ | Si |

[a] Same as the S or L configuration in this instance.
[b] Same as the R or D configuration in this instance.

"post malonate exchange" by Sedgwick and Cornforth.[23] McInnes *et al.* have now shown that the loss of isotopic *H occurs from malonate predominantly.[9] This is also true for brefeldin A biosynthesis.[5]

The experimental method we used to determine the $^{13}CD_3CO_2H$-induced isotopic labeling stereochemistry of fatty acids produced by *P. brefeldianum* is based on the known stereochemistry of desaturation in the biosynthesis of unsaturated long chain fatty acids in other living systems. This process converts stearic acid (**3**) to oleic acid (**4**), for example, by stereospecific removal of the 9 *pro R* and 10 *pro R* hydrogens (Fig. 4) in bacteria (*Corynebacterium diphtheria*[13]) and in algae (*Chlorella vulgaris*[14]). The configuration of chiral $R^1CHDR^2$ centers in the saturated fatty acids labeled by $CD_3CO_2H$ or $CD_2(CO_2H)_2$ is the same at all even-numbered positions;[19,20] therefore, the retention or loss of D from C-10 of **3** on its oxidation to **4** reflects the absolute stereochemistry of these chiral centers. We assumed that the desaturation of **3** to **4** *in vivo* by the fungus has the stereospecificity of H removal shown in Fig. 4, which is a reasonable assumption based on the literature procedents.[13-15] Therefore, in **3** the $^2H$ label at C-10 had the *R*(**D**) configuration and was lost in the formation of **4** by desaturation.

Our results show that the H-isotope labeling pattern of stearic acid (**3**) and oleic acid (**4**) in *P. brefeldianum* mirrors that seen for the two of the other four eukaryotic fatty acid synthetases (Table 2). We could not, however, accurately determine if C-10 of **3** was partially racemized because of the low S/N ratio in the $\{^1H, ^2H\}^{13}C$ NMR spectrum due to insufficient sample, and the partial overlap of the C-10 $^{13}C^2H$ resonance with the C-9 $^{13}C^1H$ resonance at 25.2 MHz (Table 1).

These data reveal that the labeling stereochemistry at C-6 and C-8 of brefeldin A (**1**) is opposite to that in the corresponding positions of the saturated mycelial fatty acids produced by the same organism, which negates our earlier conclusion.[6] If the configuration of C-6 and C-8 of **1** is determined as we stated earlier in this paper, then two things about the stereo-

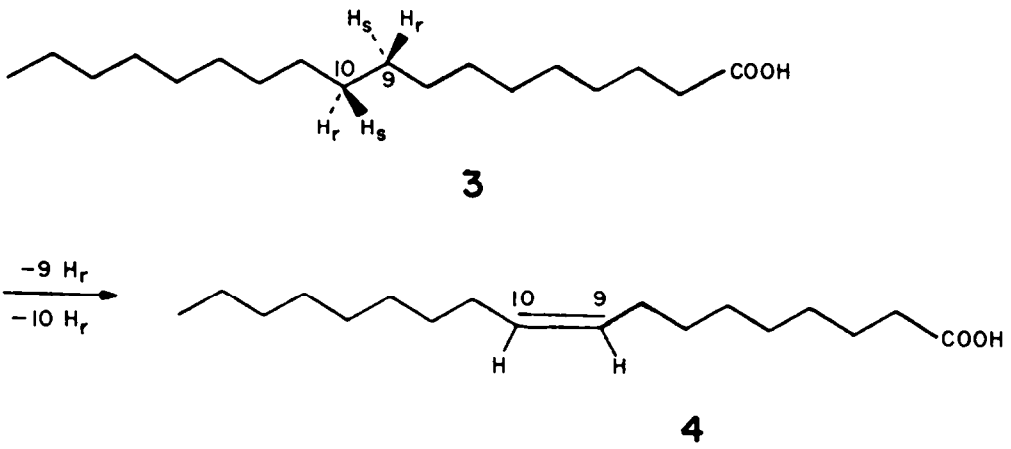



Fig. 4. The stereochemistry of the desaturation of long chain fatty acids in algae and bacteria.

chemistry of brefeldin A biosynthesis are unique to the secondary metabolic pathway: the stereospecificity of carbonyl reduction at C-15,[6] and of enolylSEnz protonation at C-6 and C-8.[6] On the other hand, the C-6 and C-8 configuration could be determined in some other way, such as a result of the as yet unexplained mechanism of cyclopentane ring formation. If this were true (which we doubt), then it would weaken our argument supporting the hypothesis for the latter mechanism[6]: *viz*, that the C-4 configuration and $^2H$ labeling retention from $CD_3CO_2H$ are consistent with its being the product of C-4,5 olefin oxidation and transannular cyclization (Fig. 3). In any event it is clear that one has to be cautious in drawing mechanistic conclusions about secondary metabolic processes by comparison with primary metabolic pathways.

## EXPERIMENTAL

*Feeding experiment.* Slant medium: malt extract, 20 g; dextrose, 20 g; peptone, 1 g; water, 1L; agar, 20 g. Inoculum medium: yeast extract 10 g; dextrose, 10 g; corn steep liquor, 5 g; water, 1 L. Production medium as described in Ref. 4.

Spores from two slants of *Penicillium brefeldianum* Sandoz 464 were transfered into the inoculum medium (50 ml per flask) in four 250 ml Erlenmeyer flasks. These cultures were grown 4 days at 25°–26° on the NBS (G-25) shaker at 250 rpm. The contents of each of the flasks then were homogenized three times by a Waring blender for 3 sec and poured into 2 L Erlenmeyer flasks containing 350 ml of the production medium. [2-$^{13}C$, 2-$^2H_3$] acetate (93% $^{13}C$, 98% $d_3$), 168 mg, and [1-$^{14}C$] acetate (5 $\mu$Ci) were added to each flask, and the cultures were incubated for 24 hr as before. Trial feeding experiments with [$^{14}C$] acetate had shown that this protocol resulted in the highest incorporation of $^{14}C$ into the crude mycelial lipids.

After incubation, the mycelial mass was removed from each flask by suction filtration through Whatman No. 1 filter paper, combined and air-dried at 45–50° for 3 days. This yielded 15.1 g of dry mycelia.

*Isolation of stearic* (**3**) *and oleic* (**4**) *acids.* The dry mycelia was extracted continuously with EtOAc for 3 days in a Soxhlet extractor. The EtOAc extract was washed with water, dried over $Na_2SO_4$, and evaporated to an oily residue (2.9 g). This material was saponified with 10% KOH and MeOH (50 ml) by refluxing for 1 hr. The reaction mixture was neutralized with 6N HCl, extracted with an equal volume of Skelly B (hexane) which was discarded, acidified with 6N HCl to pH 2, re-extracted with diethyl ether three times, the combined ether extracts dried over $Na_2SO_4$, and then evaporated to give a crude mixture of fatty acids (726 mg). This mixture (720 mg) was re-dissolved in diethyl ether and treated with an etheralethanolic solution of $CH_2N_2$ at 5°–15° until the pale yellow color of excess $CH_2N_2$ persisted. After destruction of the excess $CH_2N_2$ with glacial AcOH, the solvents were evaporated and the resulting crude mixture of fatty acid methyl esters was purified by thick-layer chromatography on silica gel plates (400 cm$^2$) containing 20% (w/w) $AgNO_3$ and developed in the dark with hexane–diethyl ether (2:8). The bands corresponding to the methyl stearate and methyl oleate were visualized under UV light after spraying with 0.2%, 2′,7′-dichlorofluorescein in $H_2O$–EtOH (1:1). The esters were obtained by elution with diethyl ether from the recovered adsorbent and solvent evaporation to give the methyl-ester of **3** (107 mg) and of **4** (78 mg).

The remainder of the crude mixture of free fatty acids (*ca* 6 mg) was converted to the mixture of *p*-bromophenacyl esters by refluxing 1 hr with *p*-bromophenacyl bromide (6 mg) in acetone (2 ml) containing $Et_3N$ (15 $\mu$l). The crude reaction products were purified as before to give crystalline samples of the corresponding esters of **3** and **4**. These compounds were identical with authentic reference standards by m.p. and TLC analysis.

A sample of the [$^2H$, $^{13}C$, $^{14}C$]-methyl stearate (5 mg) was diluted with unlabeled methyl stearate (400 mg), saponified, and converted to its *p*-bromophenacyl ester as described above. This sample was recrystallized four times to a constant $^{14}C$ specific activity ($1.76 \times 10^4$ dpm $^{14}C$/mmole). Since the $^{14}C$ specific activity of the labeled acetate solution added to the production medium was $6.3 \times 10^6$ dpm/mmole, this value represents a specific incorporation of 22.5%, or 2.5% per $C_2$ unit, of the precursors into **3**, after correction for the dilution by the unlabeled methyl stearate.

*Spectral analyses.* These were done at Halifax as described in Refs. 9 and 18.

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## REFERENCES

[1]U. Handschin, H. P. Sigg and Ch. Tamm, *Helv. Chim. Acta* **51**, 1943 (1968).
[2]R. G. Coombe, P. S. Foss and T. R. Watson, *Chem. Commun.* 1229 (1967).
[3]R. G. Coombe, P. S. Foss, J. J. Jacobs and T. R. Watson, *Austral. J. Chem.* **22**, 1943 (1969).

[4]C. T. Mabuni, L. Garlaschelli, R. A. Ellison and C. R. Hutchinson, *J. Am. Chem. Soc.* **101**, 707 (1979).
[5]C. R. Hutchinson, I. Kurobane, C. T. Mabuni, R. W. Kumola, A. G. McInnes and J. A. Walter, *Ibid.* **103**, 2474 (1981).
[6]C. R Hutchinson, I. Kurobane, D. E. Cane, H. Hasler and A. G. McInnes, *Ibid.* **103**, 2477 (1981).
[7]M. Sunagawa, T. Ohta and S. Nozoe, *Heterocycles* **13**, 267 (1979).
[8]A. Hori and C. R. Hutchinson, unpublished results.
[9]A. G. McInnes, J. A. Walter and J. L. C. Wright, this issue **Ms. S8.**
[10]A. G. McInnes, J. A. Walter and J. L. C. Wright, *Tetrahedron Letters* 3245 (1979).
[11]B. Samuelsson, *J. Am. Chem. Soc.* **87**, 3011 (1965).
[12]P. D. G. Dean, P. R. Ortiz de Montellano, K. Block and E. J. Corey, *J. Biol. Chem.* **242**, 3014 (1967); J. D. Willett, K. B. Sharpless, K. E. Lord, E. E. van Tamelen and R. B. Clayton, *ibid.* **242**, 4182 (1967).
[13]G. J. Schroepfer and K. Bloch, *J. Biol. Chem.* **240**, 54 (1965).
[14]L. J. Morris, R. U. Harris, W. Kelly and A. T. James, *Biochem. Biophys. Res. Commun.* **28**, 904 (1967).
[15]R. H. White, *Biochemistry* **19**, 9 (1980).
[16]G. E. Maciel, P. D. Ellis and D. C. Hafer, *J. Phys. Chem.* **71**, 2160 (1967).
[17]J. B. Stothers, *Top. Carbon-13 NMR Spectrosc.* **1**, 229 (1974).
[18]A. G. McInnes, J. A. Walter and J. L. C. Wright, *Lipids* **15**, 609 (1980).
[19]K. Saito, A. Kawaguchi, Y. Seyama, T. Yamakawa and S. Okuda, *Eur. J. Biochem.* **16**, 581 (1981).
[20]K. Saito, A. Kawaguchi, Y. Seyama, T. Yamakawa and S. Okuda, *J. Biochem.* **90**, 1967 (1981).
[21]B. Sedgwick and C. Morris, *J. Chem. Soc.* Chem. Commun. 96 (1980).
[22]A. Kawaguchi, T. Yoshimura, K. Saito, Y. Seyama, T. Kasama, T. Yamakawa and S. Okuda, *J. Biochem.* **88**, 1 (1980).
[23]B. Sedgwick and J. W. Cornforth, *Eur. J. Biochem.* **75**, 465 (1977).