THE USE OF ISOTOPIC HYDROGEN AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC TECHNIQUES FOR THE ANALYSIS OF BIOSYNTHETIC PATHWAYS[1]

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The progress of basic research on the biosynthesis of natural products, whether they are primary metabolites common to the biochemistry of all living organisms or secondary metabolites having a largely species-specific distribution and an uncertain role in the producing organism's ontogeny, has depended greatly on the use of the isotopes of C, H, O and other elements found in living systems. Thereby, scientists have been able to deduce probable precursor-product relationships in complex biochemical pathways and to determine the mechanism of enzymatically catalyzed transformations by following the fate of molecules whose isotopic "label" allows their distinction among the other molecules present in the system. Sometimes the labeling isotope imparts subtle differences to the way in which the molecules interact with their environment, enabling deductions about the character of the transition state of enzymatically catalyzed reactions by comparison of the reaction rates of substrates (1). More often the isotope simply affords distinction of the labeled substrate molecules from all the otherwise identical molecules in the system without perturbing how these molecules react. If the labeling isotope is present at a significantly higher concentration than its natural abundance, then analysis of its presence and/or exact location within the product molecules reveals information about the probable events experienced by all of the substrate molecules during their biochemical processing. This is the basis for most experimental studies of the biosynthesis of secondary natural products, e.g., alkaloids, antibiotics and terpenoids, since the study of the enzymology of secondary natural products like these is not well-advanced.

The use of radioactive isotopes (^{14}C, ^{3}H, ^{32}P) in such biosynthetic studies predominated during the period between 1946 and 1970, even though ^{2}H was the isotope first used to study metabolic processes (2,3). The main reason for their preferential use over stable isotopes is sensitivity. It is possible to detect and quantify accurately compounds labeled with radioactive isotopes after admixture up to 10^{9} times (^{14}C) with their non-labeled forms. Since dilutions of 10^{6}-fold are commonly encountered when studying the biosynthesis of secondary natural products found in plants, for example, it is often necessary to use isotopes that can be detected with high sensitivity. On the other hand, the use of radioactive isotopes has several disadvantages; two of which—the tedious methods necessary for determination of labeling regiochemistry (4) and the inability to analyze intramolecular events during bond making-and-breaking processes at sensible levels of isotopic enrichment—have hindered the progress of research in the field.

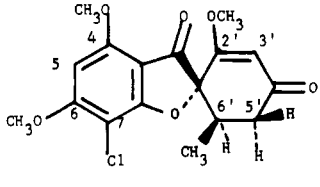
I illustrate by this eclectic review of the use of hydrogen isotopes in biosynthetic studies how to developments during the decade of 1970 have resulted in a marked increase in the use of stable isotopes (^{13}C, ^{2}H, ^{15}N and ^{18}O) to study biochemical processes governing the formation of secondary natural products. First, the problem of low sensitivity for the detection and quantification of stable isotopes, characteristic of the period before 1970, has been overcome by the commercial availability of mass and nuclear magnetic resonance (nmr) spectrometers having high resolution and sensitivity adequate for the accurate measurement of stable isotopes at low levels (enrichments only 50% $\geq$ natural abundance). Today it is

[1]Presented as a plenary lecture at the joint meeting of the American Society of Pharmacognosy and the Society for Economic Botany, Boston, Massachusetts, July 13–17, 1981.

possible to measure atom percent excesses over natural abundance of 0.55% for ^{13}C and 0.015% for ^{2}H in 0.1 mmoles of a compound with a molecular weight less than 500 in a few hours by nmr analysis with a modern super-conducting nmr Fourier transform spectrometer. Second, the results of some of the earliest uses of ^{13}C labeled compounds to probe percursor-product relationships among the polyketides (5)-natural products which are formed by the repeated condensation of simple fatty acids via poly-*beta*-ketone reaction intermediates—have revealed a wealth of new biosynthetic information in the analysis of intramolecular bond formation and rupture processes. The latter events are readily observed by the appearance or disappearance of unique multiplicities due to the coupling of neighboring ^{13}C atoms in the broad-band proton-decoupled ^{13}C nmr spectrum of multiply ^{13}C-enriched natural products. These two facts and related advances in the analysis of isotopic enrichment by nmr spectroscopy, particularly, have accelerated the pace of research into the biosynthesis of natural products. The following examples, which highlight the use of ^{2}H as a probe of biochemical transformations, show the power of stable isotopes in uncovering important clues about the intricate ways evolved in nature for the assembly of structurally complex substances.

Griseofulvin

Griseofulvin (**1**) has a significant position in the history of the investigation of natural product biosynthesis. It was one of the earliest fungal products to be examined experimentally (6) and one of the few such natural products containing a halogen atom. The results of studies with ^{14}C-labeled precursors have established that **1** is a heptaketide (6,7) and that compounds **2** (8) and **3** (9) are probable intermediates of its biosynthetic pathway. During the past five years two research groups have used nmr spectroscopy to analyze the labeling of **1** by $C^{2}H_3CO_2H$ (10), $^{13}C^{2}H_3CO_2H$ (11), and $^{2}H_2O$ (9,11) in *Penicillium* species. While the results of the latter studies using $^{2}H_2O$ clearly reveal the stereospecificity of hydrogen addition during the reduction of **3** to **1** in vivo, those of the two former studies cannot be interpreted completely from the data available (10,11). For example, the incorporation of $C^{2}H_3CO_2H$ resulted in the presence of $C^{2}H^{1}H_2$ groups on the three oxygen atoms at C–2′, C–4 and C–6 of **1** (10). Since the *O*-methyl groups of **1** must originate from the *S*-methyl groups of methionine without significant exchange of the methyl hydrogens (12), there must have been transfer of ^{2}H label from $C^{2}HC_3O_2H$ to the *S*-methyl group of methionine. This transfer must have been indirect and have involved deuterons since growth of *P. urticae* in $^{2}H_2O$ resulted in $C^{2}H_2{}^{1}H$ groups at C–2′, C–4 and C–6 of **1** (11). Perhaps $C^{2}H_3CO_2H$ gave [3-$^{2}H^{1}H$] serine from which [*SMe*-$^{2}H^{1}H_2$] methionine resulted via N^{5},N^{10}-[$^{2}H^{1}H$]methylene and N^{5}-[$^{2}H^{1}H_2$]methyl tetrahydrofolic acid (13). Answers to questions like this will not be available until more research is done on the dynamics of primary metabolism in microorganisms.



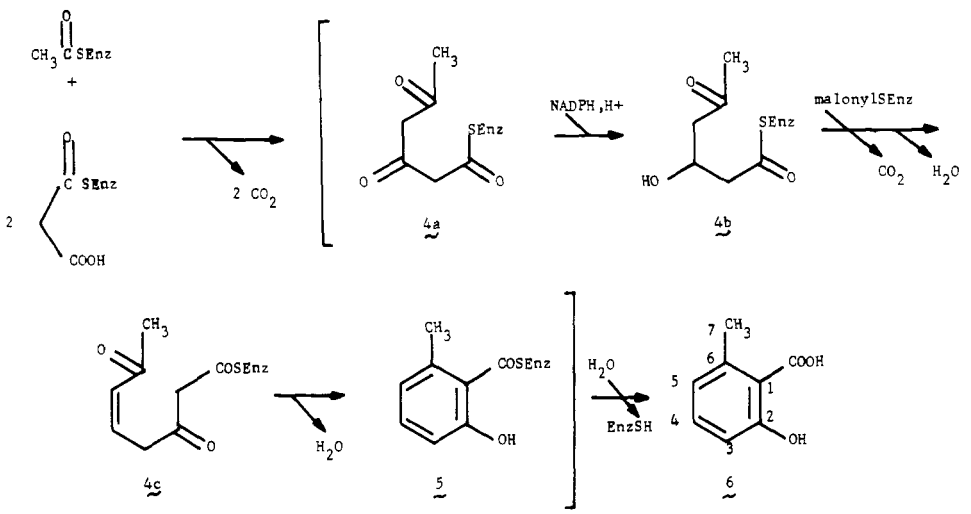
1 2 3

6-Methylsalicylic acid

The fungal metabolite, 6-methylsalicylic acid (**6**), is the prototype polyketide. The demonstration of its derivation from one acetate and three malonate molecules was the first experimental verification of the polyketide hypothesis (14), and led Feodor Lynen to formulate the biochemical "polyacetate rule" (15), in which

he proposed that carbon chain assembly of all polyketides begins with a starter unit like acetate or propionate, continues with carbon chain extension by the α-carboxy analogue of the starter unit (malonate is the most common chain-extending unit), and involves only enzyme-bound intermediates. The results of a large number of biosynthetic experiments during the past twenty-six years support this idea for the formation of aromatic (16) and non-aromatic (17) polyketides.

There is not great depth to our understanding of polyketide biochemistry since most of the research has been done in intact microorganisms. Information about the enzymology and molecular biology of polyketide formation thus is scanty, the best evidence coming from studies of 6-methylsalicyclic acid (6-MSA) synthetase (18) and patulin, a fungal metabolite derived from **6** by a series of oxidative transformations (19). The physical properties, structural organization, and substrate-cofactor requirements of 6-MSA synthetase are very similar to those of the fatty acid synthetases found in the same organism (18,20,21) and in yeast. These facts and other information are consistent with the scheme drawn below for the formation of **6** by the purified enzyme in vitro.

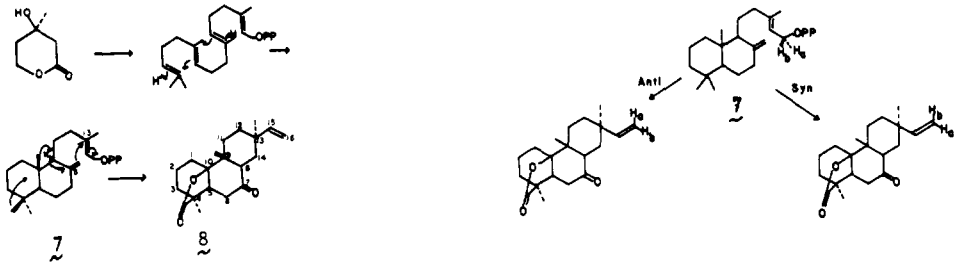


The results of biosynthetic studies of **6** done in whole microorganisms agree very well with the above hypothesis, and substantiate the general belief that the results of in vivo experiments are a valid reflection of the implicit biochemical events. Nonetheless, there are limits to what we can learn in this way. This is well-illustrated by the results of a recent study of the labeling of **6** in vivo by $C^2H_3{}^{13}CO_2H$ (22). The authors demonstrated the utility of a new technique, the β-isotopic shift of carbon resonances in the ^{13}C nmr spectrum, for observing the ^2H labeling of natural products resulting from precursor incorporation. Using this method they determined that incorporation of the acetate precursor into **6** by *Penicillium griseofulvum* although regio-specific, was accompanied by partial loss of ^2H. The observed losses, C-3 (20%), C-5 (30%) and C-7 (5%), were unequal and disagreed somewhat with earlier data obtained by chemical degradation of **6** labeled biosynthetically with $C^3H^1H_2{}^{14}CO_2H$ (23). The authors suggested that the non-uniformity of ^2H loss could be due to different degrees of random exchange during the process of carbon chain assembly, or to different mechanisms of ^2H loss at C-3 and C-5 during the steps of cyclization and aromatization (**4a**$\rightarrow\rightarrow\rightarrow$**5**). It is reasonable to expect that ^2H would be lost by exchange from the methylene carbon of enzyme-bound malonate or from C-3 and C-5 of **4a** and **4b**, since all of these positions should be acidic based on the ease of their enolization in vitro (24,25). Yet the proof of the authors' hypothesis will be gained only by studies with 6-MSA synthetase in vitro where observations can be done directly and some

control can be placed on the possibilities for exchange of isotopic hydrogen with the enzyme or environment.

Rosenonolactone

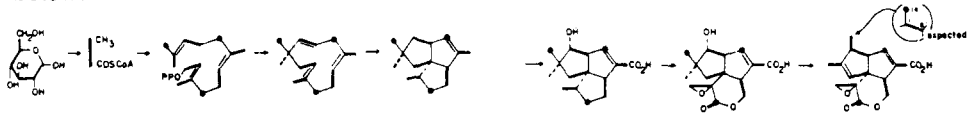
Rosenonolactone (**8**) is a fungal diterpene whose biosynthesis proceeds from mevalonic acid via geranylgeranyl pyrophosphate and **7**. Cane and Murthy have investigated the biochemical transformation of **7** into **8**, wherein they examined two mechanisms for the formation of ring C of **8** by analysis of the incorporation of (5*R*) and (5*S*)-[5-$^2H_1$] mevalonate into **8** in vivo (26). As shown below, **7** can undergo two modes of ring closure: both are formally S_N2' processes, one involving syn, and the other anti, departure of the C–16 pyrophosphate relative to the *si* face of the allylic pyrophosphate that is attacked by the electrons of the terminal methylene at C–8. The authors made the distinction between these two possibilities by determining which of the two diastereotopic hydrogens at C–16 of **7** becomes cis and which becomes trans to the C–C bond in the terminyl vinyl group of **8**. Since incorporation of each of the two diastereotopimers of [5-^{2}H] mevalonate into **8** resulted in stereochemically unique ^{2}H labeling at C–16 of **7**, which could be correlated to the corresponding ^{2}H labeling of C–6 as an internal reference, determination of the ^{2}H labeling regiochemistry at C–16 of **8** by ^{2}H nmr analysis at 41.44 MHz ($\Delta\delta_D$ H-16*Z* and H-16*E* 0.07 ppm!) gave the authors the necessary data. These results established that this biochemical S_N2' reaction occurs with overall anti stereochemistry. It would have been very difficult to deduce this fact by any means other than the nmr spectroscopic analysis of ^{2}H labeling stereochemistry.



7 8

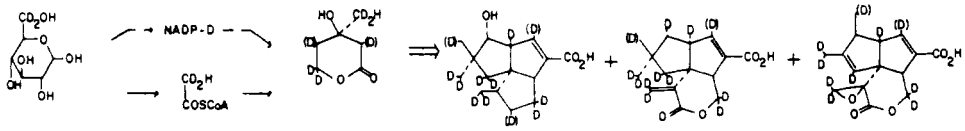
Pentalenolactone

Pentalenolactone (**11**) is an antibiotic produced by a *Streptomyces* species and presumably is formed from mevalonic acid via farnesyl pyrophosphate, humulene, and the tricyclic intermediates, **9** and **10**, as shown below. However, Cane and co-workers found that **11** was not labeled in biosynthetic experiments with radioisotopically labeled acetate and mevalonate despite numerous feeding regimens (27). This frustration led them to test a new approach for providing labeled mevalonate in vivo. Feeding of [*U*-$^{13}C_6$] glucose in admixture with unlabeled glucose to suppress the intramolecular ^{13}C-enrichment of precursor pools feeding the pathway to **11** was anticipated to result in the formation of $^{13}CH_3{}^{13}CO_2H$ via aerobic glycolysis. This uniformly labeled acetate should then have given the three intermolecularly ^{13}C-labeled forms [^{13}C-1,2; ^{13}C-3,3'; ^{13}C-4,5] of mevalonate by precedented biochemical reactions. ^{13}C nmr analysis of the ^{13}C labeling pattern of **9** and **11** confirmed the correctness of these two presumptions and the derivation of **11** from mevalonate as indicated by the thickened lines in the structures drawn below.



9 10 11

The results of a subsequent feeding experiment with [6-$^2H_2$] glucose were less clear than the above findings for establishing the mevalonoid origin of **11**. Catabolism of the [6-$^2H_2$] glucose via aerobic glycolysis was expected to give [2-$^2H_2$,^1H] acetate largely, thence a mixture of [2-^2H], [4-^2H] and [3^1-$^2H_2$] mevalonate. However, ^2H labeling also must have been present at C–5 of mevalonate since the ^2H nmr spectra of **11** and the two other terpenoid metabolites shown below revealed a multiplicity of ^2H labeling patterns, which could be rationalized only in this way. The authors pointed out that a recent demonstration in yeast and *E. coli* of the rapid equilibration of C–1 and C–6 ^{13}C-labeled hexose substrates compared to the overall glycolytic flux (28) permits an explanation of their results. [6-^2H] Glucose must have given [1-^2H] glucose, which is known to serve as a source of [4-^2H] NADPH by the action of glucose-6-phosphate dehydrogenase. This coenzyme provides the two biochemical hydride equivalents introduced at C–5 of (*R*)-mevalonate in its biosynthesis from (*S*)-3-hydroxy-3-methylglutaryl CoA. In spite of the unexpected experimental difficulties, some of the data obtained from the ^2H nmr spectra of **9**, **11** and **12** were useful to the authors in making important deductions about the mechanism of the biochemical cyclization of farnesyl pyrophosphate to humulene (27).

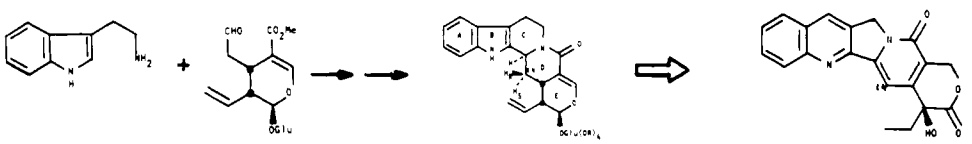


9 12 11

Camptothecin

We have studied the biosynthesis of the alkaloid camptothecin (**15**) for several years, using spectroscopic analysis and chemical degradation to obtain the data necessary for a thorough understanding of its formation in the plant, *Camptotheca acuminata*. One result from our earliest experiments (29) puzzled us for sometime since it seemed inconsistent with our hypothesis that **15** is assembled in vivo from tryptophan and secologanin (**13**) via strictosamide (**14**) as outlined below. Since ring D of **14** or a subsequent biochemical intermediate must become aromatized during the formation of **15**, one-half of the C–14 isotopic label if situated equally in the *pro R* and *pro S* hydrogens should necessarily have been lost from **14** upon its conversion to **15**. This will be observed if the removal of either diastereotopic hydrogen label occurs by a stereospecific and presumably enzymatically catalyzed process; if it does not, then the observed loss of label may be much less than 50% depending on the extent of substrate-to-product conversion and the magnitude of the kinetic isotope effect. We found a ^3H loss of only $7 \pm 2\%$ relative to the ^{14}C reference label despite a fairly good conversion of **14** to **15**, but could not verify that the [14-^{3}H,5-^{14}C]-**14** had labeled only C–14 of **15** with ^3H because of the lack of suitable chemical degradations. Since this shortage of data left several possibilities open for the reason(s) behind the small ^3H loss, we repeated feeding experiments using [14-^{2}H, 14-^{3}H]-**14** and ^2H nmr analysis of the precursor and labeled **15** to ascertain three things (30). One, that **14** was labeled only in the diastereotopic C–14 hydrogens; two, that each of these two positions carried an equal amount of ^2H label; and three, that the resulting [^{2}H, ^{3}H]-**15** had been ^2H labeled only at C–14. Since it is sensible to assume that the ^3H and ^2H labels experienced identical events during precursor's labeling and incorporation, as reflected in their nearly identical specific incorporations into **15**, we are left with one reasonable explanation for the small loss of ^3H from [14-^{3}H, 5-^{14}C]-**14**. The label must have been lost by a non-stereospecific, probably non-enzymatically catalyzed process that was accompanied by a large kinetic isotope effect. This was observable only because (a) the precursor was necessarily intermolecularly labeled with ^3H and

^{14}C, as are almost all radioisotopically labeled compounds used in biosynthetic experiments, and (b) less than 100% of the precursor was converted to [^{3}H, ^{14}C]-**15** in vivo. Our conclusion is thus reasonable, precedented by the results of at least two other biosynthetic studies (30), and does not invalidate the central role that **14** plays in the biosynthesis of **15** within the limits of experiments carried out in intact plant tissues.



13 **14** **15**

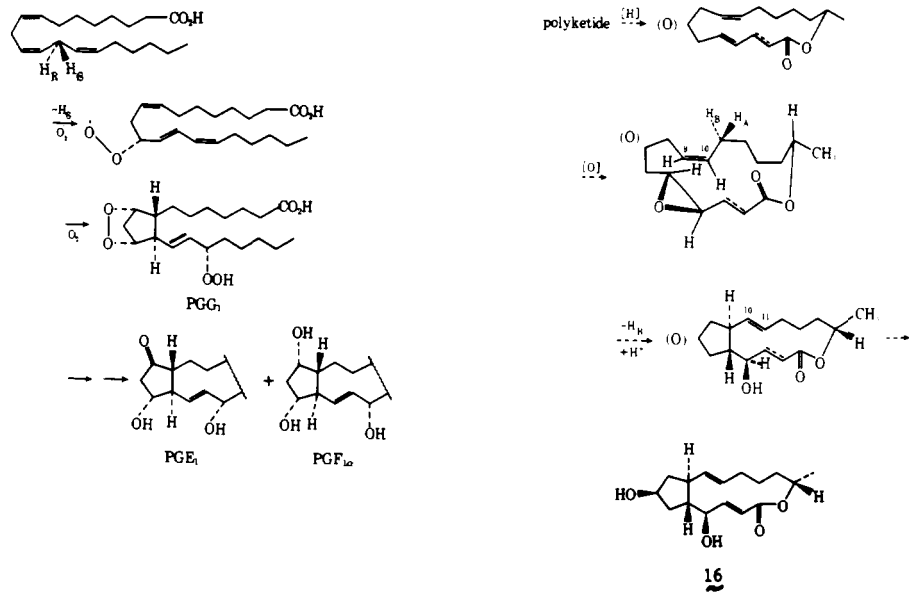
The use we made of ^{2}H labeled precursors and nmr spectroscopic analysis was the first demonstration of the applicability of these techniques to biosynthetic studies carried out in a plant. S. R. Jensen and co-workers have used this approach subsequently to study the biosynthesis of cornin, an iridoid glycoside found in *Verena officinalis* (31).

Brefeldin A

Since the biosynthesis of macrolide antibiotics involves the assembly of acetate, propionate or butyrate into molecules containing multiple C_2 to C_4 sub-units, they usually are classified as polyketide metabolites. They differ from most of the other polyketides because their structures contain many saturated sub-units and seldom contain aromatic rings (17). Consequently, macrolide antibiotic biosynthesis is characterized more by the sequence of precursor assembly and biochemical reduction than by the regiochemistry of the cyclization of poly-*beta*-ketone intermediates, which typifies the biosynthesis of aromatic polyketides (16).

Brefeldin A (**16**) is an intriguing macrolide antibiotic since its structure closely resembles the E, F and G type of prostaglandins. This fact stimulated one group of researchers to test the idea that a C_{16} fatty acid could be the biosynthetic precursor of **16**, based on the knowledge that eicosatrienoic acid serves as the specific biochemical precursor of PGE_1 and $PGF_{1\alpha}$ via PGG_1 through an oxidative cyclization involving both atoms of molecular oxygen (33) as shown below. Although their initial results (32) supported the hypothesis of a biosynthetic commonality between **16** and the prostaglandins, another research group later reached the opposite conclusion (33) concerning the role played by a C_{16} fatty acid in the biosynthesis of **16**. We also have provided information pertinent to this question, which removes a fatty acid origin of **16** from further serious consideration, and which provides some insight about the possible mechanism of cyclopentane ring formation (34) as we suggest below.

Our study of brefeldin A biosynthesis provided new information of a kind different than above about the comparative biochemistry of fatty acid and macrolide antibiotic biosynthesis, when we studied the flux of isotopic hydrogen labels through the relevant pathways in vivo. We have made a comparison of the regiochemistry (35) and stereochemistry (36) of ^{2}H labeling of **16** resulting from incorporation of $^{13}C^{2}H_3CO_2H$ and $C^{2}H_3CO_2H$ by *Penicillium brefeldianum* with the literature data from similar studies of fatty acids in *E. coli*, yeast and algae. Saturated fatty acid biosynthesis from $C^{2}H_3CO_2H$ results in a $C^{2}H_3$ unit at the ω-end (plus varying amounts of $C^{2}H_m{}^{1}H_{3-m}$ units due to partial exchange of ^{2}H) and $C^{2}H^{1}H$ units at all even-numbered positions. These alternate methylene groups exhibit different amounts of exchange of ^{2}H label (30–60% loss of ^{2}H) and have different overall absolute stereochemistries depending on the organism studied (35,36). Brefeldin A labeled by the incorporation of $C^{2}H_3CO_2H$ has ^{2}H at all the even-numbered positions except C–12, exhibits a loss of ^{2}H between 43 and 56% per even-numbered position but only 13% at the ω-methyl group, and



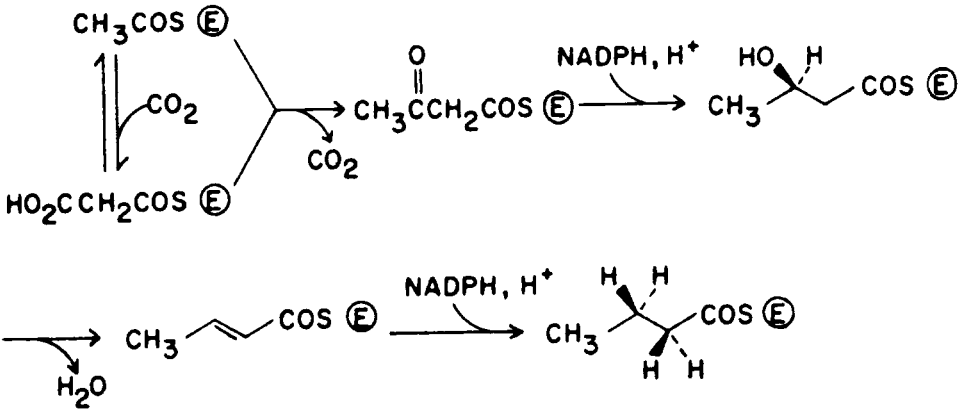
has an absolute stereochemistry for the ^2H at C–6 and C–8 which is identical to that found for the chiral methylene groups of C_{16}–C_{18} fatty acids in *E. coli* and algae. We believe these data support the hypothesis that the mechanism of saturated fatty acid and "polyacetate" macrolide (like **16**) biosynthesis is very similar. The observed differences are due to special features of the species-specific mechanisms of macrolide formation.

It is instructive to point out, in line with the purpose of this review, that we were able to analyse the complete ^2H labeling of brefeldin A by nmr techniques in a few weeks, whereas it required six months to accomplish this for only four positions of the antibiotic by chemical degradation of **16** labeled by the incorporation of $C^3H^1H_2CO_2H$!

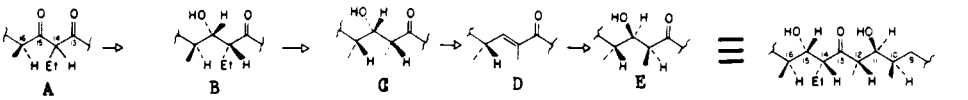
Lasalocid A

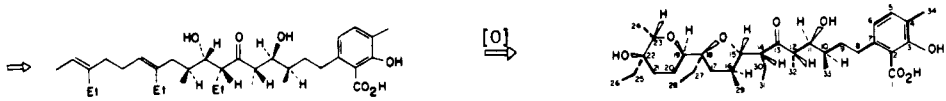
Lasalocid A (**18**) and the other polyether antibiotics whose biosynthesis has been examined (37) also are polyketide metabolites. The entry of at least three different simple fatty acids (acetate, propionate and butyrate) into the carbon chain assembly processes and the presence of heterocyclic oxygen-bearing, alicyclic, or aromatic rings are characteristics of the biosynthesis of polyether antibiotics. Thus the biochemistry of their formation is more diversified than that of macrolide antibiotics, which often uses only one fatty acid precursor and results in only one large lactone ring. We are interested in the biosynthesis of polyether antibiotics for two reasons. One, what are the biochemical mechanisms that control the absolute stereochemistry at alkyl and oxygen-bearing methine carbons; two, what is the biochemical recognition process that determines the sequence of precursor assembly during carbon chain formation? Since the structures of the known polyether antibiotics (38) exhibit an irregular absolute stereochemistry at such sites and a variable sequence of C_2 to C_4 sub-units in their contiguous carbon skeletons, yet are presumed to be formed by enzymology analogous to that governing fatty acid biosynthesis—a highly regular process as outlined below—we believe these questions are very intriguing.

Very recently we have obtained data from our first experimental investigation of lasalocid A biosynthesis that gives some insight about the stereochemical control of its formation by *Streptomyces lasaliensis*. The C–9 to C–16 subfragment of **18** contains two methine carbons (C–11 and C–15) that bear an oxygen sub-



stituent. Their absolute stereochemistry is enantiotopic and they are situated *threo*[2] to the alkyl group at the respective vicinal positions, C–10 and C–14. One hypothesis for the biochemical formation of this portion of **18** is drawn below. If the C–15 carbonyl of substructure A, which results from the addition of a butyrate-derived C_4 unit to the C–15 end of the growing carbon chain and thereby has a defined C–14 absolute stereochemistry, is reduced stereospecifically to B then this fixes the absolute stereochemistry found at C–14 and C–15 in **18**. Subsequently, a propionate-derived C_3 unit must be added to C–11 of the growing carbon chain followed by reduction to give substructure C. If these two processes occur with the same stereospecificity as before, then C will have the absolute stereochemistry shown below. Since **18** has the enantiotopic stereochemistry at these two positions, a likely process for the stereochemical inversion of C would be its dehydration to D followed by stereospecific rehydration from the opposite face of the C–10, C–11 double bond to give E. The latter substructure has the absolute stereochemistry found at C–10 and C–11 in **18**. Our working hypothesis had more significance than just the biosynthesis of **18** since similar vicinally situated oxygen atoms and alkyl groups in many macrolide and polyether antibiotics have such an enantiotopic *threo* relationship.





17 18

To test our hypothesis we chose to determine the fate of isotopic hydrogen and oxygen labels in the α-position (^2H) and carboxyl (^{18}O) of propionate and butyrate, the two precursors of the C–9 to C–16 portion, during their incorporation into **18** in vivo. J. W. Westley and co-workers had shown earlier that acetate, propionate and butyrate were the specific biochemical precursors of **18**, and had postulated that a late event in the biosynthetic pathway was the oxidation of **17** and stereoselective intramolecular cyclization of its C–18, C–22 *bis*-epoxide to **18**

[2]This nomenclature is correct if the molecule is drawn in the Fischer convention for those four carbons that define the subunit bearing the alkyl and hydroxyl or oxygen substituents.

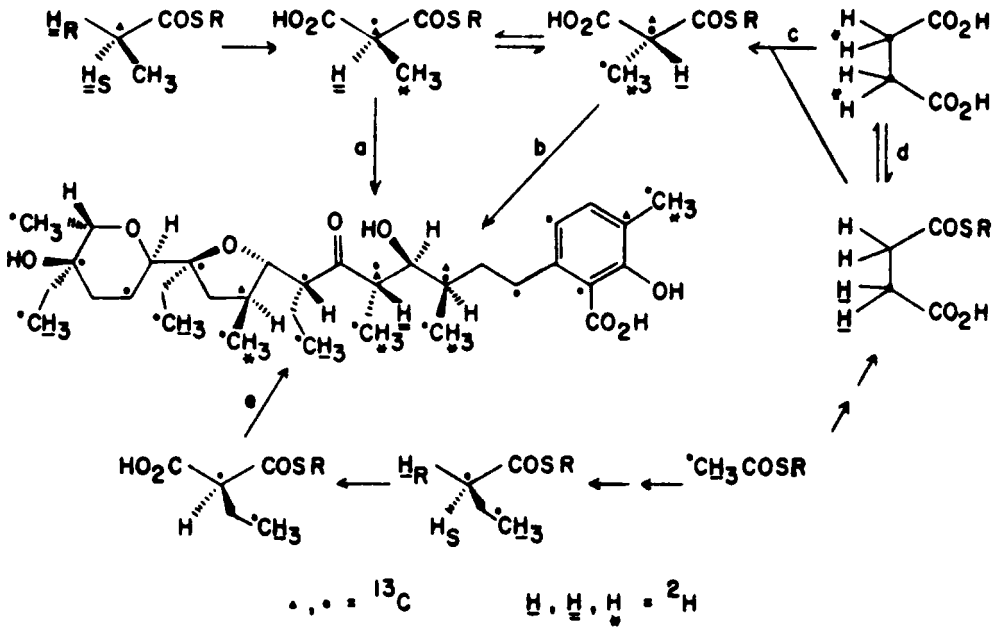
(39–41). In our case we wanted to learn if the incorporation of $CH_3CH_2{}^{13}C^{18}O_2H$ into **18** resulted in ^{18}O labeling at C–3 and C–15 but not at C–11, the result predicted by our hypothesis. We also wanted to learn if $CH_3{}^{13}C^2H_2CO_2H$ labeled C–12 and $CH_3CH_2{}^{13}C^2HCO_2H$ labeled C–14 with ^{13}C and ^2H, but C–4, C–10, C–16, C–18 and C–22 with only ^{13}C according to the respective precursors of the C_3 or C_4 subunits containing these carbons.

When the isotopically enriched samples of **18**, or simple chemical derivatives of it, obtained from the appropriate precursor feeding experiments were analyzed by ^{13}C nmr spectroscopy using two new spectral techniques based on α-isotope shifts of carbon resonances developed by our two research collaborators in Canada, J. C. Vederas and A. G. McInnes, we learned that our working hypothesis was partly invalid (42). ^{18}O label from the propionate was present at C–3, C–11 and C–15 of **18**; similarly, $CH_3CH_2CH_2{}^{13}C^{18}O_2H$ had labeled **18** at C–13 and C–1 (due to the $CH_3{}^{13}C^{18}O_2H$ produced from the butyrate by β-oxidation). The retention of ^{18}O relative to the ^{13}C reference label in **18** was $94 \pm 7\%$ and thus no significant amount of ^{18}O label had been lost by dehydration (C→D) or by any other mechanism during precursor incorporation. There also was significant retention of ^2H label at C–12 but not at C–10 of **18** from the exogenous propionate presumably due to its conversion to (*S*)-[2-^{2}H, 2-^{13}C] methylmalonate, but not at C–10 from the (*R*)-[2-^{2}H, 2-^{13}C, *methyl*-^{2}H] methylmalonate formed endogenously from the exogenous $^{13}C^2H_3CO_2H$ via succinate (43).

The above results strongly disfavor our initial idea that stereospecific dehydration-enone rehydration was the mechanism controlling the absolute stereochemistry at C–10/C–11 and C–14/C–15 during the biosynthesis of lasalocid A. It is unlikely that these events could have taken place without loss of most or all of the ^{18}O label introduced by the incorporation of propionate. Consequently, some other process such as apparently enantiodivergent reduction of C–11 and C–15 carbonyl containing intermediates must underlie the stereochemical control of lasalocid A biosynthesis at these sites. There are two reasonable ways such control could be achieved: by the operation of two oxidoreductase enzymes exerting enantioselective reduction of a carbonyl, or of one such enzyme operating on opposite faces of the carbonyl as a function of substrate conformation on the enzyme's surface. A distinction between these two hypotheses must await the results of further work.

The complex labeling pattern of lasalocid A resulting from the incorporation of $^{13}C^2H_3CO_2H$ and $CH_3{}^{13}C^2H_2CO_2H$ is difficult to interpret securely. The pictorial rationalization shown below thus should be regarded as tentative until more data are available, even though it is based on well-precedented biochemistry. We believe that the retention of ^2H label at C–12 of **18** from incorporation of the propionate supports entry of the latter into **18** via path a. In all other systems studied so far, propionate is carboxylated directly to (*S*)-methylmalonyl CoA, and in our case, this would result in the *S* configuration at C–12 of **18** if bond formation between C–12 and C–13 occurs with inversion of configuration at C–2 of the enzyme-bound (*S*)-[2-^{2}H, 2-^{13}C] methylmalonate (the intermediate condensation product, $RCO^{13}C^2H(CH_3)CO\text{-}SEnz$, has the *R* configuration, but C–12 must be designated *S* upon reduction at C–11 by the Cahn-Ingold-Prelog notation). The absence of ^2H but presence of ^{13}C at C–10 from the propionate implies that (*R*)-methylmalonate serves as the immediate precursor of this position in **18** (path b), because ^2H would be lost from C–2 of the (*S*)-[2-^{2}H, 2-^{13}C] methylmalonate on epimerization to its *R* enantiomer. We expected supporting evidence for this conclusion from the feeding experiments with $^{13}C^2H_3CO_2H$ since it can give (*R*)-[2-^{2}H, 2-^{13}C, *methyl*-^{2}H] methylmalonyl CoA via succinate and the operation of methylmalonyl CoA mutase (path c). However, the incorporation of ^2H at C–10 via the acetate was too low to measure accurately by the spectroscopic techniques. A similar reason may explain why we did not observe ^2H at C–14 of **18** from in-

corporation of the acetate via butyrate (path e). There is another explanation of this result, namely loss of ^{2}H label from the butyrate during its α-carboxylation if $^{13}C^{2}H_3CO_2H$ gives (2*R*)-]2,4-^{2}H, 2,4-^{13}C] butyrate in vivo as we suggest in the diagram. We conclude (with reservation until more work is done) that the configuration of the methylmalonate intermediates determines the absolute configuration of C–10, C–12 and C–14 of **18** by enantiospecific substrate utilization and stereospecific carbon-carbon bond formation processes during its biosynthesis. It is reasonable to speculate that these two processes also govern the absolute stereochemistry at the structurally analogous sites in all other macrolide and polyether antibiotics, such as erythromycin A (44) and monensin (45), during their biosynthesis. We await the clear experimental validation of this hypothesis with enthusiasm.



ACKNOWLEDGMENTS

I am indebted to my graduate students and co-workers whose names are given in the literature citations for their enthusiasm and experimental contributions to the studies described here. The National Institutes of Health provided partial financial support for this research (GM 25799) for which we are appreciative. We also enjoyed the continued enthusiasm of our external research collaborators, D. E. Cane, A. G. McInnes and J. C. Vederas, whose help with the spectroscopic analyses made much of this work possible.

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