chemical shifts were in agreement with those predicted from the data for amino acids and peptides, and therefore apparently contained very little information of conformational origin. However, monitoring the response of the ^{13}C chemical shifts to titration of the α-amino group of cystine provided evidence for a conformational transition at the isoleucyl residue due to alteration of the disulfide dihedral angle.[40c]

The NT_1 values of Figure 7 provide a dynamical map of almost every carbon in the molecule. The near-equality of those of the backbone α carbons in the cyclic portion demonstrate that no rapid torsional oscillations occur; the rapid increase in those of the α carbons of the terminal tripeptide with increasing distance from the point of attachment to the ring are indicative of rapid segmental motion. These data eliminate any model for the overall conformation which has the terminal glycinamide residue bound rigidly to a residue in the cyclic portion, in which case the segmental motion would be severely restricted. The NT_1 value of the prolyl β carbon is twice as large as that of either the α or δ carbons due to rapid internal motion in the prolyl ring. The mobilities of the methyl groups of isoleucine and valine depend upon the particular environment of each group. Finally, some evidence for rotation of the aromatic moiety of tyrosine about either of the aryl-C-β or the C-α-C-β bonds is found in the larger NT_1 values for the aromatic carbons relative to that of C-α.

Similar conclusions have been drawn for lysine-vasopressin[40e] and for oxytocin in $(Me)_2SO$.[40b] More recently a detailed analysis of the corresponding data for angiotensin II has been made in terms of models with isotropic or anisotropic overall motion of the oligopeptide with and without internal segmental motion.[42a] In this case conclusive evidence for rapid internal motion of the aromatic ring of phenylalanine about the aryl-C-β bond was obtained. By use of a 68-MHz spectrometer, a similar dynamical analysis for the decapeptide hormone luteinizing-hormone-releasing hormone has been completed.[44]

Conclusion

There is little doubt that ^{13}C NMR provides an invaluable source of conformational information for large molecules of biological interest. Its principal advantages are the wide range of chemical shifts and the possibility for proton decoupling. Spin–lattice relaxation times provide an extremely powerful monitor of molecular dynamics, but are at present limited in the detail they can provide due to lack of rigorous but convenient theoretical models. After a quiet period, considerable contemporary interest in the more theoretical aspects of the problem is evident,[42a,45] and we have every reason to believe that the immediate future will be very exciting.

We are deeply grateful to our colleagues who participated in the experiments, theoretical interpretation, and discussions leading to this Account. Without their advice and criticism this multidisciplinary study would not have been possible.

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Use of Carbon-13 Magnetic Resonance Spectroscopy for Biosynthetic Investigations†

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NMR spectroscopy offers many advantages when applied to biosynthetic problems. It is the only technique which can separately detect the presence of isotopes of all biosynthetically useful elements (^1H, ^2H, ^3H, ^{13}C, ^{15}N, ^{17}O, etc.), and the only nondestructive method capable of directly determining the locations and concentrations of isotopic labels in a metabolite. Spin–spin coupling effects can be used to provide *direct* evidence for the incorporation of intact biogenetic units, and for biosynthetic processes involving bond formation and cleavage, whereas such information can only be deduced indirectly by other methods.

The first applications[1,2] of NMR to biosynthetic problems in the late sixties traced the fate of ^{13}C-enriched precursors by increases in the intensi-

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ties of ^{13}C–H satellites in the ^{1}H NMR spectrum. This method was seriously limited by the inability to provide information for carbons not directly bonded to hydrogen, and was soon supplanted[3,4] by continuous wave (CW) ^{13}C NMR spectroscopy. This permitted every enriched site to be identified directly from differences in the intensities of corresponding resonances in the ^{13}C NMR spectra of naturally occurring and labeled metabolite. However, the major impetus was provided by the increase in sensitivity resulting from the development of pulse Fourier transform (PFT) ^{13}C NMR. This powerful technique has been instrumental in resolving many previously intractable biosynthetic problems, and will enable new areas of biogenetic interest to be explored.

As several excellent reviews[5–10] cover developments up to the middle of 1974, this Account is confined to a few examples which indicate the diversity of biogenetic information attainable by the ^{13}C method and illustrate significant new trends which have added another dimension to biosynthetic methodology.

^{13}C Methodology

Labeling Techniques. In work with radiotracers, the efficacy of precursors is measured by either isotopic incorporation or isotopic dilution, but only the latter is important when stable isotopes are used. Procedures for labeling with radioisotopes and stable isotopes are similar, but differ in that larger amounts of a ^{13}C-enriched precursor must be administered. This is necessary because lower isotopic dilutions are essential to offset the lower sensitivity of the ^{13}C method, coupled with the fact that enrichments are measured against a 1.1% ^{13}C natural abundance background.

Change in metabolic pool size resulting from the use of higher precursor concentrations introduces the risk of metabolic distortion and is frequently alluded to in listing the disadvantages of the ^{13}C method, although it has seldom been demonstrated. Changes in the labeling of showdomycin[11] due to precursor pressure is one of the few examples reported, and was readily explained by a knowledge of intermediary metabolism.

The amount of ^{13}C-labeled product required depends on the degree of enrichment. As enrichment improves, a lower yield can be tolerated, and vice versa, but usually about 50 μmol of metabolite will suffice. Addition of unlabeled carrier, which is a useful aid in isolating radioactive products, is obviously undesirable with ^{13}C labeling.

^{13}C NMR

Books and reviews on the principles and applications of ^{13}C NMR are available,[12] so comments here are limited to those aspects which are important from a biosynthetic viewpoint.

Sensitivity. Although ^{13}C, like ^{1}H, has spin ½, its low natural abundance and small magnetogyric ratio result in ^{13}C signals which are about 6000 times weaker than those of ^{1}H. This sensitivity problem has been partially circumvented by signal averaging, PFT spectroscopy[13] (broad-band excitation and detection), and ^{1}H noise (wide-band) decoupling (pnd). Thus PFT ^{13}C spectra can be obtained in about one-hundredth the time required for equivalent CW (narrow-band excitation and detection) spectra, and pnd ensures that all ^{13}C resonances are observed as singlets (Figure 1A).

Although the ^{13}CH satellite method has been largely superseded, it can sometimes provide useful supplementary enrichment information. Also, PFT ^{1}H NMR, in contrast to PFT ^{13}C NMR, offers the possibility of obtaining biosynthetic information on microgram quantities of material.

Assignments. The quality of biosynthetic information depends entirely on the unequivocal assignment of ^{13}C resonances, and ideally these should be made independent of biochemical assumptions. ^{13}C enrichment may simplify assignments: chemical shifts and coupling patterns (e.g., in off-resonance decoupled and high-resolution spectra) may be more easily identified following enrichment, and ambiguities due to partial overlap of signals can often be resolved. Selective biosynthetic enrichment has been used to assign resonances in the complex spectrum of gramicidin,[14] and chemical introduction of ^{13}C labels was used[15] to distinguish the resonances of the four meso carbons in protoporphyrin IX. Biosynthetic labeling which produces ^{13}C–^{13}C spin–spin coupling is a particularly valuable assignment aid (vide infra). Incorporation of labels, chemically or biochemically, which will induce isotope chemical shifts[16] (e.g., ^{2}H) or changes in spin–spin coupling (e.g., ^{15}N, ^{17}O) can also be useful.

Measurement of Isotopic Enrichments. The procedure whereby enrichments are obtained from differences between signal intensities in the pnd ^{13}C spectra of enriched and unenriched metabolite (Figure 1A,B) raises obvious difficulties when errors in measurement are of the same order as the differences. Part of the problem arises because intensities of ^{13}C resonances for different carbons vary considerably due to differences in spin–lattice relaxation times and nuclear Overhauser enhancements (NOE), but is compounded by instrumental fluctuations which cause uncontrollable intensity variations in successive PFT ^{13}C spectra recorded under the same

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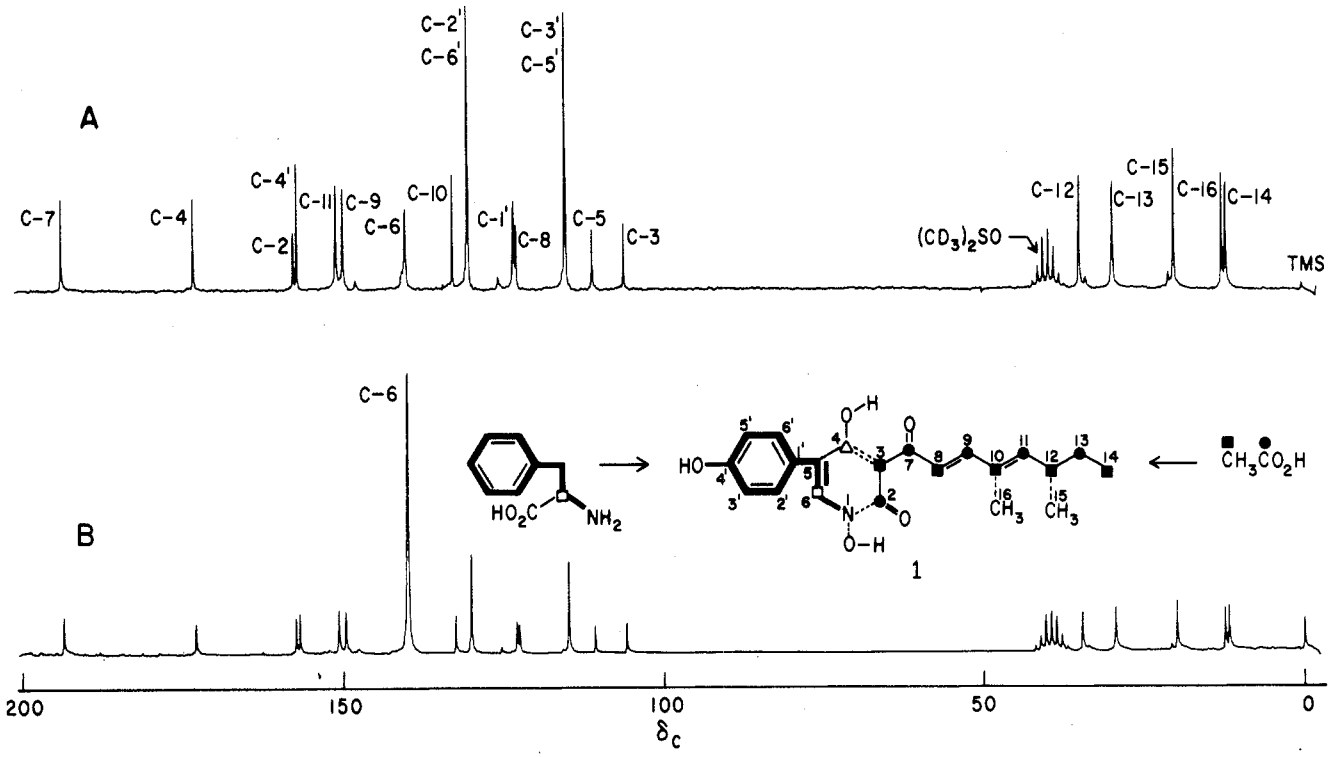


Figure 1. The proton noise decoupled pulse Fourier transform 25.2-MHz ^{13}C spectra of tenellin (1): (A) natural abundance ^{13}C; (B) ^{13}C-enriched with [2-^{13}C]phenylalanine (90% ^{13}C). Positions labeled by [1-^{13}C]- and [2-^{13}C]acetates, as well as [1-^{13}C]phenylalanine (△), are also shown in 1.

operating conditions. Thus enrichments must usually be about 0.5–1.0% ^{13}C above natural abundance to be detectable by the difference method; i.e., the permissible isotopic dilution is limited to ~1:100 when the precursor is 90% ^{13}C enriched. Gated decoupling[17] and relaxation reagents,[18] which promote more uniform intensities, may improve enrichment data for poorly relaxed carbons, but are limited in their applicability, and do not overcome the inherent disadvantages of the difference method.

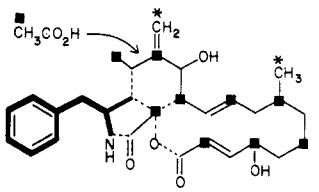
The low probability (10^{-4}) of two adjacent carbons being ^{13}C isotopes usually precludes the observation of ^{13}C–^{13}C satellites due to spin–spin coupling in the pnd ^{13}C spectrum of a compound at natural abundance. The introduction of precursors containing ^{13}C–^{13}C units will increase satellite intensities, and this can be effectively used to measure enrichments.[19] The ^{13}C–^{13}C satellite method, used in a study of bikaverin[20] (vide infra) to obtain reliable quantitative enrichment at the 0.4% level, probably extends the permissible range of isotopic dilution to ~1:500.

Single ^{13}C-Labeling

The use of the less sensitive difference method to measure enrichments and the inability to directly establish when intact biogenetic units have been incorporated or bonds have been cleaved are inherent disadvantages of singly labeled precursors. Nevertheless, the following examples show that such precursors can provide valuable biosynthetic information.

Labeling Patterns. The simplicity and elegance of the ^{13}C method are illustrated by a recent study on tenellin (**1**; Figure 1), a new metabolite isolated from the fungus *Beauveria bassiana*.[19,21] Incorporation of singly labeled precursors showed that the entire skeleton [except for two methyl groups (C-15, C-16) which originated from methionine] was formed by condensation of a ten-carbon polyketide chain with phenylalanine. As [2-^{13}C]phenylalanine labeled C-6 (Figure 1B) and [1-^{13}C]phenylalanine labeled C-4, it follows that the carboxyl carbon is transferred from C-2 to C-3 of the amino acid during biosynthesis. Precedents for this type of rearrangement are known.[22]

Cytochalasin B (**2**), a member of a group of compounds displaying cytostatic activity, is also partially derived from phenylalanine, though in this case ^{14}C studies showed that the amino acid is incorporated without rearrangement.[23] [*Me*-^{14}C]Methionine (*) and [^{14}C]acetate are also incorporated, but the distri-



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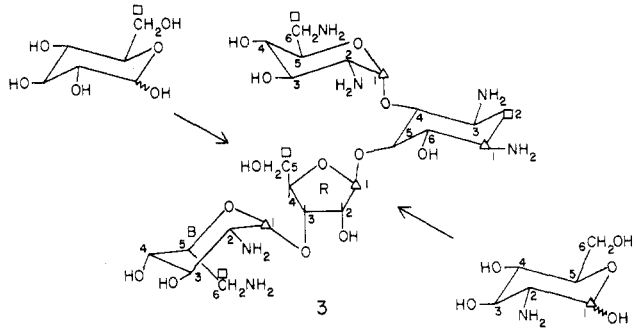
bution of label from acetate was only partially revealed by degradation steps. In contrast, all sites enriched by [2-^{13}C]acetate were readily detected by ^{13}C NMR[24] and confirmed the hypothesis that **2** is formed by condensation of phenylalanine with a nonaketide followed by oxygen insertion at some undetermined stage in the biosynthesis.

Radiotracer studies on the commercially important antibiotic neomycin B (**3**) were curtailed by a lack of

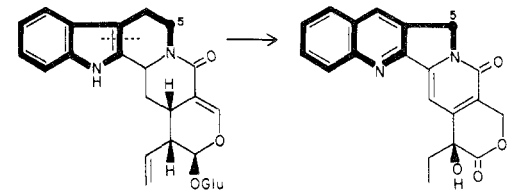


3

crystalline derivatives and a suitable degradation scheme. In a subsequent ^{13}C NMR study[25] it was found that [1-^{13}C]glucosamine and [6-^{13}C]glucose enriched corresponding positions in the two neosamine residues; this indicated incorporation via a common intermediate possessing their intact carbon skeleton. The labeling pattern in the ribose moiety was also readily rationalized by the well-known hexose monophosphate (C-6 glucose → C-5 ribose) and glucuronate (C-1 glucosamine → C-1 glucose → C-1 glucuronic acid → C-1 ribose) pathways. However, labeling of adjacent sites in the deoxystreptamine portion by the two precursors was both unexpected and inexplicable by known biosynthetic mechanisms. Cyclization of glucose to form a deoxyinosose which would yield deoxystreptamine upon amination was suggested as a possible explanation, and this hypothesis is presently under investigation.

Because of good precursor incorporation and high yield of product, most applications of ^{13}C NMR have been confined to metabolites produced by microorganisms. In fact, any system which produces a sufficient yield of reasonably enriched material can be used. Improvements in instrumentation and a wider acceptance of the method have enabled ^{13}C labeling studies to be conducted with animals[16] and higher plants.[26,27]

The incorporation of various radioactively labeled species of **4** into the plant alkaloid camptothecin (**5**) supported the premise[26] that the lactam **4** was a biosynthetic intermediate. Confirmation of isotope incorporation without randomization was not forthcoming due to a lack of suitable degradation schemes, and ^{13}C NMR was used to obtain the necessary proof. [5-^{13}C]-**4**, prepared from [1-^{13}C]tryptamine by established procedures, was wick-fed to *Camptotheca acu-*

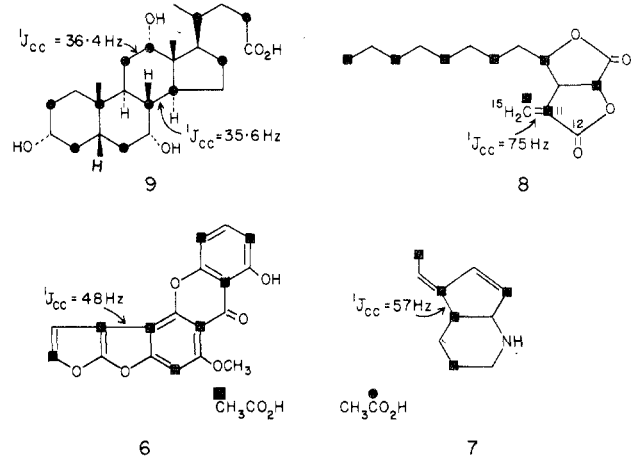


4 **5**

minata. ^{13}C NMR examination of labeled camptothecin (**5**) showed that only C-5 was enriched, confirming that **4** is an authentic intermediate in the biosynthetic pathway.

Condensations, Rearrangements, Composite Pathways, etc. In acetogenins adjacent carbons are occasionally derived from the same carbon of acetate, and when this occurs ^{13}C–^{13}C satellite resonances are produced due to spin–spin coupling ($^{1}J_{CC}$). The intensity of such satellites is proportional to the product of the ^{13}C concentrations at the two sites.

For example, labeling with [2-^{13}C]acetate and observation of one-bond ^{13}C–^{13}C coupling[5] indicated a rearrangement step during formation of sterigmatocystin (**6**) and cyclization of a polyketide chain to yield an odd-numbered carbocyclic ring during the biosynthesis of latumcidin [28] (isolated as the dihydro derivative **7**). In avenaciolide (**8**), coupling resulted from participation of acetate in the tricarboxylic acid cycle (C-15, C-11, and C-12 are derived from succinate or another intermediate in the cycle).[29] Condensation of two *trans*-farnesyl pyrophosphate molecules, derived from [1-^{13}C]acetate, yields labeled squalene which upon cyclization to form cholic acid (**9**) results in two pairs of adjacent labeled positions.[16]



9 **8**

6 **7**

Stereospecific Information. Most details of cephalosporin (**10**) and penicillin (**11**) biosynthesis are well understood,[30] but until recently it was not known whether the isopropyl residue of valine was introduced stereospecifically. It has now been established, by incorporation of the chirally labeled precursors (2*RS*,3*R*)-[4-^{13}C]- (**12**[31]) and (2*S*,3*S*)-[4-

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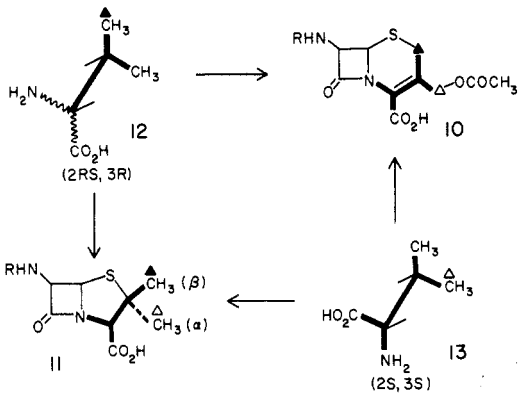
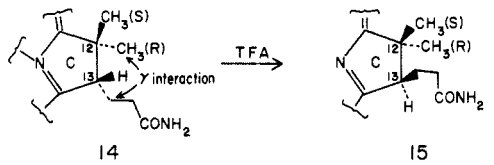


^{13}C]valine (**13**^{32}) into **10** and **11**, that the amino acid is introduced stereospecifically, and the isopropyl moieties are incorporated with retention of configuration. It would have been extremely difficult to accomplish this using radiotracers; in the case of the penicillins (**11**) it would require chemical differentiation of the two methyl groups.

One methyl group in ring C of vitamin B-12 (**14**) is derived from methionine, and the other by decarboxylation of an acetic acid side chain. Conversion of vitamin B-12 to neocobinamide with trifluoracetic acid changes **14** to **15**, thereby removing a γ interaction



which chemically shields the (*R*)-methyl carbon. The (*R*)-methyl carbon at C-12 of neocobinamide (**15**) should therefore resonate at lower field than the corresponding carbon in vitamin B-12 (**14**). When this procedure was applied to vitamin B-12 labeled with [*Me*-^{13}C]methionine,[7] one of the enriched methyl carbon resonances was so affected. Therefore the (*R*)-methyl group on ring C of vitamin B-12 originates from methionine, and is inserted from the α face of the corrin nucleus.

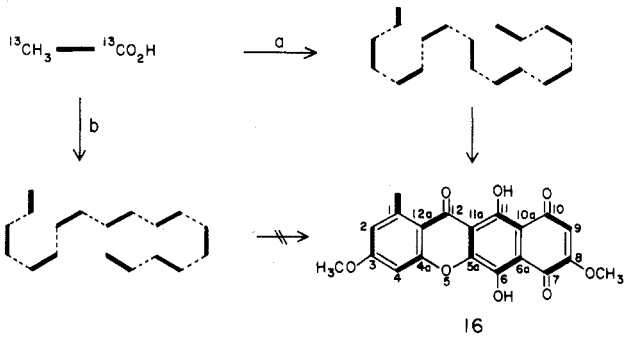
Labeling of prochiral centers in a precursor with ^{3}H (detection by scintillation counting) and ^{2}H (detection by mass spectrometry) has already proved to be a powerful technique for determining the stereochemical features of a biosynthetic process.[33] It is worth reiterating that such isotopes can be observed directly by NMR or indirectly via coupling effects or isotope chemical shifts in the ^{13}C spectrum. The first application of ^{3}H NMR to a biosynthetic problem was recently reported.[34]

Double ^{13}C Labeling

When a precursor is labeled with ^{13}C at two positions, either adjacent or nonadjacent, which are spin–spin coupled, its fate can be determined by the absence or presence of this coupling in the enriched metabolite. If coupling is preserved in the ^{13}C spectrum of the labeled product it is convincing evidence that part, at least, of the precursor is incorporated intact. Loss of $^{1}J_{CC}$ coupling accompanied by enrichment is proof of bond scission, and changes in the type of coupling (e.g., $^{1}J_{CC} \rightarrow {}^{3}J_{CC}$) may indicate a rearrangement. Where precursor units can occupy adjacent positions (e.g., acetogenins, porphyrins), incorporation levels are often controlled so that most metabolite molecules contain only one doubly labeled unit, thus eliminating spectral complications which could arise from coupling between units. As incorporation of each doubly labeled unit produces satellites, their intensities for a given enrichment are much higher than those arising from incorporation of singly labeled precursors. As we have already indicated, the creation of new signals for enriched sites enhances the sensitivity and accuracy of the ^{13}C method.

The following examples illustrate the scope of this powerful labeling procedure in deducing folding patterns of polyketides, rearrangements, distinguishing biogenetic alternatives, and as an aid in structural elucidation.

Polyketide Chain Folding. [1,2-^{13}C]Acetate was used in a very recent study[20] to determine the arrangement of acetate units in the assembly of bikaverin (**16**). The use of this precursor was mandatory



because small enrichments (<0.5%) from singly labeled acetates could not be unequivocally detected by the difference method, and ^{13}C–^{13}C couplings were essential aids to ^{13}C assignments. The enrichment measured from ^{13}C–^{13}C satellite intensities (Figure 2) for each position was 0.4 ± 0.07% and illustrates the ability of this procedure to provide good quantitative data at dilutions >1:200. The usual procedure of matching coupled pairs of carbons from ^{13}C–^{13}C satellite spacings was impracticable because spin–spin couplings had similar values, and several factors including a poor signal/noise ratio produced a lower data accuracy than that defined by the spectrometer operating parameters (±0.15 Hz). For the first time in a biosynthetic study the matching process was facilitated by homonuclear ^{13}C decoupling (inserts in Figure 2).

All resonances in the ^{13}C spectrum of labeled bikaverin (**16**) (except those for the methoxyl groups which were labeled by methionine) were accompanied by ^{13}C–^{13}C satellites, so the benzoxanthone ring system is assembled entirely from nine intact acetate units. Although the participation of an orsellinate starter or condensation of orsellinic acid with an unsymmetrical naphthoquinone intermediate is not excluded by these results, the arrangement of ^{13}C–^{13}C

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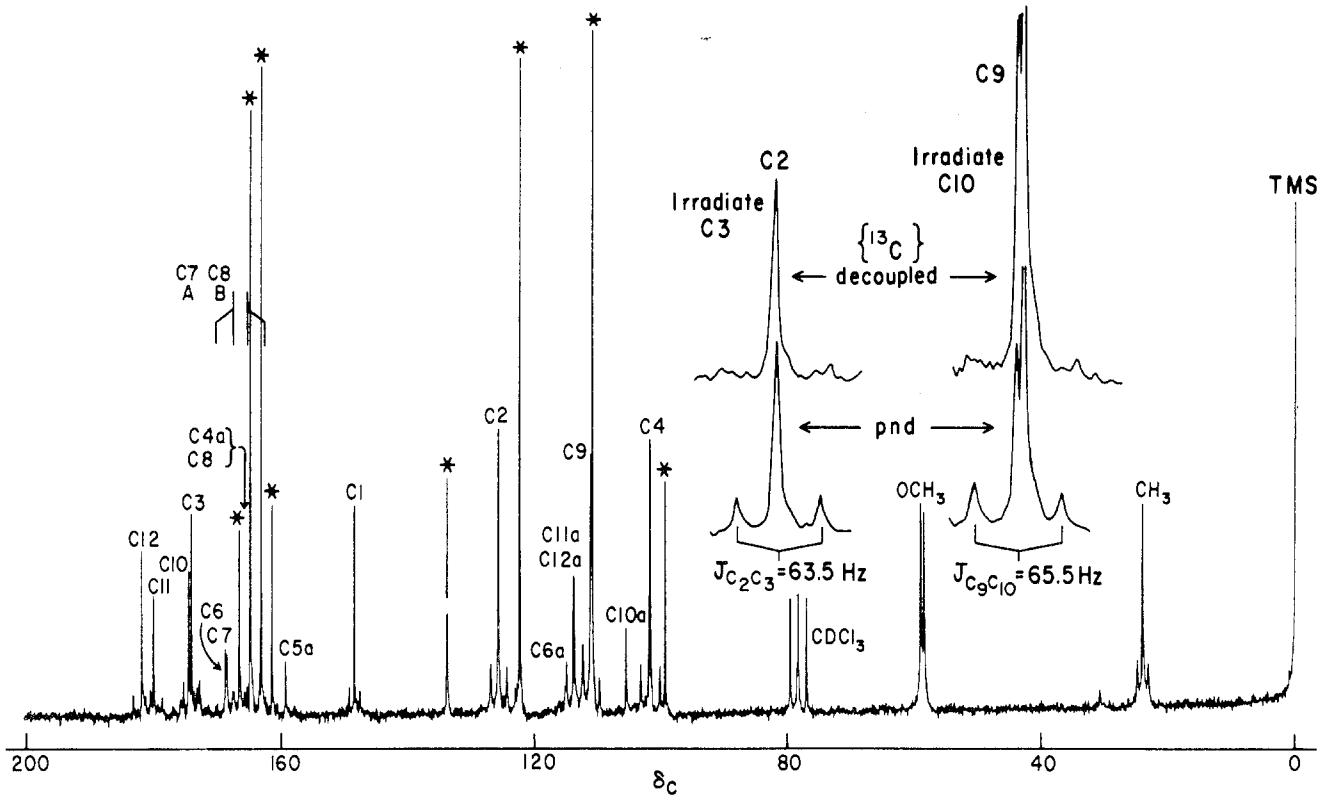
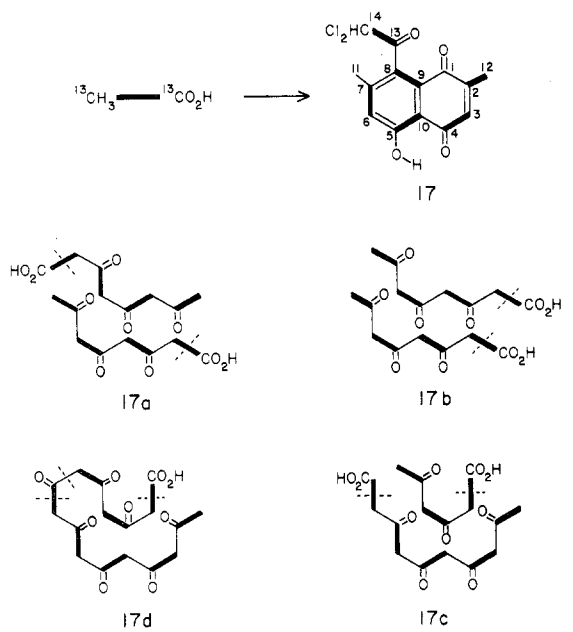


Figure 2. The pnd PFT ^{13}C spectrum of bikaverin (**16**), produced by *Fusarium oxysporum*, and enriched with [1,2-^{13}C]acetate (90% ^{13}C). Asterisks identify resonances arising from the solvent CF_3COOD. Inserts show two examples of ^{13}C–^{13}C homonuclear decoupling ($H_2/2\Pi$ = 70 Hz) with simultaneous ^{1}H decoupling.

units in **16** is consonant with participation of an 18-carbon polyketide chain folded as in pattern a. Pattern b, or a symmetrical naphthoquinone intermediate, is excluded. Thus it is now possible to deduce the directional mode of polyketide folding to generate a fused carbocyclic structure.

Biogenetic Alternatives. When [1,2-^{13}C]acetate was fed to cultures of *Mollisia caesia*, a poor yield and enrichment of mollisin (**17**) resulted in a low signal/noise ratio in the ^{13}C spectrum, so that only carbons present in ^{13}C–^{13}C units and bonded to hydrogen produced observable satellite resonances.[35] As all such carbons, except C-11, were accompanied by ^{13}C–^{13}C satellites, it was concluded that biosynthesis of **17** proceeded via polyketide **17c** rather than by **17a** or **17b**, which were previously suggested[36] but have the wrong disposition of ^{13}C–^{13}C units. However, as participation of two biogenetic units is only established by showing they have a different origin (e.g., differently enriched) or that there is more than one starter unit, the latest ^{13}C results do not necessarily require that **17** be assembled from two chains. A single polyketide such as **17d** which undergoes scission after chlorination at an activated site[37] is a plausible alternative.



Rearrangements. The double labeling technique was used to great advantage in a study[38] on ascochlorin (**18**). A biosynthetic proposal suggested formation of an orsellinic aldehyde portion (**19**) from acetate and a terpenoid fragment produced from mevalonate via *all-trans*-farnesyl pyrophosphate (**20**). [1,2-^{13}C]Acetate would be expected to introduce four ^{13}C–^{13}C units into **19** and six ^{13}C–^{13}C units into **20.** The remaining three positions in **20** would be singly labeled as a result of bond scission in mevalonic acid during generation of isopentenyl pyrophosphate. The ^{13}C spectrum of enriched **18**, produced by feeding double-labeled acetate to cultures of *Nectria coccinea*, showed nine of the expected ^{13}C–^{13}C units were present, and five other positions (C-1, C-4, C-8, C-12,

(35) H. Seto, L. W. Carey, and M. Tanabe, *J. Chem. Soc., Chem. Commun.*, 867 (1973).
(36) R. Bentley and S. Gatenbeck, *Biochemistry*, 4, 1150 (1965).
(37) D. R. Morris and L. P. Hager, *J. Biol. Chem.*, **241**, 1763 (1966); L. P. Hager, D. R. Morris, F. S. Brown, and H. Eberwein, *ibid.*, **241**, 1769 (1966).
(38) M. Tanabe and K. T. Suzuki, *J. Chem. Soc., Chem. Commun.*, 445 (1974).

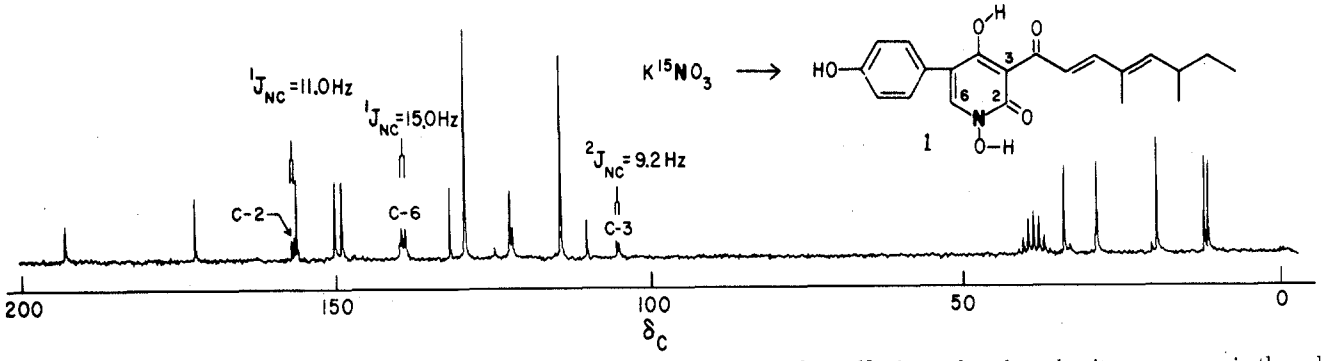
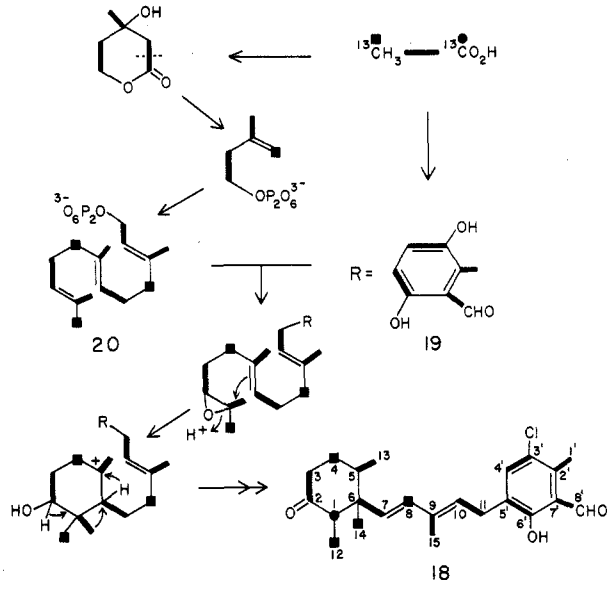


Figure 3. The pnd PFT 25.2-MHz ^{13}C spectrum of tenellin (1) 95% enriched with ^{15}N ($K^{15}NO_3$ used as the only nitrogen source in the culture medium).

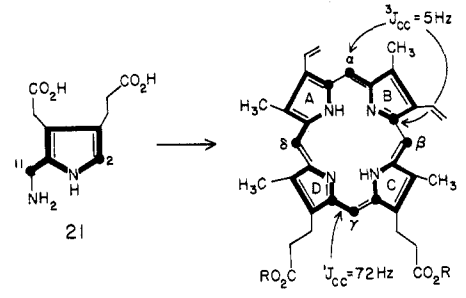


and C-14) were enriched but not accompanied by satellites. Three of these positions (C-4, C-8, C-12) were singly labeled as predicted from a knowledge of terpenoid biogenesis, but the remaining two (C-1, C-14) could only arise through bond scission accompanying methyl migration as shown.

Structure Elucidation. Additional labeling experiments combined with these already discussed provided important structural information on tenellin (1; Figure 1). The carbons bonded to hydrogen were readily assigned from chemical shift trends and the usual ^{13}CH decoupling procedures. Matching ^{13}C–^{13}C satellites in the spectrum of tenellin enriched with [1,2-^{13}C]acetate showed that five intact two-carbon units had been incorporated.[21] A carbonyl was directly bonded to a quaternary carbon in one unit and a second carbonyl was coupled to C-8 in another unit. This enabled the carbonyls at C-2 and C-7, as well as the quaternary carbons C-3 and C-5, to be distinguished. Three carbons (C-2, C-3, and C-6) were also coupled to nitrogen in the spectrum of tenellin enriched with ^{15}N (Figure 3), and the magnitudes of the J_{CN} couplings enabled their positions, relative to nitrogen, to be deduced;[21] geminal couplings would be expected to be small except through a carbonyl group. This information combined with that from the labeling experiments with phenylalanine (Figure 1) permits the main structural features of tenellin to be deduced.

Nonadjacent ^{13}C Labeling

In series III porphyrins the arrangement of substituents is out of sequence in ring D, and numerous mechanisms have been proposed to account for this inversion. In a reinvestigation of the problem[39] 5-amino-[5-^{13}C]levulinic acid was first converted enzymically to 90% ^{13}C-enriched [2,11-^{13}C]porphobilinogen (21; PBG). By means of a series of enzyme preparations, 21 was first converted to uroporphyrinogen III and then protoporphyrin IX (22). Labeled



PBG (21) was diluted with unlabeled material to ensure that molecules of 22 only contained one doubly labeled unit. The ^{13}C spectrum of enriched 22 showed the α-, β- and δ-meso carbons were 5-Hz doublets ($^3J_{CC}$; incorporation of an intact PBG unit) centered on singlets, whereas the doublet component for the γ carbon had a spacing of 72 Hz ($^1J_{CC}$; rearrangement). Consequently rings A, B, and C and their corresponding meso carbons each originate from an intact PBG unit. The contiguous location of labels in ring D can only be explained by an intramolecular rearrangement of a PBG unit during construction of the porphyrin ring system.

Mixtures of Singly Labeled Precursors

Incorporation of a mixture of singly labeled precursors may result in labeling of a metabolite such that spin–spin coupling occurs, and this information is useful in delineating condensation and cyclization processes. Components of such a mixture can be chemically dissimilar (e.g., $CH_3{}^{13}COOH$ and $NH_2{}^{13}CH_2COOH$) or isotopic species of the same molecule (e.g., $CH_3{}^{13}COOH$ and $^{13}CH_3COOH$). Although the former approach has not been exploited, it has obvious potential for compounds of a mixed bi-

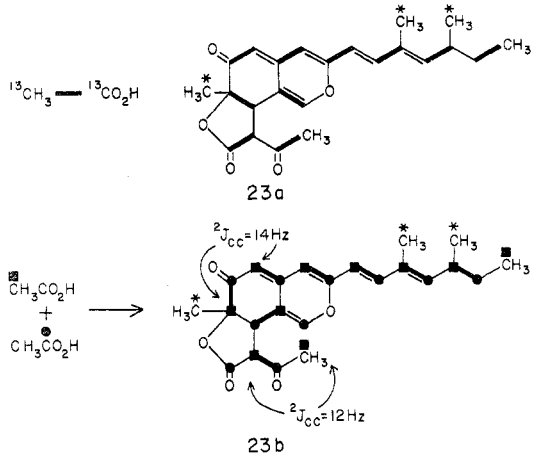
(39) A. R. Battersby, E. Hunt, and E. McDonald, *J. Chem. Soc., Chem. Commun.*, 442 (1973).

ogenesis. The latter technique has been employed in a few cases while studying polyketide biogenesis.

A limiting factor to this labeling approach is the need for low isotopic dilution (lower than for a doubly labeled precursor) in order that the ^{13}C–^{13}C satellites become visible. This is particularly true for a precursor mixture containing isotopic species of the same molecule since one precursor dilutes the other. With polyketides the problem is enhanced by the fact that only one condensation in four produces adjacent labels. A mixture of [1-^{13}C]- and [2-^{13}C]acetate will obviously introduce different pairs of coupled carbons into a polyketide compared with [1,2-^{13}C]acetate, provided the isotopic dilution of the latter is adjusted so that molecules of the metabolite only contain one ^{13}C–^{13}C unit.

When this is not done, the ^{13}C spectrum of a metabolite labeled with [1,2-^{13}C]acetate will contain all the spin–spin coupling originating from and between ^{13}C–^{13}C units. Furthermore, all satellites arising through coupling between ^{13}C–^{13}C units will be more intense since in this case every condensation between such units will produce adjacent ^{13}C labels. Coupled pairs of carbons in the metabolite will probably have to be matched by homonuclear ^{13}C decoupling because of the complexities introduced into the spectrum. Where decoupling facilities are not available, similar information can be provided by labeling first with [1,2-^{13}C]acetate (diluted with unenriched material) and then with the mixed acetate precursor.[40]

The mixed-precursor technique was elegantly used in a study of ochrephilone (**23**), a new pigment produced by *P. multicolor,* and is another example in which the structure and biosynthesis were established simultaneously.[41] ^{13}C–^{13}C satellites in the PFT ^{13}C NMR spectrum of **23** enriched from [1,2-^{13}C]acetate established ten acetate units had been incorporated (**23a**), while the spectrum of **23** enriched from a mixture of [1-^{13}C]- and [2-^{13}C]acetate contained eight ^{13}C–^{13}C units, arising through head-to-tail condensation of ten acetate units (**23b**). An additional experiment with [2-^{13}C]acetate enabled two geminal couplings through carbonyls ($^2J_{CC}$) to be observed, as well as identifying those sites derived from the methyl group of acetate. The *C*-methyl groups (denoted by asterisks) which were not labeled in these experiments probably arise from methionine by analogy with earlier radiotracer results on related compounds.[42]



Summary and Future

The recent availability of moderately priced (≈$60,000) PFT spectrometers overcomes one of the obstacles to more widespread use of the ^{13}C method. Sensitivity limitations, however, are more basic. Instrumental developments will undoubtedly improve this situation, but a further gain in sensitivity analogous to that obtained by the introduction of the FT technique is unlikely.

On the other hand, the range of isotopic dilution can probably be extended to ~1:1000 by growing an organism on a carbon source almost devoid of ^{13}C (e.g., 99.95% ^{12}C) and supplying a highly ^{13}C-enriched precursor during the growth cycle. Metabolites obtained by this ^{13}C-depletion method should, in principle, give ^{13}C spectra containing resonances for only the ^{13}C-labeled positions. The ability to observe only signals arising from selected sites in proteins, enzymes, or nucleic acids is particularly attractive, and could facilitate studies on enzyme–substrate or drug–receptor interactions by spin–lattice relaxation techniques. The procedure is being used in this laboratory to study biosynthetic processes in algae, which can assimilate $^{12}CO_2$ photosynthetically, and the same approach is being used elsewhere[7] for studies with higher plants. Also, an algal hydrolysate, prepared from ^{13}C-depleted algae, can serve as a carbon source for microorganisms, thus extending the range of biosynthetic problems which could be tackled by the ^{13}C-depletion method. It should be noted that the use of highly ^{12}C-enriched (>99.9%) precursors for biosynthetic studies is impracticable in spite of claims to the contrary.[7,43] Such precursors would require unrealistically low isotopic dilutions (a dilution of 1:2 would only halve the natural-abundance ^{13}C signal) in order to monitor incorporation.

NMR is superior to other methods for investigating biosynthetic problems, the only limitations being sample size and degree of enrichment.

(40) H. Seto, T. Sato, and H. Yonehara, *J. Am. Chem. Soc.*, **95**, 8461 (1973).
(41) H. Seto and M. Tanabe, *Tetrahedron Lett.*, 651 (1974).
(42) A. J. Birch, A. Cassera, P. Fitton, J. S. E. Holker, H. Smith, G. A. Thompson, and W. B. Whalley, *J. Chem. Soc.*, 3583 (1962).
(43) S. B. W. Roeder, *J. Magn. Resonance*, **12**, 343 (1973).