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BIOSYNTHETIC STUDIES WITH ^{13}C LABELED PRECURSORS

Urs Séquin[+] and A. Ian Scott*

[+]Institut für Organische Chemie, Universität of Basel, Switzerland

*Sterling Chemistry Laboratory, Yale University, New Haven, Conn. 06520

Carbon-13 double labeling experiments are now widely used in the investigation of biosynthesis.

Due to the ease with which carbon-13 nuclear magnetic resonance spectra can be obtained nowadays, the use of carbon-13 labeled precursors for the elucidation of biosynthetic pathways has become a well established procedure, as reflected by the increasing number of papers reporting applications of this technique. Since our last report (1), which covered about 60 papers published between 1966 and 1974, some 60 new articles dealing with this topic have appeared, among them several reviews. Two detailed reviews by Tanabe (who was the first to enter the field with his study on griseofulvin (2)) cover developments up to 1973 (3). In an excellent article McInnes and Wright discuss the different techniques commonly used, giving examples from their

own laboratory as well as from the literature (4), while Neuss has focussed attention on the biosynthesis of antibiotics (5).

This report is intended to update our previous review (1), to highlight the trends in this rapidly expanding field, and to provide chemists interested in this topic with a comprehensive and up-to-date reference list. The basic principles of feeding carbon-13 labeled precursors and of the detection of the labeled sites in the metabolite by ^{13}C NMR spectroscopy have been discussed elsewhere (1, 4 and refs. therein).

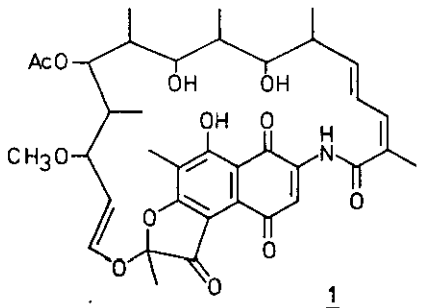
Optimization of Feeding Conditions

When the intermediacy of a putative and complex precursor in the biosynthesis of a metabolite is in doubt, it is advisable to do some preliminary experiments with ^{14}C as a label. Incorporation of ^{14}C can be detected at a much lower level than is possible with ^{13}C due to the almost nonexisting background of natural abundance (only 183.6 dpm/mol carbon for ^{14}C vs. 1.1% for ^{13}C). Thus it can be seen whether any incorporation takes place and feeding/fermentation conditions can be optimized.

It must be emphasized, however, that feeding conditions which give optimum incorporation with ^{14}C cannot always be used for ^{13}C experiments. It is obvious, that if exactly the same experimental conditions are used in both experiments, similar incorporation should result. But, as ^{14}C is more easily detected, precursors labeled with this isotope are usually fed in small amounts, but

with high specific activity. The difficulty in detecting incorporation of ^{13}C makes it necessary that large amounts of precursors (cf. 6) be fed despite the fact that most ^{13}C labeled substrates available today are ≥ 90% enriched in specific sites. As a consequence, if feeding/fermentation conditions are to be optimized for a ^{13}C experiment, in the preliminary ^{14}C experiments the same relatively large amounts of precursors should be added as will be used in the later assay. The difficulty which may arise is that large amounts of precursor may be toxic for the organism under investigation. One possible solution is to add the labeled substrate sequentially ("Pulse Feeding") rather than in one large batch.

This pulse feeding technique has been successfully used in a study of rifamycin S (1) biosynthesis (7), where acetate or propionate levels of more than 200 µg/ml proved to be toxic to Nocardia mediterranei. By feeding the microorganism twice a day over 96 hours, adequate incorporation of the precursors could be obtained. A second example is islandicin (2), where cultures of Penicillium islandicum were pulsed daily with 8 mg/ml [1,2-$^{13}C_2$] sodium acetate from day 7 through day 16 (8).



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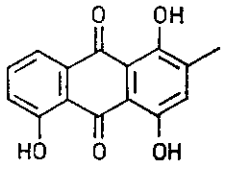

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This technique is thus of special importance in ^{13}C experiments as it might enable one to get the precursor concentration below the toxic level.

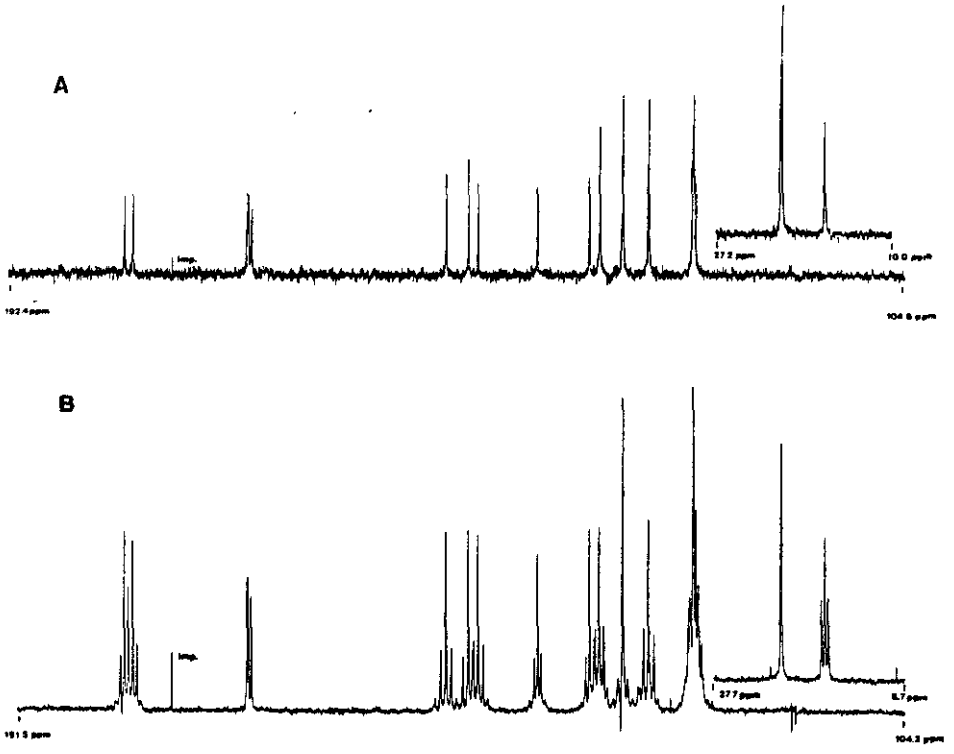


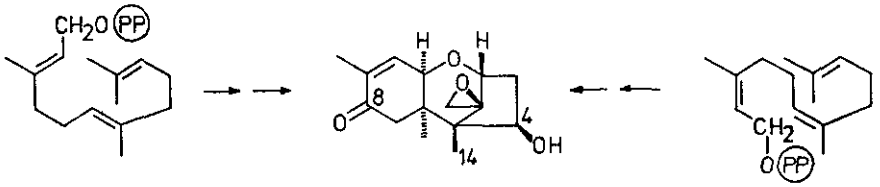
Figure: Proton noise-decoupled FT ^{13}C NMR spectrum of islandicin triacetate: (A) from ^{13}C natural abundance, (B) from $^{13}CH_3{}^{13}CO_2Na$ enrichment [from (8), courtesy of the American Chemical Society].

For the ^{13}C labelling experiments feeding and fermentation conditions should be established in such a way that the NMR signals corresponding to labeled sites in the metabolite isolated show a distinct enhancement over those at natural abundance. With today's equipment if spectra are measured under rigorously identical conditions for labeled and unlabeled metabolites, if care is taken to exclude any errors in peak intensities which might arise from nuclear Overhauser enhancement (NOE), spin-lattice relaxation times (T_1) or digitization of data (cf. (1)) and if the enrichment at each site is calculated from normalized peak intensities (vide infra), labeled centers can be detected unambiguously when their signal intensity in the spectrum of the biosynthetically enriched metabolite is ≥ ca. 1.5 times the intensity in the natural abundance spectrum (corresponding to a dilution value of ca. 160, assuming 90% enriched precursors).

^{14}C Spiking Technique

Certain authors prefer to add to the ^{13}C labeled precursors small amounts of material labeled with ^{14}C at the same sites. This provides them with an easy means of monitoring the incorporation and getting quantitative values even at low incorporation rates. This spiking technique (9) was first used by Hanson et al. in a study on trichothecolone (3) (10). To cultures of Trichothecium roseum was added [2-^{13}C, 2-^{14}C] mevalonic acid (90% ^{13}C,

25 μCi ^{14}C, corresp. 12.33 μCi/mmol). Five days later trichothecin -



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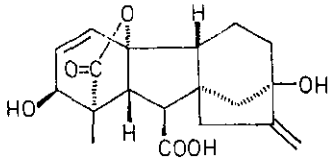
the crotonate of trichothecolone - was isolated and hydrolyzed to afford trichothecolone (3). From the ^{14}C activity of the obtained metabolite, a *incorporation of* 0.3% *per labeled site* was determined. The ^{13}C spectrum of the enriched trichothecolone was in good agreement, showing three signals to be enriched 0.35% and corresponding to carbons 4, 14 and 8. The fact that C(8) was labeled suggests that the farnesyl pyrophosphate is folded as shown in 4 rather than as in 5, as was assumed before.

Two other recent investigations on gibberellic acid (6) (11) and islandicin (2) (8) respectively also used ^{14}C spiking.



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Reporting the Extent of Labeling

Much confusion can arise when reporting the extent of labeling of a metabolite as no common terminology has yet been adopted in connection with ^{13}C experiments. For the same reason incorporation data from ^{14}C studies are difficult to compare with data from ^{13}C studies (12). In ^{14}C work the following terms are encountered [cf. also (13) and (14)]:

The absolute incorporation, per cent incorporation or simply incorporation (I_{abs}) is the ratio of the total activity (in Ci or dpm) of the metabolite isolated (A_{metab}) and the total activity of the precursor fed (A_{prec}) expressed in per cent:

$$I_{abs} = \frac{A_{metab}}{A_{prec}} 100\%$$

The specific incorporation (I_{spec}) is the ratio of the specific activities of metabolite and precursor (e.g. in dpm/mmol) expressed in %. The specific activity (a) is obtained by dividing the absolute activity by the molar quantity (M):

$$I_{spec} = \frac{a_{metab}}{a_{prec}} 100\% = \frac{A_{metab}/M_{metab}}{A_{prec}/M_{prec}} 100\% = I_{abs} \frac{M_{prec}}{M_{metab}}$$

The dilution value (D), finally, is given by:

$$D = \frac{a_{prec}}{a_{metab}} = \frac{100\%}{I_{spec}} = \frac{100\%}{I_{abs}} \times \frac{M_{metab}}{M_{prec}}$$

Note that when radiocarbon is used as the tracer, the specific activities are usually thought of as molecular, not atomic parameters (12).

In ^{13}C experiments icorporation data can be obtained from mass spectral analysis [cf. (12) and e.g. (15)] but is usually taken from the enhancement of ^{13}C NMR signals corresponding to labeled sites. In cases where this enhancement is clearly evident (i.e. when peaks in the spectrum of the labeled metabolite attain ca. threefold or greater intensity of the corresponding signals in the natural abundance spectrum) usually no quantitative measurements are made. Sometimes an enhancement factor is given, which can be symbolized by P and defined as:

$$P = \frac{i_{lab}}{i_{nat\ abund}} \qquad \text{or also} \quad P = \frac{i_{lab}}{i_{unlab}}$$

The i's denote the peak intensities (it usually makes little difference whether peak heights or peak integrals are used), with i_{lab} standing for the intensity of the signal corresponding to a labeled center in the spectrum of the enriched metabolite,

$i_{nat\ abund}$ for the intensity of the same signal in a natural abundance spectrum and i_{unlab} for the peak intensity of an unlabeled center in the spectrum of the enriched metabolite. When P has to be calculated from the spectrum of the enriched metabolite alone (using i_{lab} and i_{unlab}) care must be taken to avoid errors from differencies in nuclear Overhauser enhancement and spin-lattice relaxation times [cf. (16)].

When incorporation is low, a special effort is necessary to assign the labeled sites unambiguously and calculate incorporation rates. In these cases it is advisable to normalize the peak intensities in some way. Hanson and coworkers have done this (10, 11) by dividing all the peak intensities in each, the natural abundance spectrum and the spectrum of the enriched metabolite, by the respective intensity of a reference line, belonging to a carbon atom which with high certainty has not been labeled or affected by scrambling of the tracer:

$$\text{normalized intensity} = \frac{\text{observed intensity}}{\text{observed intensity of reference line}}$$

A more complicated procedure of normalization was recently proposed by Holker et al., in which all the positions expected to remain unlabeled were together taken as reference (6).

The peak intensities, whether the observed values or the normalized ones, can serve for the calculation of further incorporation data. The total ^{13}C content, X, at a specific site [Campbells uncorrected enrichment factor (12)] can be obtained (16), remembering that X at a center with ^{13}C only at natural abundance is 1.1%:

$$X = \frac{i_{lab}}{i_{nat\ abund}} \quad 1.1\% = P \times 1.1\%$$

The enrichment factor, EF, for a specific site, i.e. the excess label above natural abundance can be calculated with the following approximation:

$$EF = X - 1.1\% = \frac{i_{lab} \times 1.1\%}{i_{nat\ abund}} - 1.1\% = \frac{i_{lab} - i_{nat\ abund}}{i_{nat\ abund}} 1.1\%$$

$$= (P \times 1.1\%) - 1.1\% = (P - 1) \times 1.1\%$$

Again, one can substitue in these equations $i_{nat\ abund}$ by i_{unlab}, bearing in mind the potential errors mentioned above. A more accurate way to calculate enrichment factors corrected for natural abundance was recently given by Campbell (12). The atomic specific incorporation, I^*_{spec}, and dilution value, D*, can now be obtained:

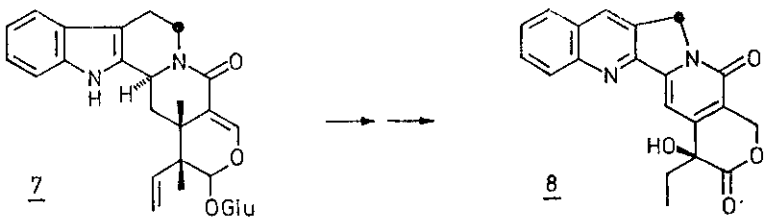
$$I^*_{spec} = \frac{EF_{metab}}{EF_{prec}} 100\% \qquad D^* = \frac{EF_{prec}}{EF_{metab}}$$

If the molecular parameters are to be calculated - for comparison with ^{14}C work - the numbers of labeled sites in the precursor and metabolite, respectively, (denoted in the formula with 1's) have to be taken in account (17):

$$I_{spec} = \frac{EF_{metab} \times l_{metab}}{EF_{prec} \times l_{prec}} 100\%$$

Studies Involving Higher Plants

Due to the relatively high level of incorporation which has to be attained for a ^{13}C NMR study (dilution should not be more than ca. 100, with an upper limit of 250, as stated elsewhere in this article), this experimental technique has so far largely been confined to biosynthetic investigations with microorganisms or partially purified enzyme systems (18, 19). Studies involving higher plants or animals have only very recently been reported. Early in 1974 a paper was published on the *in vivo* incorporation of ^{2}H-^{13}C-labeled ethanol into bile acids in the rat (20) and a few months later Hutchinson *et al.* reported the successful incorporation of a ^{13}C labeled indole alkaloid precursor (7) into camptothecin (8), an anti-tumor alkaloid produced by *Camptotheca acuminata* (21). At the same time Battersby and coworkers fed [1-^{13}C]autumnaline (9) to *Colchicum autumnale* (22) and proved that the label was incorporated into position 7 of



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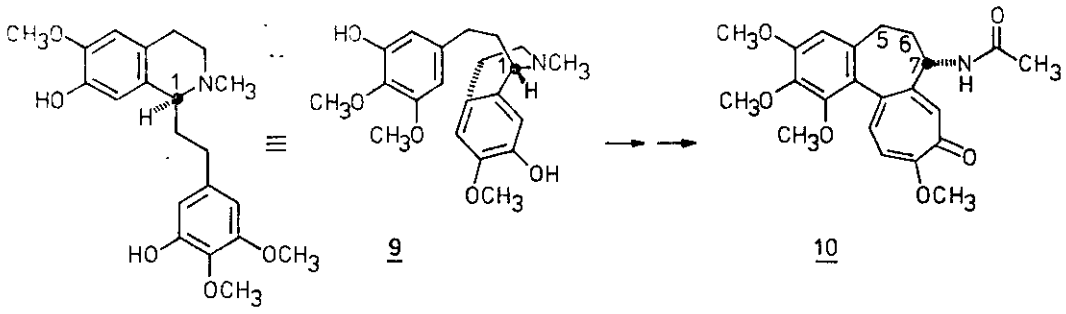
colchicine (10) since the ^{13}C NMR spectrum of the enriched metabolite showed a 2.5 fold enhanced signal for C(7). Although autumnaline (9) has been labeled in many different positions, this is the first time that a 1-labeled specimen was administered.



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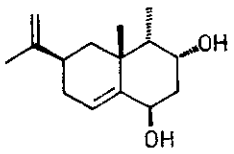
The problem of getting a high enough incorporation of the precursor was solved in a most spectacular way in a study of the biogenesis of capsidiol (11), a phytoalexin produced by

sweet peppers (Capsicum frutescens) (23). Use was made of an



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earlier observation (24) that the production of capsidiol in sweet peppers was greatly enhanced when the fruits were infected with the fungus Monilinia fruticola. Thus peppers were incubated with the fungal spores in the presence of [1,2-$^{13}C_2$]acetate and the capsidiol isolated was highly enough enriched to permit analysis of the labeling pattern by NMR. It is evident, however, that the infection of a higher plant with a microorganism is not a generally applicable method to get better incorporation.

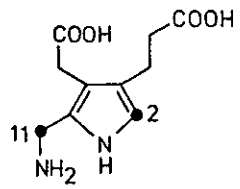
In this connection it should be mentioned that incorporation of ^{13}C labeled precursors has not only been used for the elucidation of the pathways leading to secondary metabolites but also for other purposes; a few examples are given below. The metabolism of relatively small molecules was e.g. studied in soybean ($^{13}CO_2$) (25), algae ($^{13}CO_2$) (26) or the yeast Candida utilis ([^{13}C]glucose) (27). Biosynthesis was also an efficient method to prepare fatty acyl chains specifically labeled in

alternating positions (28) or uniformly labeled sugars (29). Chlorophyll biosynthetically enriched with ^{13}C was used in a study of chlorophyll-chlorophyll interactions (30).

Double Labeling Experiments

Among the newer techniques the most spectacular growth has been in the field of double labeling experiments. As was outlined in our earlier paper (1), the use of a doubly labeled precursor such as [1,2-$^{13}C_2$]acetate will lead to coupled resonances in the ^{13}C NMR spectrum every time the precursor is incorporated as an intact unit, i.e. when no bond between the two labels has been broken up prior to incorporation. Many experimentors have recognized by now that when investigating acetate derived metabolites, the information gained from a single experiment with [1,2-$^{13}C_2$]acetate is much greater than with a singly labeled precursor, and thus easily compensates for the relatively high cost of the doubly labeled acetate. Furthermore, such an experiment is very helpful in structure elucidation, since pairs of neighboring carbon atoms can be found [cf. (1, 31)]. Investigators have so far mostly worked with [1,2-$^{13}C_2$]acetate because it is commercially available. Battersby and coworkers, however, prepared a doubly labeled porphobilinogen (12) and were able to deduce the assembly pattern of protoporphyrin IX (13)(18) and of a heptacarboxylic porphyrin (32) from the couplings

observed in the ^{13}C NMR spectra of the enriched metabolites. And very recently the successful incorporation of [4,5-$^{13}C_2$]



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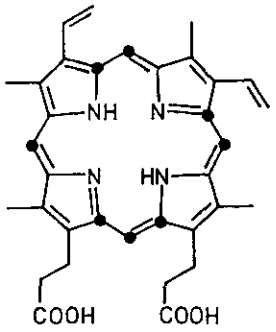

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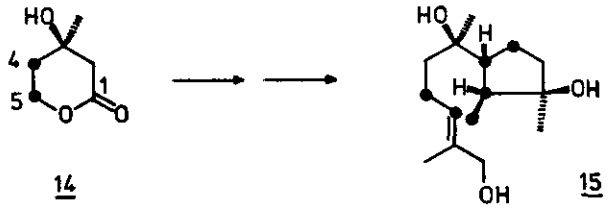
mevalonic acid (14) into the sesquiterpene cyclonerotriol (15) was reported (33).



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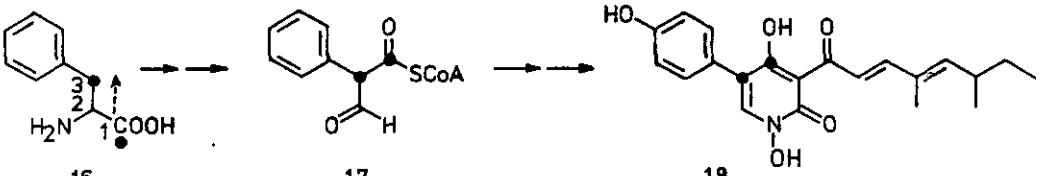
A very elegant study, which was just published, used 1,3-labeled phenylalanine (16) to show that the skeleton of this amino acid undergoes an intramolecular rearrangement prior to its fusion [presumably as α-formylphenylacetyl coenzyme A (17)] with a polyketide to give ultimately tenellin (18) (34). This is the first time that a pr- sor was doubly labeled in such a way

as to give vicinal labels after biological transformation. If the rearrangement is intermolecular, the molecules of the metabolite isolated will no longer contain two labels simultaneously, and therefore no 1,2-coupling will be observed (35). After an intramolecular process, however, vicinal labeling within the same molecule of metabolite is obtained and strongly coupled resonances will be observed in the CMR spectrum, as was the case with tenellin.



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Another advantage of the use of [1,2-$^{13}C_2$]acetate has been pointed out recently (4, 36, 37): incorporation of this doubly labeled precursor is more easily detected with NMR than an equal incorporation of a singly labeled molecule. With singly labeled precursors incorporation can be detected whenever there is a distinct peak enhancement over the background of natural abundance. The limit of detecting incorporation with doubly labeled acetate, however, is given by the observability of the split resonances due to the coupling [cf. (1)]. Since commercially available substrates contain usually 90% ^{13}C in each labeled position,

and hence 81% of the molecules of [1,2-$^{13}C_2$]acetate contain two labels, these satellite signals will become enriched more prominently than the corresponding parent signal. Whether they can be observed or not is a question of the attainable signal-to-noise ratio and the complexity of the spectrum (satellites may be hidden by overlap with other main signals). Note that the intensity of each of these satellites due to the natural abundance ^{13}C content of the metabolite is only 0.0055 times the intensity of the corresponding main signal at natural abundance. The signal-to-noise ratio of the spectrum is a function of the amount of metabolite available and the sensitivity of the spectrometer used. From the above it can be seen that, whereas the permissible dilution for single label experiments is in the range of 100 - 300, this value might be 500 or even more for experiments with [1,2-$^{13}C_2$]acetate or other doubly labeled substrates. It is evident, that if the two labeled centers in a precursor are too far apart from one another to give rise to easily observable coupling of their resonances, criteria other than this have to be used to confirm incorporation of the intact substrate (lack of scrambling, radioactive tracer experiments) as was recently demonstrated in a study from the field of corrinoid biosynthesis (38).

An indepth theoretical treatise of the application of multiply labeled precursors and of the information that can be gained from the multiplets produced in the ^{13}C NMR spectra has recently been published by Matwiyoff and coworkers (26). This paper shows that when a detailed quantitative analysis of the observed multiplets is made, much more information on the biosynthetic pathway can be obtained than just the mere locating of adjacent C-atoms or the proof for the incorporation of intact acetate units. E.g. discrepancies between expected (calculated) and observed multiplet patterns shed light on precursor dilution with endogenous material.

In most cases where [1,2-$^{13}C_2$]acetate was fed, the precursor was diluted with unlabeled acetate [see e.g. (39 - 42)] in order to lower the probability of simultaneous incorporation of two labeled acetate units into adjacent positions of the same molecule of the metabolite, which would lead to more complex splitting patterns in the NMR spectrum (26, 43), as was e.g. observed with islandicin triacetate obtained after administration of [1,2-$^{13}C_2$]acetate (see figure)(8). It is obvious that in biological systems where such a dilution occurs in vivo through endogenous material, the addition of unlabeled precursor is no longer necessary (26, 37).

When matching of the corresponding pairs of doublets is difficult due to similar magnitude of the coupling constants, ^{13}C - ^{13}C homonuclear decoupling may help. The first example of this technique was recently reported from McInnes' laboratory (37).

When studying terpene biosynthesis with [1,2-$^{13}C_2$]acetate yet another feature has to be mentioned. Besides getting matching pairs of carbon atoms derived from unbroken acetate units, all the C-atoms coming from C(2) of mevalonic acid can easily be picked out: they do not show coupling as their partners, the C(1)'s of mevalonic acid, were lost during formation of the isoprene units (39, 41, 44).

The complement to the [1,2-$^{13}C_2$]acetate experiment, the feeding of a 1 : 1 mixture of [1-^{13}C]- and [2-^{13}C]acetates (1, 26) has, since its introduction by Seto (45), been applied once again in the same laboratory (42).

In the context of double labeling experiments we should also mention an investigation where the in vivo incorporation of ethanol into bile acids in the rat was measured (20). The precursor fed was [1-^{13}C,1,1-$^{2}H_2$]ethanol. Besides coupling with deuterium, carbon atoms bound to this isotope experience an isotopic shift and usually resonate at higher field than the corresponding protonated carbons. Thus the signals corresponding to labeled centers of the bile acids were identified by the appearing of the

isotope shifted satellites. In order to make these more readily observable the spectrum was recorded with both, ^{1}H and ^{2}H decoupling thus collapsing to singlets the otherwise split resonances of the carbons bound to deuterium.

It must be pointed out, however, that commercially available ^{13}C NMR spectrometers are usually not fitted with the equipment necessary for simultaneous ^{1}H and ^{2}H decoupling or for ^{13}C - ^{13}C homodecoupling experiments. This equipment is optional or has to be custom built.

Conclusion

There seems no doubt that many double labeling experiments will be carried out in the future, since they provide much information from one experiment. Besides the versatile and commercially available [1,2-$^{13}C_2$]acetate one can predict an increasing number of more complex precursors, synthesized to fit the specific needs of many different experiments.

Some time ago Roeder suggested that ^{12}C would be a suitable marker for biosynthetic studies (46) using precursors depleted of natural abundance ^{13}C content in specific sites. Two authors have in the meantime pointed out that this concept is hardly feasible as it would require extremely high incorporation (14, 47). However, the strategy described in our earlier paper (1) of growing

an organism on a medium depleted of ^{13}C or with $^{12}CO_2$ as the only carbon source, thus lowering the background of natural abundance in studies with ^{13}C labeled precursors, is now being used to investigate metabolic pathways in algae (4).

The concluding pages of this article contain a list of papers reporting the use of ^{13}C labeled precursors in biosynthetic studies and the detection of the labeled centers by ^{13}C NMR spectroscopy. The table contains all the articles which have come to the authors' attention before December 1975. Papers referenced in our earlier review (1) are, generally, not listed.

It is our hope that this second instalment in the fascinating development of the use of ^{13}C in biosynthesis will be both useful and stimulating to workers in this field and the authors would welcome suggestions or comments for future articles on this theme.

Table: Metabolites Investigated and Precursors Fed

Organism Metabolite	Precursors*	Refs.
Escherichia coli mutant		
Phosphatidylethanolamines	[1-^{13}C]ac, [2-^{13}C]ac	(48)
Lactobacillus delbrueckii		
L-Lactate	[$^{13}C_6$]glucose	(26)
Propionibacterium shermanii		
Vitamin B_{12}	[α,γ-$^{13}C_2$]uro'gen III	(38)
	[2-^{13}C]ALA, [5-^{13}C]ALA	
	[8-^{13}C]PBG, [$^{13}CH_3$]met	
	[^{13}C]uro'gen mixture	(16)
Nocardia mediterranei		
Rifamycin W	[1-^{13}C]ac, [1-^{13}C]prop,	
	[2-^{13}C]prop, [3-^{13}C]prop	(49)
Rifamycin S (1)	ethyl [2-^{13}C]malonate,	
	[1-^{13}C]glucose	(50)
	[1-^{13}C]glycerate, [1-^{13}C]-	
	glucose	(51)
Streptomyces griseus		
Streptomycin (19)	[6-^{13}C]glucose	(52)

S. Venezuelae		
Chloramphenicol (20)	[6-^{13}C]glucose	(53)
S. nigrifaciens		
Nigrifactin (21)	[1-^{13}C]ac	(54)
S. reticuli var. latumcidicus		
Dihydrolatumcidin (22)	[1,2-$^{13}C_2$]ac, 1:1 mixture of [1-^{13}C]ac and [2-^{13}C]ac	(45)
S. refuineus		
Anthramycin (23)	[$^{13}CH_3$]met, [1-^{13}C]tyr	(55)
S. luteoreticuli		
Aureothricin (24)	[3-^{13}C]prop	(56)
Aureothin (25) and related compounds	[2-^{13}C]ac, [3-^{13}C]prop	(57)
S. mobaraensis		
Piericidin A (26)	[1-^{13}C]ac, [2-^{13}C]ac, [1-^{13}C]prop	(58)
S. kitasatoensis		
Leucomycin A_3 (27)	[1-^{13}C]ac, [2-^{13}C]ac, [1,2-$^{13}C_2$]ac, [1-^{13}C]prop, [1-^{13}C]butyrate, [1,4-$^{13}C_2$]succinate	(59)

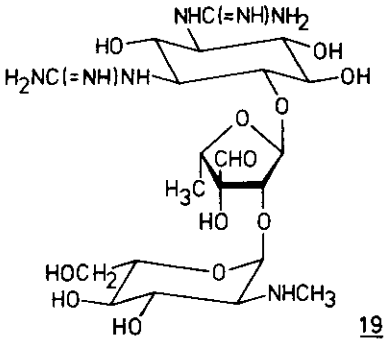
S. Longisporus ruber		
Metacycloprodigiosin (28), Undecylprodigiosin (29)	[1-^{13}C]ac, [2-^{13}C]ac, [1-^{13}C]DL-pro, [2-^{13}C]gly, [3-^{13}C]DL-ser	(60)
Serratia marcescens		
Prodigiosin (30)	[1-^{13}C]ac, [2-^{13}C]a-	(61)
Aspergillus amstelodami		
Echinuline (31)	[1-^{13}C]gly, [2-^{13}C]gly	(62)
Cryptoechinuline A (32)	[1-^{13}C]gly	(63)
A. terreus		
Terrein (33)	[1-^{13}C]ac, [1,2-$^{13}C_2$]ac	(64)
A. melleus		
Pyrone metabolite (34)	[1-^{13}C]ac, [2-^{13}C]ac [1,2-$^{13}C_2$]ac	(43)
A. variecolor		
Tajixanthone (35)	[1-^{13}C]ac, [2-^{13}C]ac	(6)
A. parasiticus		
Averufin (36)	[1-^{13}C]ac	(65)
Aflatoxin B_1 (37)	[1-^{13}C]ac, [2-^{13}C]ac	(9)
A. versicolor		
Sterigmatocystin (38)	[1,2-$^{13}C_2$]ac	(40, 66)

A. flavus		
Aflatoxin B_1 (37)	[2-^{13}C]ac, [1,2-$^{13}C_2$]ac	(67)
Penicillium cyclopium		
Penicillic acid (39)	[1,2-$^{13}C_2$]ac	(68)
P. chrysogenum		
Penicillin V (40)	[2RS,3S)-[4-^{13}C]val	(69)
P. multicolor		
Multicolic acid (41)	[1-^{13}C]ac, [2-^{13}C]ac, [1,2-$^{13}C_2$]ac	(31)
P. islandicum		
Islandicin (2)	[1,2-$^{13}C_2$]ac	(8)
Nigrospora sphaerica		
Aphidicolin (42)	[1,2-$^{13}C_2$]ac	(41)
Fusicoccum amydali		
Fusicoccin (43)	[1-^{13}C]ac, [2-^{13}C]ac	(70)
Oospora virescens		
Virescenol B (44)	[1,2-$^{13}C_2$]ac	(44)
Phyllosticta sp.		
Epoxydon (45)	[1-^{13}C]ac, [2-^{13}C]ac	(71)
Gibberella fujikuroi		
Gibberellic acid (6)	[2-^{13}C]mevalonate	(11)
Fusarium oxysporum		
Bikaverin (46)	[1,2-$^{13}C_2$]ac, [$^{13}CH_3$]met	(37)

F. culmorum		
Cycloerotriol (15)	[4,5-$^{13}C_2$]mevalonic acid	(33)
Periconia macrospinosa		
Metabolites (47), (48)	[1-^{13}C]ac, [2-^{13}C]ac, [1,2-$^{13}C_2$]ac	(72)
Stenphylium radicinum		
Radicinin (49)	[1,2-$^{13}C_2$]ac, 1:1 mixture of [1-^{13}C]ac and [2-^{13}C]ac	(42)
Cercospora kikuchii		
Cercosporin (50)	[^{13}C]formate, [1-^{13}C]ac, [2-^{13}C]ac	(73)
Phialophora lagerbergii		
Scytalone (51)	[1-^{13}C]ac	(74)
Phoma spec.		
Cytochalasin B (Phomin) (52)	[2-^{13}C]ac	(15)
Zygosporium masonii		
Cytochalasin D)53)	[1-^{13}C]ac, [2-^{13}C]ac	(15)
	[1,2-$^{13}C_2$]ac, [1-^{13}C]prop, [1-^{13}C]myristic acid, [1-^{13}C]palmitic acid	(75)
Pseudeurotium ovalis		
Ovalicin (54)	[1,2-$^{13}C_2$]ac	(39)
	[4-^{13}C]mevalonate	(76)

Helminthosporium ravenelii		
Ravenelin (55)	[1-^{13}C]ac, [1,2-$^{13}C_2$]ac	(77)
Beauveria bassiana		
Tenellin (18)	[1,3-$^{13}C_2$]phe	(34)
Capsicum frutescens infected with *Monilinia fructicola*		
Capsidiol (11)	[1,2-$^{13}C_2$]ac	(23)
Camptotheca acuminata		
Camptothecin (8)	Indole alkaloid precursor	(21)
Colchicum autumnale		
Colchicine (10)	(RS)-[1-^{13}C]autumnaline	(22)
Tissue cultures of *Isodon japonicus*		
Oleanene- and ursene-type triterpenes e.g. (56)	[4-^{13}C]mevalonate	(78)
	[1,2-$^{13}C_2$]ac	(79)
Avian erythrocyte enzyme system		
Heptacarboxylic porphyrin	[2,11-$^{13}C_2$]PBG	(32)
Rat		
Bile acids	[1-^{13}C,1,1-$^{2}H_2$]ethanol	(20)

* Abbreviations: ac = acetate, prop = propionate, ALA = δ-amino-levulinic acid, PBG = porphobilinogen, uro'gen = uroporphyrinogen, met = methionine, tyr = tyrosine, pro = proline, ser = serine, val = valine, phe = phenylalanine.



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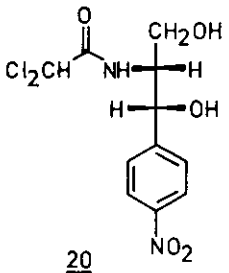

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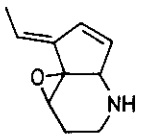

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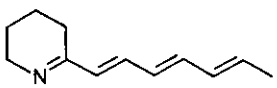

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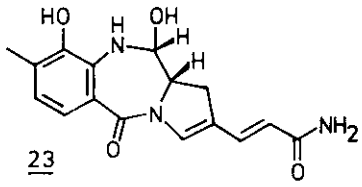

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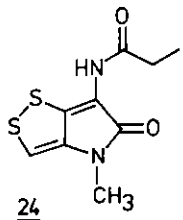

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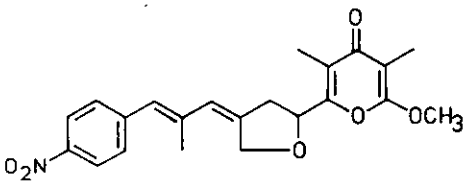

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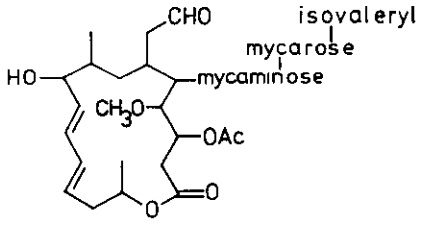

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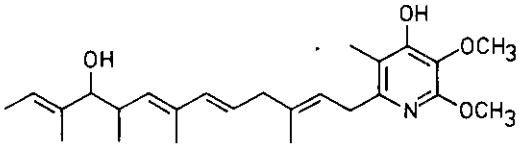

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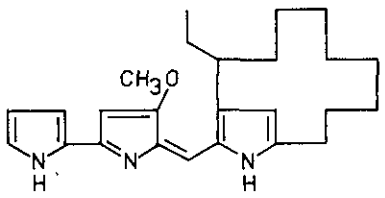

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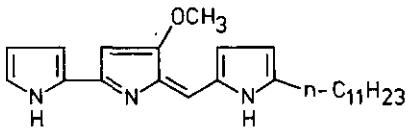

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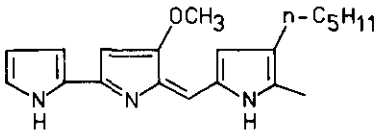

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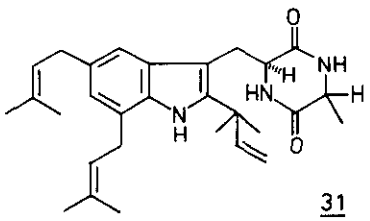

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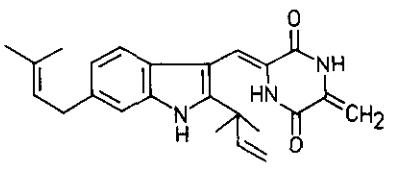

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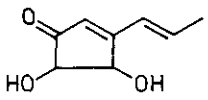

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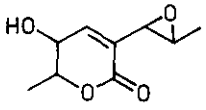

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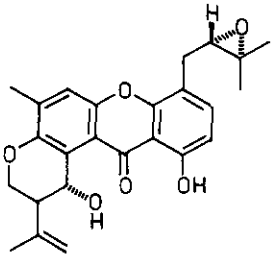

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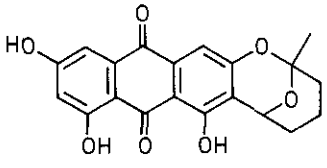

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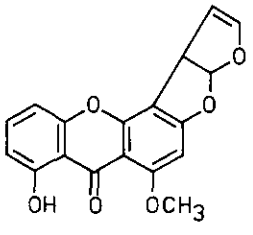

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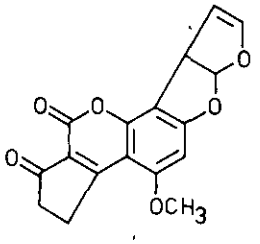

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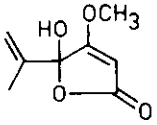

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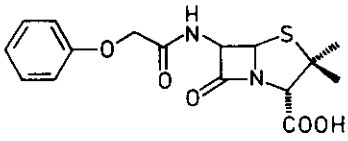

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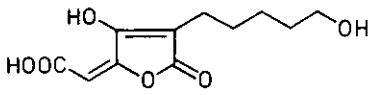

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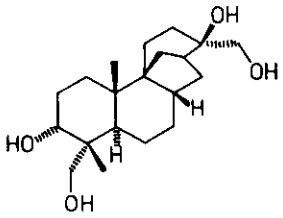

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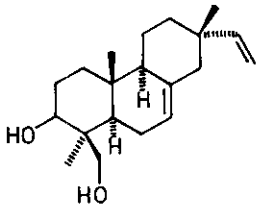

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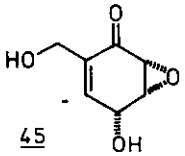

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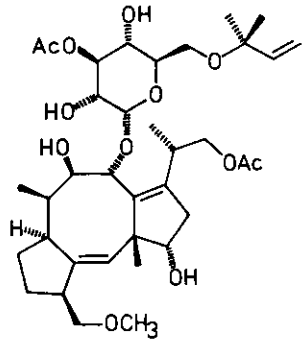

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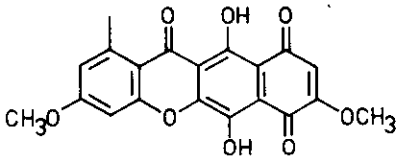

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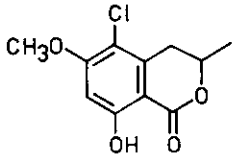

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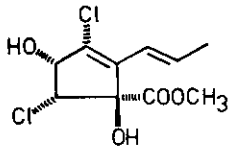

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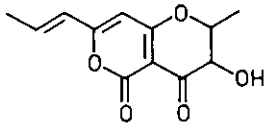

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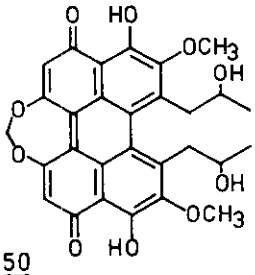

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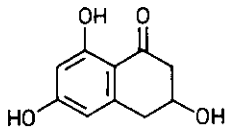

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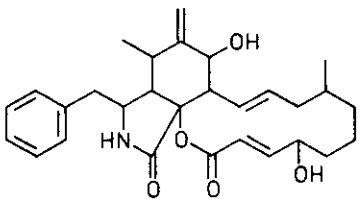

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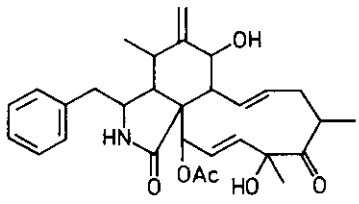

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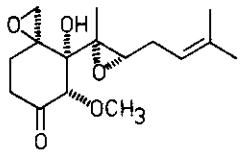

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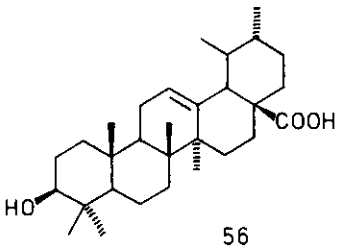

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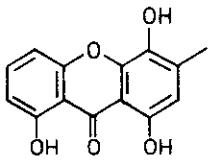

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17. Note that the term $EF_{metab} \times l_{metab}$ is equal to the expression $x_1 + 2x_2 + \ldots + nx_n$, which was used in (15).

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35. Note that some coupling may occur from the statistical introduction of two labels into adjacent positions of the same molecule of the metabolite.

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