Retrobiosynthetic NMR Studies with $^{13}$C-Labeled Glucose

FORMATION OF GALLIC ACID IN PLANTS AND FUNGI*

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The biosynthesis of gallic acid was studied in cultures of the fungus Phycomyces blakesleeanus and in leaves of the tree Rhus typhina. Fungal cultures were grown with $[1-{^{13}}C]$glucose or with a mixture of unlabeled glucose and $[U-{^{13}}C_6]$glucose. Young leaves of R. typhina were kept in an incubation chamber and were supplied with a solution containing a mixture of unlabeled glucose and $[U-{^{13}}C_6]$glucose via the leaf stem. Isotope distributions of the fungus Rhus typhina and in leaves of R. typhina were analyzed by one-dimensional $^1$H and $^{13}$C NMR spectroscopy. A quantitative analysis of the complex isotopomer composition of metabolites was obtained by deconvolution of the $^{13}$C$^{13}$C coupling multiplets using numerical simulation methods. This approach required the accurate analysis of heavy isotope chemical shift effects in a variety of different isotopomers and the analysis of long range $^{13}$C$^{13}$C coupling constants. The resulting isotopomer patterns were interpreted using a retrobiosynthetic approach based on a comparison between the isotopomer patterns of gallic acid and tyrosine. The data show that both in the fungus and in the plant all carbon atoms of gallic acid are biosynthetically equivalent to carbon atoms of shikimate. Notably, the carboxyl group of gallic acid is derived from the carboxylic group of an early intermediate of the shikimate pathway and not from the side chain of phenylalanine or tyrosine. It follows that the committed precursor of gallic acid is an intermediate of the shikimate pathway prior to prephenate or arogenate, most probably 5-dehydroshikimate. A formation of gallic acid via phenylalanine, the lignin precursor, caffeic acid, or 3,4,5-trihydroxycinnamic acid can be ruled out as major pathways in the fungus and in young leaves of R. typhina. The incorporation of uniformly $^{13}$C-labeled glucose followed by quantitative NMR analysis of isotopomer patterns is suggested as a general method for biosynthetic studies. As shown by the plant experiment, this approach is also applicable to systems with low incorporation rates.

The genetic manipulation of phenylpropanoid-derived metabolites has been proposed as a prospective target for crop improvement (1). A fundamental understanding of the key enzymes of the various phenylpropanoid branching pathways is therefore of increasing interest.

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Gallic acid (3,4,5-trihydroxybenzoic acid, compound 7, Fig. 1) serves as a fundamental precursor for gallotannins and ellagitannins, abundant classes of plant secondary metabolites. Gallic acid is also formed in substantial amounts by some fungi. A considerable number of studies on the biosynthesis of tannins has been reported (for review, see Gross (2) and Haslam (3)). Surprisingly, the biosynthesis of gallic acid, the phenolic unit of this important class of natural products, is still incompletely understood. Based mainly on radiolabeling studies, it has been proposed that gallic acid could be formed from phenylalanine (compound 5) via caffeic acid (compound 8), 3,4,5-trihydroxy-cinnamic acid (compound 9), or protocatechuic acid (compound 6, Fig. 1) (4–9). This would imply that the carboxyl group of gallic acid is derived from the $\beta$ carbon atom of phenylalanine (compound 5), and ultimately from the enolpyruvyl group of chorismate (compound 3, Fig. 1). On the other hand, it has been argued that the carboxyl group of gallic acid is biosynthetically equivalent to the carboxyl group of shikimate (10–15). This would imply that gallic acid is formed from an early shikimate intermediate, e.g. directly from 5-dehydroshikimate (compound 4) by dehydrogenation or via protocatechuic acid (compound 6) as an intermediate (Fig. 1) (16). It was also proposed that the controversial results could indicate the existence of alternative biosynthetic routes in the same organism (17–20).

This report describes studies with shake flask cultures of the fungus, Phycomyces blakesleeanus, and with young leaves of the tree, Rhus typhina, which were supplied with exogenous $^{13}$C-labeled glucose. Gallic acid and amino acids were obtained by hydrolysis of cell material, and the $^{13}$C distribution was analyzed by NMR spectroscopy. A central aspect of this approach was a retrobiosynthetic comparison of labeling patterns of gallic acid with those of amino acids. The results show unequivocally that gallic acid is biosynthesized in both organisms from an early intermediate of the shikimate pathway, but not via phenylalanine.

**EXPERIMENTAL PROCEDURES**

*Materials—* $[U-{^{13}}C_6]$Glucose (99% $^{13}$C) was purchased from Isotec (Miamisburg, FL), and $[1-{^{13}}C]$glucose was purchased from Micromer (South Bend, IN).

*Fungal Strain—* Spores of P. blakesleeanus (NRRL1555(--)) were obtained from P. Galland, Marburg, Germany.

*Plant Incubation—* Young leaves of R. typhina were cut from a local tree between May and August.

*Culture Conditions for P. blakesleeanus—* Spores of P. blakesleeanus were activated by heat treatment (48 °C) for 2 h. The culture medium contained, per liter, 2.0 g of asparagine in H$_2$O, 5.0 g of KH$_2$PO$_4$, 0.5 g of MgSO$_4$·7H$_2$O, 0.2 ml of 14% (w/w) CaCl$_2$ solution, 2 mg of thiamine hydrochloride, 2 mg of citric acid in H$_2$O, 1.5 mg of Fe(NO)$_3$·9H$_2$O, 1 mg of ZnSO$_4$·7H$_2$O, 0.5 mg of MnSO$_4$·H$_2$O, 50 µg of CuSO$_4$·5H$_2$O, 50 µg of NaMoO$_4$·2H$_2$O, pH 4.7. A sterile solution of 6 g of $[1-{^{13}}C]$glucose in 5 ml of water was added to the autoclaved medium (300 ml). Alternatively, a mixture of $[U-{^{13}}C_6]$glucose (320 mg) and unlabeled glucose (7.7 g) in 5 ml of water was added to the autoclaved medium (400 ml). Erlenmeyer flasks were autoclaved at 121 °C for 20 min.

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flasks (1 liter) containing 400 ml of culture medium were seeded with one loopful of spores and were incubated with shaking at 25 °C for 6 days. The mycelium was harvested by filtration and was washed with a small amount of water.

**Plant Culture**—The stems of young leaves of *R. typhina* (with a length of 3–4 cm) were immersed into a sterile solution of 1% (w/w) of glucose consisting of 96% of unlabeled glucose and 4% of [U-\textsuperscript{13}C\textsubscript{6}]glucose. They were incubated in a dark compartment (Heraeus Vötsch climate chamber) at 18 °C and 60% humidity for 33 days. Small sections of the stem were removed with a sharp razor at intervals of 1 or 2 days.

**Isolation of Gallic Acid from *P. blakesleeanus* Culture**—The culture medium from experiments with *P. blakesleeanus* was adjusted to pH 1, treated at 96 °C for 6 h, and extracted with 150 ml of diethyl ether for 12 h using a liquid/liquid perforator. Fungal biomass was treated with 1M sulfuric acid (100 ml) at 96 °C for 6 h. The solution was filtered and was then continuously extracted with 150 ml of diethyl ether for 12 h using a liquid/liquid perforator. The organic phases obtained by extraction of culture medium and of cell mass hydrolysate of *P. blakesleeanus* were combined. The aqueous solution obtained by acidic treatment of biomass and subsequent extraction with ether was set aside for isolation of tyrosine and phenylalanine.

The organic phase was extracted twice with 75 ml of saturated sodium bicarbonate. The aqueous solution was adjusted to pH 1 by the addition of concentrated sulfuric acid and was again subjected to continuous extraction with 150 ml of diethyl ether. The organic phase was concentrated to a small volume (about 3 ml) under reduced pressure.

The solution was applied to a column of silica gel (Merck, 1.5 × 35 cm). The column was developed with a mixture of diethyl ether/ethyl acetate/formic acid (50:40:10, v/v/v). Gallic acid was eluted with the colored solvent front. Fractions were combined and were brought to dryness under reduced pressure. Aliquots of fractions were applied to a TLC silica gel plate (Merck, Darmstadt), which was developed with a mixture of diethyl ether/ethyl acetate/formic acid (50:40:10, v/v/v). Gallic acid migrated at a *R*\textsubscript{f} of 0.82 and was detected by fluorescence quenching (254 nm).

**Isolation of Gallic Acid from Plant Material**—Acid treatment of biomass and isolation of gallic acid was performed as described for fungal cell mass.

**High Performance Liquid Chromatography**—Reversed phase high performance liquid chromatography was performed with a column of Nucleosil RP18 (4.5 × 250 mm) which was developed with 23% aqueous methanol. The effluent was monitored photometrically (258 nm). Semi-preparative separations were performed with a Nucleosil RP18 column (16 × 250 mm). The retention volume of gallic acid was 48 ml.

**Isolation of Amino Acids**—Tryptophan was isolated from cell mass after alkaline hydrolysis as described earlier (21). Tyrosine and phenylalanine were isolated from the aqueous phase obtained after extraction of the biomass hydrolysate with diethyl ether (see above). The aqueous phase was adjusted to 6 M HCl and boiled under reflux for 24 h. The separation of the amino acids from the hydrolysate was performed as described earlier (22).

**NMR Spectroscopy**—\textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded at 500.13 and 125.76 MHz, respectively, with a Bruker DRX500 spectrom-

![Diagram](image-url)
The present study was designed to determine unequivocally whether the carboxylic group of gallic acid originates from the carboxylic group of an early shikimate precursor (e.g. 5-dehydroshikimate) or from the β carbon atom of the phenylalanine side chain. This question can be addressed by a comparison between the labeling patterns of biosynthetic gallic acid and phenylalanine or tyrosine, which are easily obtained by hydrolysis of cell protein. This retrobiosynthetic approach requires a labeling strategy conducive to significantly different labeling of the carboxylic group of early shikimate derivatives and of the β carbon atom of phenylalanine or tyrosine. Since the carboxylic group of early shikimate derivatives and the β carbon of phenylalanine are derived from different atoms (i.e. the carboxyl group and the methyl group) of pyruvate, this condition can be fulfilled by a variety of precursors, and unequivocal results were obtained with [1-13C]glucose in P. blakesleeanus. In leaves of R. typhina, the incorporation rate of [1-13C]glucose was too low to afford unequivocal results. However, this problem could be addressed by incorporation experiments using a mixture of [U-13C6]glucose and unlabeled glucose. It will be shown that the sensitivity of this technique is substantially better as compared with the single-labeled precursor technique.

Experiments with Cultures of P. blakesleeanus—A shake flask culture of P. blakesleeanus (300 ml) was supplied with 6 g of [1-13C]glucose (99.5% enrichment). The culture was incubated for 6 days. Gallic acid (5 mg) was isolated from the culture fluid and from the cell hydrolysate as described under "Experimental Procedures." Phenylalanine (3 mg), tyrosine (4 mg), and tryptophan (2 mg) were obtained by hydrolysis of cell mass (8 g, wet weight). The absolute 13C abundance of all carbon atoms of gallic acid (compound 7), phenylalanine (compound 5), tyrosine (compound 10), and tryptophan (compound 11) from P. blakesleeanus cultured with [1-13C]glucose was determined as described under "Experimental Procedures." (Fig. 2).

Due to the symmetry of the aromatic rings of phenylalanine, tyrosine, and gallic acid, the respective ring carbon atoms 2/6 and 3/5 yield only an averaged 13C abundance value, although they have different biosynthetic origins (C-4 of erythrose 4-phosphate/C-3 of phosphoenolpyruvate and C-1/C-3 of erythrose 4-phosphate, respectively). Tryptophan reflects the original, i.e. nonsymmetrical, labeling patterns of the shikimate ring system, and the labeling pattern of erythrose 4-phosphate (compound 2) can thus be reconstructed by a retrobiosynthetic approach on basis of the tryptophan biosynthetic pathway (Fig. 2). The labeling pattern of phosphoenolpyruvate (compound 1) can be deduced unequivocally from the labeling pattern of the side chains of phenylalanine and tyrosine (Fig. 2).

The observed labeling patterns are explained on basis of carbohydrate metabolic pathway. Label from [1-13C]glucose is diverted to C-3 of triose phosphate type metabolites by the glycolytic pathway (compounds 14 and 15, Fig. 3). This results in the high 13C enrichment of the β position of phenylalanine and tyrosine reflecting C-3 of phosphoenolpyruvate (23.4% 13C abundance, Fig. 2). Decarboxylation of [1-13C]glucose 6-phosphate by the oxidative branch of the pentose phosphate pathway yields 13CO2 and unlabeled ribulose 5-phosphate (compound 13, Fig. 3). Erythrose 4-phosphate (compound 2) generated from the unlabeled ribulose 5-phosphate via the nonoxidative pentose phosphate pathway should be devoid of 13C label. However, the observed 13C labeling in the 4-position of erythrose 4-phosphate (18.5% 13C abundance) can be explained by futile cycling via the glycolytic/glucogenic pathways and/or via the mannitol pathway (Fig. 3). Specifically,
regeneration of glucose from triose phosphate isomerase could divert $^{13}$C label to the 6-position of glucose. Similarly, the reversible conversion of glucose to mannitol (compound 16) could also divert label to the 6-position of glucose. The resulting glucose or $[^{13}C]$glucose is indicated by asterisks. Hypothetical contiguous $^{13}$C-labeling from $[^{13}C]$glucose is indicated by bold lines (for details, see text).

The labeling pattern of the benzenoid ring of gallic acid (compound 7) agrees closely with the ring labeling of phenylalanine (compound 5) and tyrosine (compound 10, Fig. 2). The carboxylic group of gallic acid has less $^{13}$C abundance (2.9%) whereas the $\beta$ carbon atoms of tyrosine or phenylalanine are highly labeled (23.4%). It follows that the carboxylic group is not derived from the $\beta$ carbon of phenylalanine or tyrosine.

As shown in Fig. 1, the carboxylic atom of early shikimate precursors (e.g. 5-dehydroshikimate, compound 4) stems from C-1 of phosphoenolpyruvate (compound 1) which is virtually not labeled from $[^{13}C]$glucose (Fig. 2). Thus, a direct conversion of 5-dehydroshikimate to gallic acid would be in line with the experimental data.

Attempts to use the same experimental approach for the study of gallic acid biosynthesis in leaves of the tree, R. typhina, were unsuccessful. The amount of $^{13}$C-label diverted to gallic acid and phenylalanine was so low (less than 2%) $^{13}$C abundance in the most highly labeled carbon atoms) that it was impossible to draw firm conclusions. This is due to the fact that exogenous glucose supplied to the intact leaves via the leaf stem is poorly metabolized, and the NMR spectra are dominated by the natural $^{13}$C abundance of metabolites, which were formed prior to glucose application. A much more sensitive technique was therefore required. As shown below, the sensitivity problem can be overcome by the use of a mixture of $[^{13}C]$glucose and unlabeled glucose. This experimental approach will be described first with the fungal culture and then with plant material.

A shake flask culture of P. blakesleeanus was supplied with a mixture of $[^{13}C]$glucose and unlabeled glucose at a ratio of 1:25. The NMR signals of metabolites isolated from this culture are complex multiplets as a consequence of $^{13}$C-$^{13}$C coupling (Figs. 4 and 5). To resolve these multiplets, it was important to obtain a spectral resolution of at least 0.5 Hz. The central signals marked by asterisks represent molecules with a single $^{13}$C atom, which were formed from the natural abundance material in the proffered glucose mixture. The complex satellite patterns indicate the presence of various isotopomers that reflect biosynthetic contributions of the totally labeled glucose. A detailed analysis of the spectra indicates that the biosynthetic products are complex mixtures of different isotopomers.

The satellite patterns can be resolved by numerical simulation of the $^{13}$C-$^{13}$C coupling patterns for each respective isotopomer in the mixture. As shown in Fig. 4, the chemical shift positions as well as amplitude modulations due to higher order coupling show perfect agreement between the simulated and experimental spectra. It should be noted that the lack of symmetry of the coupling with respect to the signal of the single-labeled isotopomers is due to nonlinear coupling patterns and to chemical shift variations caused by heavy isotope shift effects. The heavy isotope shifts of individual nuclei in specific isotopomers as deduced from comparison of experimental signals with spectral simulations are summarized in Fig. 6. The various isotopomers in the biolabeled mixture are symbolized by bold lines, indicating multiple contiguous carboxylate atoms. A single adjacent $^{13}$C atom typically results in a high field shift in the range of 2 Hz; two adjacent $^{13}$C atoms are conducive to a high field shift of the observed carbon in the range of 4 Hz.

Single bond and multiple bond $^{13}$C-$^{13}$C coupling constants are complex multiplets as a consequence of $^{13}$C-$^{13}$C coupling (Figs. 4 and 5). To resolve these multiplets, it was important to obtain a spectral resolution of at least 0.5 Hz. The central signals marked by asterisks represent molecules with a single $^{13}$C atom, which were formed from the natural abundance material in the proffered glucose mixture. The complex satellite patterns indicate the presence of various isotopomers that reflect biosynthetic contributions of the totally labeled glucose. A detailed analysis of the spectra indicates that the biosynthetic products are complex mixtures of different isotopomers.

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shift of ~2.0 Hz. This results in higher order coupling between C-3 and C-5 which are no longer homotopic.

Only the coupling patterns without the actual simulations are shown in Fig. 5. However, it should be noted that the intensity modulations due to higher order coupling contributions are again faithfully reflected by the simulated data.

The fraction of each isotopomer in the mixture can be obtained by integration of the signal groups representing each individual isotopomer. Some isotopomers can be estimated from different spectral patterns. Thus, the abundance of isotopomer d can be diagnosed independently from the signatures of C-2, C-3, and C-4. The quantitative data obtained independently from different parts of the spectrum are in very good agreement.

The isotopomer composition of the biosynthetic gallic acid (compound 7) is summarized in Fig. 7. The quantitative contribution of each isotopomer is indicated by the width of the line connecting contiguous 13C atoms and also by numbers which indicate relative concentrations (mol %) of individual isotopomers. Isotopomer d indicates that approximately one-third of the erythrose 4-phosphate pool is formed via the pentose phosphate shunt or mannitol cycling (Fig. 3). Isotopomer e indicates that approximately two-thirds of the erythrose 4-phosphate pool is synthesized via futile cycling of the hexose phosphate pool. This is in line with the erythrose 4-phosphate labeling pattern from [1-13C]glucose (see above).

The complex multiplets of biosynthetic tyrosine (compound 10) obtained from the biomass grown on the mixtures of [U-13C6]glucose and unlabeled glucose was analyzed by the same approach. The isotopomer composition is again summarized in Fig. 7. It is immediately obvious that the isotopomer signatures of the benzene rings are closely similar in gallic acid and tyrosine. This is a conclusive proof for the origin of gallic acid from the shikimate pathway.

Virtually no isotopomers with contiguous 13C atoms in ring atom C-1 and the β carbon of the side chain are present in the amino acid. The conspicuous absence of this isotopomer type reflects the assembly of the amino acid from the ring atoms of shikimate and the side chain contributed by a second molecule, i.e., phosphoenolpyruvate.

On the other hand, the isotopomer composition of gallic acid is dominated by the [carboxyl-1,6-13C3]isotopomer b (Fig. 7). If gallic acid were derived from phenylalanine or tyrosine by subsequent cleavage of the side chain, the presence of this isotopomer could not be explained. It follows again that gallic acid is not formed via phenylalanine or tyrosine in the fungus.

Experiments with Young Leaves of R. typhina—Initial experi-

![Fig. 4. 13C NMR signature of C-4 of gallic acid from P. blakesleeanus cultured with [U-13C6]glucose diluted with unlabeled glucose (1:25; w/w). A, 1H decoupled spectrum; the asterisk indicates natural abundance background. B–D, simulated signals of isotopomers based on NMR parameters shown in Fig. 6. The respective isotopomers are shown schematically by bold lines connecting contiguously adjacent 13C atoms, and the observed carbon is marked by an arrow. The amplitudes of the simulated spectra were adjusted to reflect the relative abundance of each respective isotopomer in the mixture.](image1)

![Fig. 5. 1H decoupled 13C NMR signals of gallic acid from P. blakesleeanus after incorporation of [U-13C6]glucose diluted with unlabeled glucose (1:25, w/w). A, carboxyl C; B, C-1; C, C-3; for details see legend to Fig. 4.](image2)
ments mentioned above had shown that the incorporation of [1-13C]glucose in leaves of *R. thyphina* is low. The poor utilization of exogenous nutrients had also hampered earlier studies in the literature and is probably the major reason for the conflicting reports on gallic acid biosynthesis. The preliminary experiments with fungal cultures indicated that the sensitivity problem could be addressed by the use of totally labeled glucose conducing to the formation of multiply labeled isotopomers. The signal contributions of the multiply labeled isotopomers are spread out in the frequency domain by 13C13C coupling and are thus separated from the signals of molecules representing the high background of preformed gallic acid with natural 13C abundance.

A large number of preliminary experiments were required to find experimental conditions affording appropriate transfer of 13C from exogenous glucose into the plant system. Variations in the experimental set-up concerned (i) the age of the plant tissue used as judged by the size of the leaves and the degree of unfolding, (ii) the conditions of incubation, (iii) the length of the feeding period, and (iv) the 13C abundance in the glucose used as supplement. These experiments will be described in some detail because similar considerations may be relevant in the context of other incorporation experiments with higher plants. (i) It was important to utilize very young leaves since the utilization of exogenous glucose decreased rapidly with increasing age and maturation of the tissue. (ii) Standardized conditions of temperature and humidity had to be maintained to extend the lifetime of the plant tissue as much as possible. We found 18 °C and 60% relatively humidity most appropriate for *R. typhina* leaves. Incubation in the dark resulted in higher 13C incorporation rates as compared with permanent or intermittent light. (iii) Both the utilization of glucose in *R. typhina*, and the biosynthetic capacity of the plant material after removal from the mother plant was relatively poor. It was therefore to extend the feeding period as much as possible. With appropriate care to minimize microbial growth, the leaves could be kept for more than 1 month. (iv) The most crucial feature is the labeling pattern of the precursor material. Using single 13C-labeled glucose the maximum 13C enrichment values observed in any metabolite studied was always less than 2% in these experiments. On the background of 1.1% natural 13C abundance, these small enrichment increments contributed by the 13C labeled precursor could not be evaluated with sufficient accuracy. Thus, the strategy with single-labeled glucose had to be abandoned.

Subsequent experiments were performed with [U-13C6]-, [1,2-13C2]-, and [2,3-13C2]glucose with 99% enrichment. Whereas the overall enrichment of metabolites was again low (<2% 13C), the incorporation of the precursors could be conclusively demonstrated by the presence of 13C13C coupling satellites in the spectra of gallic acid and amino acids. Actually, in the experiment with [U-13C6]glucose, 13C13C coupling was extensive throughout the molecules analyzed. In light of the low enrichment of the metabolites formed, this presented a paradox. In retrospect, it is now clear that the cut leaves retained little biosynthetic capacity, irrespective of the incubation conditions. The vast majority of the isolated metabolites had already been synthesized prior to the feeding experiments. On the other hand, the system had been flooded with the labeled precursor, and this had resulted in the assembly of three 13C-labeled precursor molecules for formation of phenylalanine. Thus, less than 1% of the total gallic acid had been synthesized from the labeled precursor during the incubation period but the
small amount of newly formed gallic acid was produced almost exclusively from exogenous glucose.

On basis of these initial data, we performed an optimized experiment with 150 young leaves of *R. typhina* (60 g) which were supplied with a solution (300 ml) containing 250 mg of [U-13C6]glucose and 6 g of natural abundance glucose. Gallic acid (50 mg) and amino acids were isolated by the standard procedure, and high resolution NMR spectra of gallic acid and tyrosine were recorded. The complex 13C NMR signals of the carboxylic carbon and ring atom C-1 of gallic acid are shown in Fig. 8. In contrast to the experiments with the fungal cultures (Figs. 4 and 5), the relative intensity of the coupling patterns is low as compared with the contribution of isotopomers carrying a single 13C atom (marked by asterisks), which represent the contribution of natural abundance gallic acid. The large fraction of natural abundance material essentially represents gallic acid which had already been present at the beginning of glucose application, whereas a minor amount was contributed biosynthetically from unlabeled glucose in the feeding mixture.

Fig. 8 also shows the carboxyl- and C-1 13C NMR signals of natural abundance gallic acid. The asterisks mark the signals of the [carboxyl-13C1]- and [1-13C1]gallic acid. The plots were arranged to give identical amplitudes for the (truncated) signals of the biosynthetic sample and the natural abundance sample. The stochastic 13C distribution in the natural abundance sample is conducive to a signal equivalent of 1.1% of the central signal for isotopomers with two adjacent 13C atoms (such as [1,2-13C2]-, and [carboxyl-1-13C2]gallic acid). These signals are readily observed in the natural abundance sample. The stochastic contribution of isotopomers with 3 and 4 adjacent carbon atoms in natural abundance material is approximately 10^-4 and 10^-6 mol %, respectively. The relative abundances of these species are therefore far below the level of detection in the natural abundance gallic acid. On the other hand, signals pertaining to multiply labeled isotopomers are apparent in the biosynthetic sample and can be identified unequivocally on basis of the isotopomer deconvolution which has been described above (Fig. 8).

A quantitative analysis of the biosynthetic gallic acid spectra yields the isotopomer composition shown in Fig. 9. The abundance of isotopomers with 2 and more adjacent 13C isotopomers was referenced to the satellites for the various [13C2]-isotopomers in the natural abundance sample. The natural abundance background was then subtracted from the labeled sample, thus affording exclusively the composition of the material which was biosynthesized de novo from the proffered [U-13C6]glucose.

The isotopomer composition of biosynthetic tyrosine was determined by the same experimental approach and is also shown in Fig. 9. The isotopomer patterns of the aromatic rings of biosynthetic gallic acid and tyrosine are similar and leave no doubt that the aromatic rings of both compounds originate from the same (i.e. the shikimate) pathway. Gallic acid shows a significant amount of the triple-labeled [carboxyl-1,6-13C3]-isotopomer in close analogy with the Phycomyces experiment. This isotopomer has no equivalent in tyrosine where the abundance of the [β,1,2-13C3] is low in accordance with the expectations based on the shikimate pathway. In analogy with the Phycomyces experiment, it can be concluded that phenylalanine and tyrosine do not serve as major intermediates in the gallic acid biosynthetic pathway.

**DISCUSSION**

Feeding with totally 13C-labeled fundamental precursors such as [U-13C]carbohydrates or [U-13C]lipid in conjunction with unlabeled material is conducive to biosynthetic formation of complex isotopomer mixtures. In earlier studies, we used two-dimensional NMR techniques to characterize multiply 13C-labeled isotopomers (24, 25). In the present study, this approach would have been difficult for reasons of sensitivity because the incorporation of glucose by leaves of *R. typhina* was...
low. However, we have shown that an even more detailed analysis of isotopomer mixture is possible by an in depth analysis of coupling multiplets in one-dimensional $^{13}$C spectra. For this approach, it is important to obtain NMR spectra at high magnetic field strength to maximize the chemical shift dispersion. Moreover, it is important to work at the maximum obtainable resolution (better than 0.5 Hz) to maximize sensitivity and to minimize overlapping of lines. Integrals of each component of the complex signals can then be determined easily. Typically, the NMR signatures of an individual carbon atom will arise by the $^{13}$C coupling multiplets of several different isotopomers as shown in Figs. 4, 5, and 8. The intuitive deconvolution of the complex multiplet superpositions is possible only in the most simple cases. Complex patterns require a stringent analysis by numerical simulation of the spectra for each contributing isotopomer.

As part of the deconvolution process, it is necessary to determine the heavy isotope shift effects and the coupling constants for each isotopomer from the complex spectra. It should be noted that molecular symmetries conducive to homotopy of nuclei can be broken by heavy isotope shift effects, and unexpected coupling patterns of higher order can result.

Whereas this process is laborious, it has the advantage to yield quantitative data for each isotopomer in the mixture. This isotopomer pattern can then be used efficiently for biosynthetic retroanalysis via comparison of isotopomer distribution patterns in different metabolites. For example, the comparison of isotopomer patterns of the benzenoid ring of gallic acid with those of phenylalanine and tyrosine leave no doubt whatsoever that gallic acid is a derivative of the shikimate pathway.

The experimental sensitivity of biosynthetic studies with single-labeled $^{13}$C precursors is limited by the inherent sensitivity of NMR instrumentation, and, more important, by the presence of natural abundance material in the numerous cases where the de novo formation of metabolites from the preformed, isotopically labeled precursor is low. The use of multiply labeled precursors and the subsequent, quantitative NMR assessment of biosynthetic isotopomers with multiple labels affords a large gain in experimental sensitivity because the relevant signals are separated from the natural abundance component via spreading in the frequency domain by $^{13}$C$^{13}$C coupling.

As mentioned above, the stochastic abundance of multiply labeled isotopomers in natural abundance material is very low (approximately $10^{-4}$ mol % for triple-labeled and $10^{-6}$ mol % for quadruple-labeled species). As a consequence, any observed signals of triple- and quadruple-labeled isotopomers can be unequivocally addressed as the result of de novo biosynthesis from the multiply labeled precursor.

In the biosynthesis of tyrosine and phenylalanine, two molecules of phosphoenolpyruvate are consumed for the biosynthesis of part of the ring system (i.e. C-6 and C-1) via shikimate and of the side chain via the enoylpyruvate side chain of chorismate. It is therefore surprising that the isotope contribution of $[^{1,6-13}$C$_2]$glucose to the side chain of tyrosine in $R$. typhina exceeds the level of $^{13}$C-labeling in the ring (specifically, the $[1,6-^{13}$C$_2]$isotopomer) by a factor of about 2. Earlier studies had shown that plant tissues can contain substantial amounts of shikimate pathway intermediates such as shikimate and quinate (26). We conclude from the labeling data that preformed early shikimate derivatives were converted to chorismate and further to tyrosine during the $[^{13}$C$_2]$glucose feeding period. This would result in the formation of the aromatic amino acid from a preformed ring system at natural $^{13}$C abundance by the addition of an enoylpyruvate side chain derived from the proffered $^{13}$C-labeled glucose mixture.

The data in this report show clearly that gallic acid is formed predominantly or entirely from an early intermediate (i.e. prior to prephenic or arogenic acid) of the shikimate pathway. Several authors have proposed that two different pathways, via an early shikimate intermediate and via one of the aromatic amino acid could be operative in the same organism (5, 17–20). More specifically, it has been claimed that gallic acid is predominantly formed via dehydrogenation of shikimate in young leaves of $R$. succedanea, but via phenylalanine in older leaves of $R$. succedanea (17). Our data show that a biosynthetic pathway via phenylalanine could have contributed less than 2% of biosynthesized gallic acid in the experiments with $P$. blakesleeanus and less than 10% in the experiments with $R$. typhina leaves.

The biosynthesis of gallic acid (compound 7, Fig. 10) by dehydrogenation of 5-dehydroshikimic acid (compound 4) in both the fungus and the plant appears to be the major pathway. However, the data cannot exclude a pathway via protocatechuic acid (compound 6) by dehydration of 5-dehydroshikimic acid followed by a monooxygenase catalyzed reaction (Fig. 10) (4). Supporting this hypothesis, a conversion of 5-dehydroshikimic acid to protocatechuic acid has been observed with crude enzyme preparations from mung bean seedlings (16).
Experiments to distinguish between the two possible routes are in progress.

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REFERENCES

1. Dixon, R. A., Lamb, C. J., Masoud, S., Sewalt, V. J. H. & Paiva, N. L. (1996) *Gene* (Amst.) 179, 61–71
2. Gross, G. C. (1992) *Basic Life Sci.* 59, 43–60
3. Haslam, E. (1992) *Basic Life Sci.* 59, 169–194
4. Kato, N., Shiroya, M., Yoshida, S. & Hasegawa, M. (1968) *Shokubutsugaku Zasshi* 81, 506–507
5. Zenk, M. H. (1964) *Z. Naturforsch.* 19b, 83–84
6. Neish, A. C., Towers, G. H. N., Chen, D., El-Basyouni, S. Z. & Ibrahim, R. K. (1964) *Phytochemistry* 3, 485–492
7. Brucker, W. & Hashem, M. (1962) *Ber. Dtsch. Bot. Ges.* 75, 3–13
8. Brucker, W. & Drehmann, U. (1958) *Arch. Mikrobiol.* 30, 396–408
9. Brucker, W. (1957) *Planta* 48, 627–630
10. Dewick, P. M. & Haslam, E. (1968) *Chem. Commun.* 673–675
11. Dewick, P. M. & Haslam, E. (1969) *Biochem. J.* 113, 537–542
12. Marigo, G., Alberty, G. & Boulot, A. (1969) *C. R. Acad. Sci. Ser. D* 269, 1852–1854
13. Cornthwaite, D. C. & Haslam, E. (1965) *J. Chem. Soc.* 3008–3011
14. Conn, E. E. & Swain, T. (1961) *Chem. Ind.* 592–593
15. Haslam, E., Haworth, R. D. & Knowles, P. F. (1961) *J. Chem. Soc.* 1854–1859
16. Tateoka, T. N. (1968) *Bot. Mag.* 81, 103–104
17. Ishikura, N., Hayashida, S. & Tazaki, K. (1984) *Bot. Mag.* 97, 355–367
18. Sajio, R. (1983) *Agric. Biol. Chem.* 47, 455–460
19. Ishikura, N. (1975) *Experientia* 31, 1407–1408
20. Zaprjometov, M. N. & Bukhlaeva, V. Y. (1968) *Biohimiya* 33, 383–386
21. Eisenreich, W., Schwarzkopf, B. & Bacher, A. (1991) *J. Biol. Chem.* 266, 9622–9631
22. Strauß, G., Eisenreich, W., Bacher, A. & Fuchs, G. (1992) *Eur. J. Biochem.* 205, 853–866
23. Eisenreich, W., Menhard, B., Hylands, P. J., Zenk, M. H. & Bacher, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6431–6436
24. Eisenreich, W., Kupfer, E., Weber, W. & Bacher, A. (1997) *J. Biol. Chem.* 272, 867–874
25. Gilchrist, D. G. & Kosuge, T. (1980) in *The Biochemistry of Plants* (Miflin, B. J., ed) Vol. 5, pp. 507–531, Academic Press, New York