Stereochemistry of the Isoprenylation of Tryptophan Catalyzed by 4-(γ,γ-Dimethylallyl)tryptophan Synthase from *Claviceps*, the First Pathway-Specific Enzyme in Ergot Alkaloid Biosynthesis†

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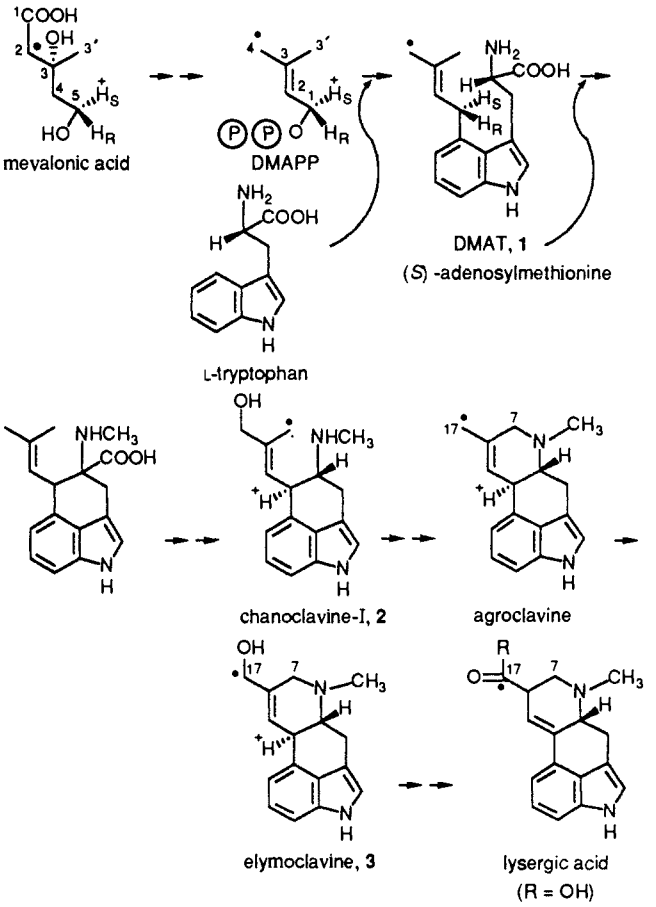
Abstract: The first pathway-specific reaction in ergot alkaloid biosynthesis, the isoprenylation of tryptophan catalyzed by 4-(γ,γ-dimethylallyl)tryptophan (DMAT) synthase, involves displacement of the allylic pyrophosphate moiety by C-4 of the indole ring with inversion of configuration at C-1 of dimethylallyl pyrophosphate (DMAPP). The geometry of the allylic double bond is retained, and no scrambling of labeled hydrogens between the two methyl groups is observed in the reaction. Occurrence of 8-10% scrambling of a ^{13}C label from C-2 of mevalonate between C-7 and C-17 of elymoclavine was confirmed, and it was shown (i) that this scrambling must take place in the formation of DMAPP from mevalonate and (ii) that it is unrelated to another partial scrambling of label, between the two hydrogens derived from C-5 of mevalonate, also observed in ergot alkaloid formation. The results are fully consistent with a mechanism for DMAT synthase involving direct attack of DMAPP on C-4 of the indole, possibly through a stabilized allylic carbocation or ion pair as intermediate.

The biosynthesis of ergot alkaloids, the derivatives of lysergic acid and their simpler progenitors, the clavines,[1] has been studied extensively in various species of the ergot fungus, *Claviceps*.[2] The four-membered ergoline ring system characteristic of these alkaloids is assembled from L-tryptophan, an isoprene unit in the form of γ,γ-dimethylallyl pyrophosphate (DMAPP) and a methyl group contributed by *S*-adenosylmethionine. The reaction sequence, as summarized in Scheme I, starts with the isoprenylation of tryptophan to 4-(γ,γ-dimethylallyl)tryptophan (DMAT, **1**),[2] which is then methylated at the amino nitrogen.[3,4] A further sequence of reactions which is not yet fully understood[2,5-9] then leads to the first tricyclic intermediate, chanoclavine-I. Cyclization of the latter via the aldehyde[10,11] gives agroclavine which is further oxidized to elymoclavine[2] and then to the lysergic acid oxidation level.[12,13] The reaction sequence involves two unique isomerizations at the allylic double bond of the isoprenoid moiety, the first between DMAT and chanoclavine-I and the second during the cyclization of chanoclavine-I to agroclavine.[14] As a consequence, an isotopic label placed at the (*E*)-methyl group of DMAT will appear in the (*Z*)-methyl group of chanoclavine-I and in the *E* position (C-17) in agroclavine, elymoclavine, and lysergic acid.[2,15]

The enzyme committing the two key substrates to this biosynthetic pathway, dimethylallyltryptophan synthase (dimethylallyl pyrophosphate–L-tryptophan dimethylallyltransferase), has been purified to homogeneity and crystallized, and some of its properties have been reported.[16,17] The enzyme is a dimer of molecular weight 70 000, and it displays both negative and positive cooperativity and is deregulated by Ca^{2+} ion. The substrate binding order is random sequential. The chemical mechanism of the reaction catalyzed by this enzyme has been the subject of considerable speculation, revolving around the fact that C-4 of an indole is not the most favored position for an electrophilic substitution. However, a number of feeding experiments and model studies have not produced supporting evidence for any of a number of proposals for activation of the 4-position or for migration of

Scheme I. Biosynthesis of Ergot Alkaloids[a]



[a] The symbols ● and + denote the predominant fate of isotopic labels from specific positions of mevalonic acid.

† This paper is dedicated to Professor Detlef Gröger, Halle, one of the pioneers of ergot alkaloid biosynthesis, on the occasion of his 60th birthday.

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(1) Hofmann, A. *Die Mutterkornalkaloide*; Enke Verlag: Stuttgart, 1964.

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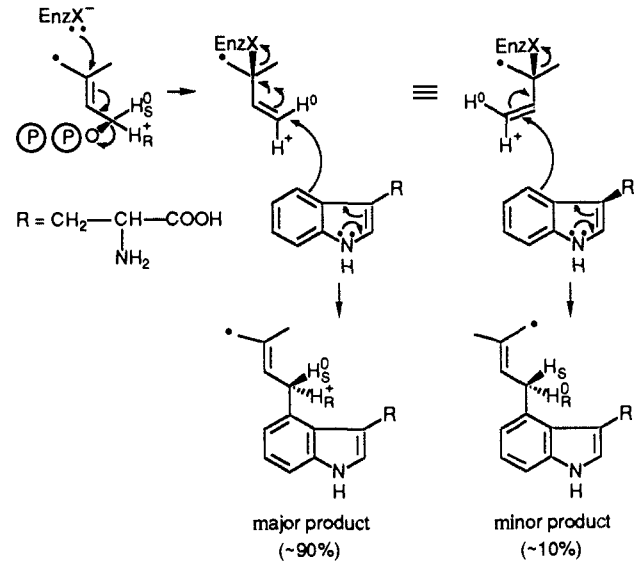
an isoprene residue from another position to C-4.[2] It is therefore concluded that the enzyme promotes a direct attack at the 4-position and prevents reaction at other, electronically more favored sites by proper alignment of the two substrates.[16]

Some additional information about the reaction catalyzed by DMAT synthase can be deduced indirectly from the results of feeding experiments on ergot alkaloid biosynthesis. When mevalonic acid carrying a ^{14}C label at C-2 was fed to ergot cultures and the resulting alkaloids were degraded to determine the ^{14}C distribution, it was found consistently by several investigators[2] that the tetracyclic alkaloids (agroclavine, elymoclavine, lysergic acid) contained the majority of the label (~90%) at C-17, but some ^{14}C (~7–10%) was located at C-7. The same scrambling of ^{14}C was seen in the chanoclavines, where the majority of the isotope resided in the C-methyl group, but 7–10% was located in the hydroxymethyl group. A similar partial scrambling of label between the two carbons derived from the two methyl groups of DMAPP was seen in a feeding experiment with [3′-$^2H_3$]mevalonic acid in which the alkaloids were analyzed by NMR spectroscopy,[10] further eliminating the possibility that the scrambling is an artefact of the chemical degradations used. However, when DMAT labeled with ^{14}C in one of the methyl groups was fed, within the limits of detection (1–2%) no scrambling was observed.[18,19] Since the reaction sequence from mevalonic acid to DMAPP in other organisms appears to be stereospecific,[20] these results seemed to suggest that the most likely source of the partial scrambling of label between the two isoprenoid methyl groups in ergot alkaloid biosynthesis is the DMAT synthase reaction. It has also been observed, both by Arigoni's[21] and our laboratory,[22] that the processing of the isoprenoid unit at the pyrophosphate-bearing carbon, arising from C-5 of mevalonic acid, may not be completely stereospecific; mevalonic acid tritiated stereospecifically at C-5 was incorporated into various clavines with retention of 5.5–13.5% of tritium from the *pro*-5*R* position and 79.5–88% from the *pro*-5*S* position. Again, the DMAT synthase reaction is a possible source of the partial scrambling of these two labels.

The following study was undertaken in order to shed further light on the mechanism of the DMAT synthase reaction, particularly to characterize it in stereochemical terms and to probe for the occurrence of isotope scrambling in the reaction.

Scheme II. A Hypothetical Mechanism of the DMAT Synthase Reaction, Which Would Explain Isotopic Scramblings Observed in Ergot Alkaloid Biosynthesis



Results

In the normal course of enzyme-catalyzed aromatic isoprenylation reactions and in the analogous chain elongation reactions catalyzed by various prenyltransferases, the allylic pyrophosphate group of a molecule of DMAPP or its C_{10} or C_{15} homolog is replaced by nucleophilic attack of a carbon of the second substrate with net inversion of configuration at the allylic carbon and without stereochemical change at the allylic double bond.[20,23] Although these reactions display the stereochemical attributes of S_N2 processes, they follow in all likelihood a different mechanism involving first dissociation of the carbon–pyrophosphate bond to a countercharge-stabilized carbocation or an ion pair, which is then approached by the second substrate on the face opposite the one from which the pyrophosphate has departed.[24] Such a mechanism could account for scrambling of label between the two methyl groups and the two allylic hydrogens by independent rotations around the C-1/C-2 and the C-2/C-3 axes in an intermediate dimethylallyl cation. Presumably, the barriers to rotations around the C-1/C-2 and C-2/C-3 bonds in such a cation could be modulated by properly positioned negatively charged groups in the enzyme active site. On the other hand, the similar magnitude of the two kinds of isotopic scrambling observed in ergoline biosynthesis, if it is not fortuitous, may suggest that both result from the same event. This could be a mechanism of the DMAT synthase reaction of the type illustrated in Scheme II, in which a 180° rotation around the C-2/C-3 axis is interspersed between two S_N2' steps. In such a mechanism the two isotopic scramblings would be interrelated, i.e., scrambling of the methyl label would be confined to molecules also experiencing scrambling of the allylic hydrogen label and vice versa. With the assumption that both S_N2' steps are suprafacial, this mechanism would predict that one product species arises with retention of double bond geometry and retention of configuration at the allylic carbon and the other with inversion of stereochemistry at both centers.

It is recognized that the mechanism in Scheme II predicts ping-pong kinetics, whereas the enzyme was reported to operate by a random Bi–Bi process.[16,17] Nevertheless, in light of the pecularities observed in the biosynthesis of ergot alkaloids we felt that the stereochemistry of the DMAT synthase reaction and the nature of the isotopic scrambling deserved closer examination. First of all we addressed the question whether the scrambling of a carbon label from C-2 of mevalonate between C-7 and C-17

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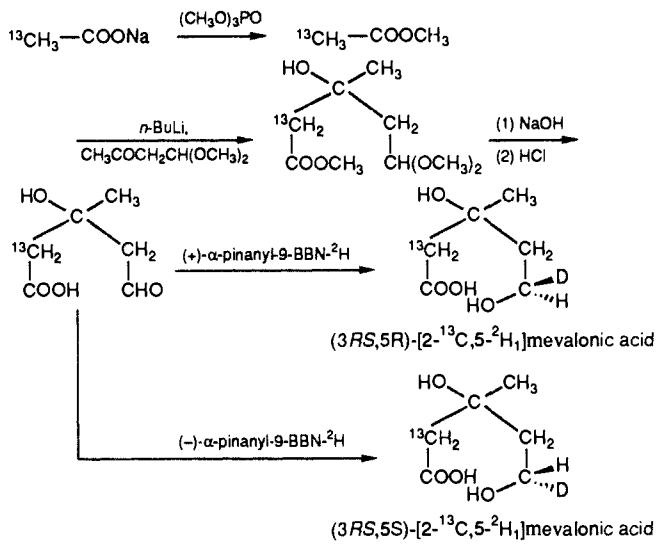
Scheme III. Synthesis of (3*RS*,5*R*)- and (3*RS*,5*S*)-[2-^{13}C,5-^{2}H$_1$]Mevalonic Acid



of ergolines and the scrambling of a hydrogen label from C-5 of mevalonate between the two allylic hydrogens are independent of each other or whether they are correlated to each other. To this end we synthesized two samples of mevalonate, each carrying ^{13}C (90 atom %) at C-2 and one atom of deuterium (89 atom %) at C-5, one with 5*R* and the other with 5*S* configuration. The synthesis is outlined in Scheme III; the stereospecific deuterium label was introduced by reduction of the C-5 aldehyde with deuterated[25] (+)- and (−)-pinanyl-9BBN.[26] Aliquots of these samples were then converted into elymoclavine (**3**) with resting cells of *Claviceps spec.*, strain SD58 essentially as described earlier.[10] To deplete the cells of preformed alkaloids and hence increase the isotope enrichment in the subsequently formed alkaloids, the washed cells were incubated in 67 mM phosphate buffer with shaking for 2 h, washed again, and then incubated in 67 mM phosphate buffer with labeled mevalonate and unlabeled L-tryptophan and L-methionine as previously described.[9,10] After 2 days the alkaloids were extracted, purified by acid-base partitioning, and analyzed by GC-MS.

On the basis of the previous results[2] one can predict slightly different isotope distributions in the samples of **3** derived from the two mevalonate samples depending on whether the scramblings of the two isotopic labels occur independent of each other or in a correlated fashion in the same step. These are shown in Scheme IV for the idealized case that the precursors are 100% enriched in ^{13}C and deuterium in the labeled positions and that each label is scrambled to the extent of 10%. Correlated scrambling will produce only two isotopomers of **3** from each precursor, whereas independent scrambling will result in four species each. These can be distinguished by mass spectrometry, looking at a diagnostic fragment ion at *m/z* 223 arising by loss of $-CH_2OH$ (C-17) from the molecular ion of **3**. Table I shows the observed isotopic compositions of this fragment ion and compares them to the calculated values for independent and correlated scrambling which are based on the enrichment of the precursor and the dilution of the precursor during incorporation determined from the fragment ion and its isotope satellites. It is evident that the observed values match the calculated ones for independent scrambling much more closely than those for correlated scrambling. This experiment thus suggests that the scrambling of ^{13}C from C-2 of mevalonate between C-7 and C-17 of **3** and that of ^{2}H from C-5 of mevalonate between the two allylic hydrogen positions of the isoprenoid unit occur independently of each other (although not necessarily in different reaction steps).

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Table I. Mass Spectral Analysis of the Isotopic Composition of **3** Biosynthesized from (5*S*)- and (5*R*)-[2-^{13}C,5-^{2}H$_1$]Mevalonate Based on the Fragment Ion F = M − CH_2OH at *m/z* 223

	relative intensity		
m/z	unlabeled **3**	**3** from (5*S*)-[2-^{13}C,5-^{2}H$_1$]-mevalonate	**3** from (5*R*)-[2-^{13}C,5-^{2}H$_1$]-mevalonate
223	100	100	100
224	18.6	76.3	23.4
225	2.0	17.1	3.5
226	0	3.4	1.1

isotopic species	obsd (%)	predicted for independent scrambling (%)	predicted for correlated scrambling (%)
	3 from (5*S*)-[2-^{13}C,5-^{2}H]Mevalonate		
F	61.7	61.7	61.7
F + 1	35.6	34.9	38.3
F + 2	2.6	3.4	0
	3 from (5*R*)-[2-^{13}C,5-^{2}H$_1$]Mevalonate		
F	94.9	94.9	94.9
F + 1	4.6	4.9	1.0
F + 2	0.5	0.2	4.1

We next determined the steric course of the isoprenylation reaction at C-1 of DMAPP, i.e., whether the pyrophosphate moiety is replaced by C-4 of tryptophan in a retention or inversion mode. Extensive attempts to solve this question by stereospecific deuterium labeling failed because various analysis schemes, although workable on a multimilligram scale, gave poor yields and/or ambiguous results when reduced to the μmolar scale dictated by the amount of DMAT synthase activity available. We therefore proceeded to synthesize (1*R*)- and (1*S*)-[1-^{3}H]DMAPP by stereospecific reduction of 3-methyl-2-butenal with tritiated (+)- and (−)-α-pinanyl-9-BBN followed by pyrophosphorylation of the alcohols by the method of Immel.[27] The requisite [^{3}H]9-BBN was synthesized from tritiated $NaBH_4$ by an adaptation[28] of the method of Brown and co-workers.[29] Since the α-pinene samples used had been purified to 100% ee[30] and since literature data[26] as well as our own control experiments[28] indicate that pinanyl-9-BBN reductions of simple unhindered aldehydes proceed with essentially complete stereospecificity, the two tritiated DMAPP samples can safely be assumed to have >98% ee 1*R* and 1*S* configuration, respectively.

The two stereospecifically tritiated DMAPP samples (1*R*, 10.2 μCi; 1*S*, 9.5 μCi) were converted into **1** with partially purified DMAT synthase and unlabeled L-tryptophan. The samples of **1** were extracted with 1-butanol and purified by reverse phase HPLC (yield: *R* 5.3 μCi, *S* 2.85 μCi). They were then degraded and analyzed for their configuration at the allylic–benzylic carbon, C-1″, as shown in Scheme V. Hydrogenation of **1** over Adam's catalyst gave 2″,3″-dihydro-DMAT which was oxidized with RuO_2 to 4-methylpentanoic acid in 35–55% overall yield on a 1-mg scale. Control experiments in which unlabeled **1** was hydrogenated under identical conditions with D_2 gas had shown incorporation of substantially more than two atoms of deuterium; however, careful analysis of the product by NMR, mass spectrometry, and degradation had also revealed insignificant (<7%) deuterium incorporation at C-1″. Hence, the stereochemical integrity of this center is not significantly jeopardized during the hydrogenation. Alternative methods for the reduction of the double bond (Wilkinson's catalyst, diimine) were tried but were ruled out by the fact that they resulted in very poor recoveries of dihydro-DMAT on a small reaction scale.

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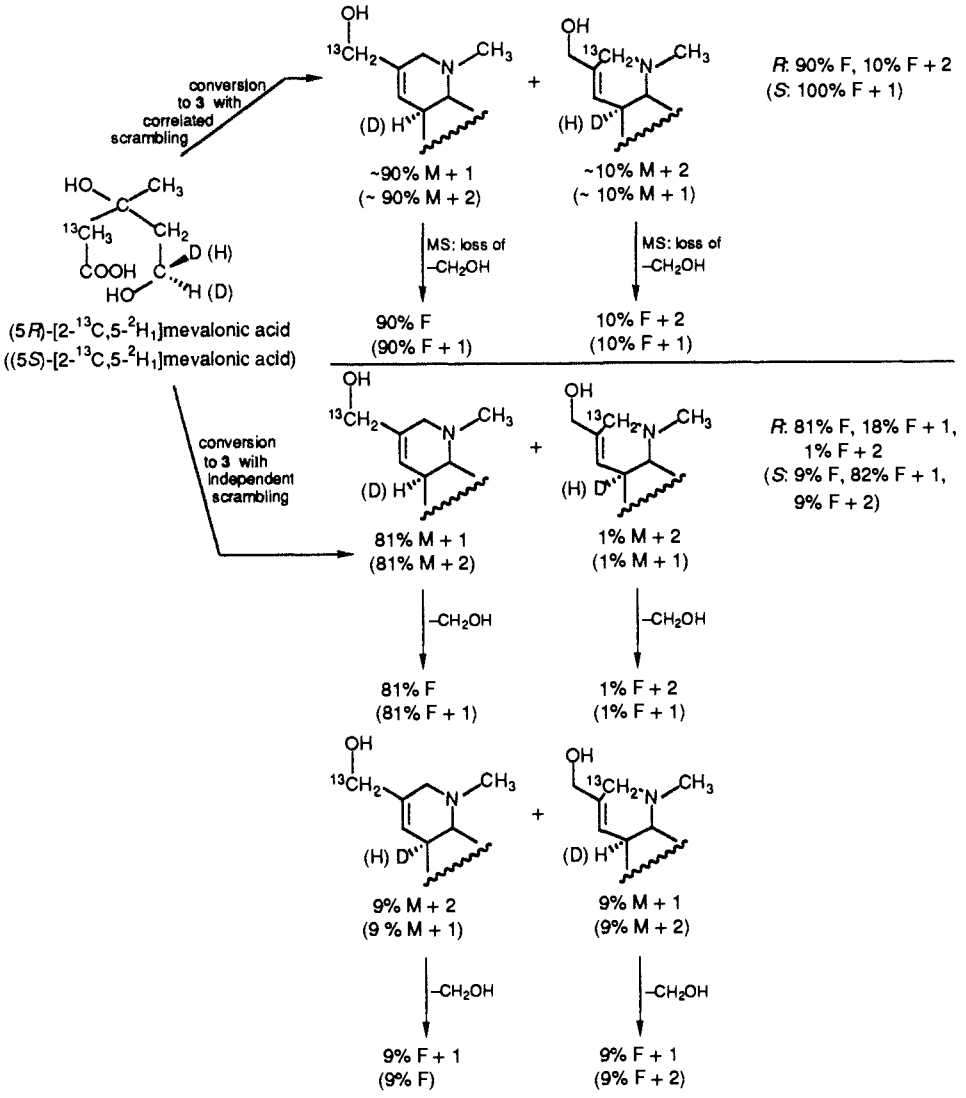
Scheme IV. Isotopomers of 3 Predicted To Arise from Correlated and Independent Scrambling of Isotopic Labels from (3*R*,5*R*)- and (3*R*,5*S*)-[2-^{13}C,5-^{2}H$_1$]Mevalonate during Ergot Alkaloid Biosynthesis[a]



[a] Assumptions: (i) each labeled position is 100% enriched and (ii) each label is scrambling to the extent of 10%.

The further degradation of the two samples of 4-methylpentanoic acid involved a Schmidt reaction which gave 3-methylbutylamine with retention of configuration.[31] This material was mixed with 3-[U-^{14}C]methylbutylamine, generated by thermal decarboxylation of L-[U-^{14}C]leucine in diphenylmethane. Aliquots were diluted with unlabeled carrier, derivatized to the 3,5-dinitrobenzamide and recrystallized to a constant ^{3}H/^{14}C ratio. The bulk of the amine samples was incubated with bovine plasma monoamine oxidase to give 3-methylbutanal, which was reduced in situ with LADH and NADH to the alcohol. The two samples of 3-methylbutanol too, were mixed with unlabeled carrier material, derivatized to the 3,5-dinitrobenzoyl ester and recrystallized to a constant ^{3}H/^{14}C ratio. The results, shown in Scheme V, indicate that the amine obtained by degradation of **1** generated from (1*S*)-[1-^{3}H]DMAPP retained 78.6% of its tritium in the monoamine oxidase reaction, whereas that produced ultimately from (1*R*)-[1-^{3}]DMAPP retained 19.4%.

Bovine plasma monoamine oxidase has been shown to remove stereospecifically the *pro-S* hydrogen from benzylamine[32] and *p*-hydroxybenzylamine[33] but seems to oxidize dopamine non-stereospecifically.[34] The enzyme from rat liver, however, oxidizes various amines with loss of the *pro-R* hydrogen.[35] In light of these earlier findings, we considered it prudent to verify the steric course of the bovine plasma monoamine oxidase reaction with the particular amine used in this work. We had available from earlier studies[36] samples of (*R*)- and (*S*)-4-methyl-[2-^{2}H$_1$]pentanoic acid (94 and 93% ee, respectively) which were subjected to the last two steps of the degradation sequence shown in Scheme V. Analysis of the resulting 3-methylbutyl 3,5-dinitrobenzoate by GC-MS for its deuterium content showed that the monoamine oxidase had removed preferentially the *pro-S* hydrogen. The analysis revealed significant scrambling, but a quantitative assessment was complicated by the possibility that isotope effects in the mass spectral fragmentation may have distorted the apparent isotopic compositions. In any case, this experiment confirmed qualitatively that bovine plasma monoamine oxidase exhibited the same stereochemistry toward 3-methylbutylamine as toward benzylamines as substrates.

The overall conclusion thus is that the isoprenylation of tryptophan involves displacement of the pyrophosphate moiety of

(31) Cf.: Eliel, E. L. *Stereochemistry of Carbon Compounds*; McGraw-Hill: New York, NY, 1962; p 119. A control experiment revealed only 5% scrambling in this step.
(32) Battersby, A. R.; Staunton, J.; Klinman, J.; Summers, M. C. *FEBS Lett.* **1979**, *99*, 297.
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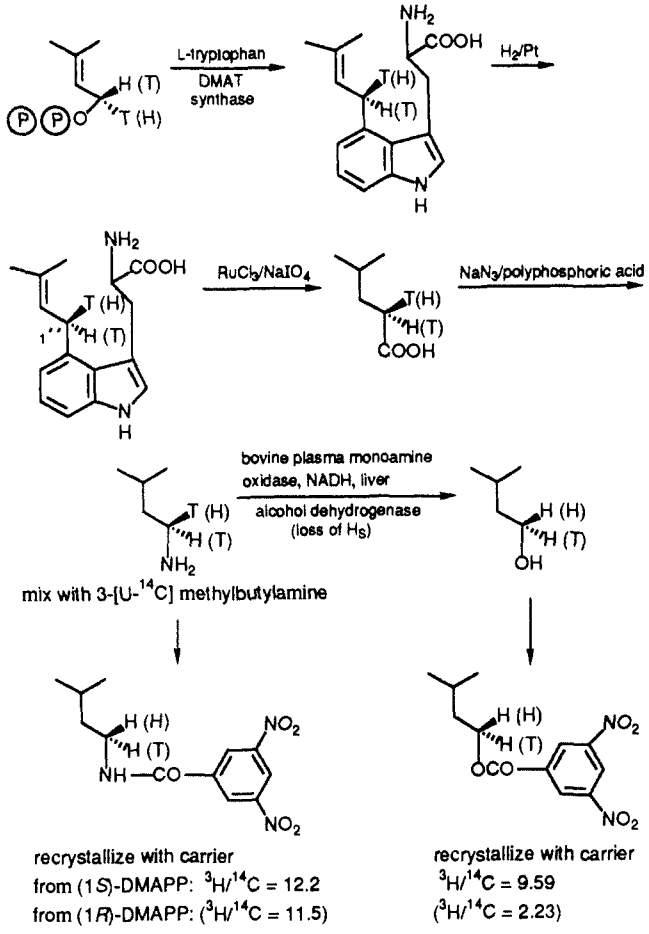
(36) Otsuka, H.; Floss, H. G. *Z. Naturforsch.* **1987**, *42C*, 449.

Scheme V. Degradation and Stereochemical Analysis of DMAT Formed Enzymatically from (1*R*)- and (1*S*)-[1-^{3}H]DMAPP

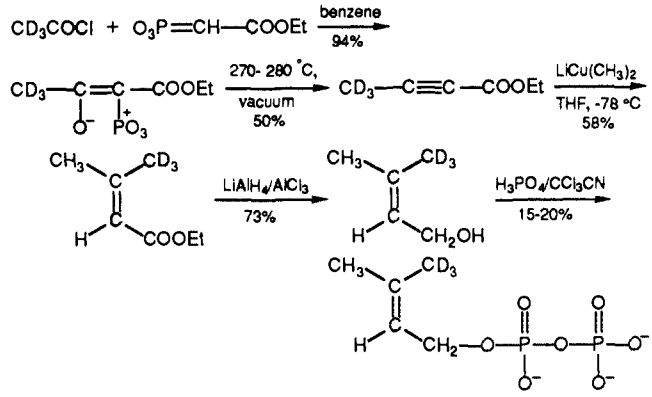


DMAPP by the indole residue in an inversion mode. The partial scrambling (~20%) of the isotopic label observed in the analysis of the tritiated samples could in principle reflect the mode of action of DMAT synthase. However, it is actually somewhat larger than the scrambling of the allylic hydrogens observed in vivo and may well be in part or entirely an artefact of the degradation. The Schmidt reaction is accompanied by a small degree of racemization, and the monoamine oxidase reaction may not be completely stereospecific either.

Indirect evidence from in vivo feeding experiments suggests that the configuration of the allylic double bond of DMAT should be the same as that of the DMAPP from which it is derived.[2] In view of the occurrence of multiple isomerizations at the isoprenoid double bond in ergot alkaloid biosynthesis it was important to confirm that the geometry at this double bond is retained in the DMAT synthase reaction. To this end we synthesized a sample of DMAPP deuterated stereospecifically in the *Z* methyl group. Enzymatic conversion of this material to DMAT would also reveal any scrambling of deuterium between the two methyl groups in the DMAT synthase reaction.

(*Z*)-3-Methyl-2-[4-$^2H_3$]butenyl pyrophosphate (**2**) was synthesized from [$^2H_3$]acetyl chloride via ethyl (*Z*)-3-methyl-2-[4-$^2H_3$]butenoate as shown in Scheme VI. The double bond geometry was established by lithium dimethylcuprate addition to the deuterated ethyl butynoate,[37,38] which at low temperature proceeds cleanly in a syn fashion.[39] Reduction of the ester with AlH_3 instead of $LiAlH_4$ avoided the otherwise co-occurring reduction of the conjugated C=C double bond. Pyrophosphorylation of the alcohol as described by Cornforth and

Scheme VI. Synthesis of (*Z*)-[*methyl*-$^2H_3$]DMAPP



Popjak[40] followed by column chromatographic purification gave DMAPP in 15–20% yield based on the alcohol. The product was free of mono- and polyphosphates, as demonstrated by chromatography and elemental analysis, and contained 3.2% of the *E* isomer, as revealed by proton NMR.

Incubation of this material with L-tryptophan and partially purified DMAT synthase gave **1** which was purified by reverse phase HPLC and subjected to proton NMR analysis. Unlabeled **1** shows two methyl signals at δ 1.748 and 1.764 ppm. Assignment of the upfield signal (1.748 ppm) to the (*E*)-methyl group rests firmly on the analysis of an authentic sample of (*E*)-[*methyl*-$^{13}C_1$]DMAT.[41] The NMR spectrum of the sample of deuterated **1** generated from (*Z*)-[*methyl*-$^2H_3$]DMAPP displayed the two methyl signals in an intensity ratio of 97.2% (1.748 ppm):2.8% (1.764 ppm), indicating that the *Z* methyl group of DMAPP gives rise to the (*Z*)-methyl group of DMAT. Hence the geometry of the allylic double bond is indeed retained in the DMAT synthase reaction. The excellent quantitative agreement of the isomer ratio in precursor and product also demonstrates that no scrambling of label between the two methyl groups takes place during the reaction; within the limits of detection DMAT synthase operates completely stereospecifically with respect to the allylic double bond of the isoprenoid unit.

The latter finding caused us to reexamine the premise that in the overall formation of ergot alkaloids some scrambling of label from C-2 of mevalonate between C-7 and C-17 of the alkaloids occurs. To this end, D,L-[2-^{13}C]mevalonate at different concentrations was converted in resting cells of *Claviceps spec.*, SD58 with unlabeled L-tryptophan and L-methionine into **3**. Mass spectral analysis of the samples of **3**, ranging in ^{13}C-enrichment from 60 to 99%, revealed that they contained from 8.0 to 10.4% of their ^{13}C in the M–CH_2OH fragment, presumably at C-7, and the remainder at C-17. Thus, the occurrence of this scrambling is confirmed by yet another independent methodology.

To verify the finding of Pachlatko and Arigoni[18,19] that no scrambling occurs after the stage of DMAT a sample (~1 mg) of (*Z*)-[*methyl*-$^2H_3$]-**1** (97.2% *Z*, 2.8% *E*) generated from (*Z*)-[*methyl*-$^2H_3$]DMAPP with DMAT synthase was likewise converted into **2** and **3** with resting cells of *Claviceps spec.*, SD58. Not unexpectedly, **3** was useless for the mass spectral analysis, because the most prominent fragmentation is loss of a hydrogen from the partially deuterated C-7 of the molecular ion. An isotope effect in this process distorts the isotopic composition of the relevant ions. However, chanoclavine-I (**2**) gave meaningful and reproducible data. Inspection of Scheme I shows that a *Z* trideuterio methyl group in **1** will predominantly produce a dideuterio hydroxymethyl group in **2**. If the next step, stereospecific dehydrogenation of chanoclavine-I aldehyde is partially reversible, as seems to be the case,[10] some of the dideuterio species will be further converted into monodeuterated molecules. Scrambling

(37) Tau, S. J. Ph.D. Dissertation, University of Georgia, 1973.
(38) We thank Prof. R. K. Hill, University of Georgia, for providing us with the experimental details of this procedure prior to publication.
(39) Posner, G. H. *Org. React.* **1972**, *19*, 45.
(40) Cornforth, R. H.; Popjak, G. *Methods Enzymol.* **1969**, *15*, 359.
(41) We thank Prof. D. Gröger, Halle, for kindly providing this sample which had originally been synthesized by Prof. H. Plieninger, Heidelberg, and his co-workers.[42]

of label between the two methyl groups of DMAT or the carbons derived from them, on the other hand, will generate **2** carrying a trideuterio methyl group. Thus, the percentage of (M + 1) + (M + 2) and (M + 3) species in **2** is a measure of the degree of scrambling. The labeled molecules in the sample of **2**, formed with 97% specific incorporation from **1** (i.e., containing only 3% unlabeled molecules), consisted of 95.9% (M + 1) + (M + 2) species (16.9% M + 1 and 74.4% M + 2) and 4.1% M + 3 species. This ratio is very close to the *Z* vs *E* isomer distribution of the precursor (97.2% vs 2.8%) indicating that not more than 1.3% scrambling has taken place between **1** and **2**.

The original conclusion of Arigoni and co-workers[18,19] that the scrambling must occur prior to or during the formation of **1** is thus confirmed. Since the preceding experiments have shown that it does not occur during the formation of **1** from DMAPP, we have to conclude that the conversion of mevalonic acid into DMAPP in *Claviceps* is not completely stereospecific. An attempt was made to prove this assertion directly by incubating resting cells with [2-^{13}C]mevalonic acid and unlabeled L-tryptophan, but the amount of **1** obtained was too small for NMR analysis.

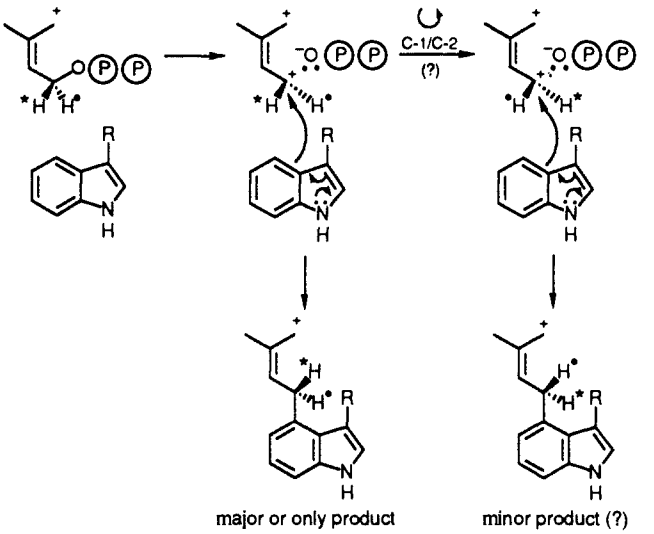
Discussion

The experimental results presented here reveal that the steric course of the isoprenylation at C-4 of L-tryptophan in the biosynthesis of ergot alkaloids follows a "classical" pattern. As in other aromatic isoprenylations[23] and in the chain-elongating prenyl transfers,[20] the allylic pyrophosphate is displaced by the incoming nucleophilic carbon in an inversion mode. In addition, the geometry of the allylic double bond is preserved in the process. This finding does not constitute evidence for an S_N2 mechanism; in fact, in analogy to the chain-elongating prenyltransferases,[24] the enzyme may well operate by a mechanism involving a stabilized carbocation or an ion pair as intermediate. The data do, however, argue against the mechanism shown in Scheme II, or variants thereof, unless one makes the assumption that the two S_N2' steps proceed with opposite stereochemistry, i.e., one syn and one anti.

The results also indicate that the isoprenylation reaction proceeds without any detectible (<1%) scrambling of label between the two methyl groups of the isoprenoid moiety. The data are inconclusive as to whether the reaction is accompanied by any scrambling of label between the two allylic hydrogens; the final analysis showed an 80:20 distribution of tritium label between the two methylene hydrogens, but this might very well be an artefact of the degradation or of the enzymatic analysis rather than the result of the DMAT synthase reaction itself. The in vivo experiments with (5*R*)- and (5*S*)-[2-^{13}C,5-^{2}H$_1$]mevalonate also indicate that the two types of isotopic scrambling mentioned above, which have been observed in the overall conversion of mevalonate into **2** and **3** are not correlated to each other. It was the hypothesis that both scramblings occur in a correlated fashion in the DMAT synthase reaction which led us to consider the mechanism shown in Scheme II. In light of the results obtained here there is no longer any need to invoke such a mechanism.

The mechanism of the DMAT synthase reaction may reasonably be portrayed as shown in Scheme VII. In the absence of any evidence supporting proposed[2] alternative modes of operation, a direct electrophilic attack at C-4 of the indole seems most plausible. The regiochemistry is probably dictated by the geometry of the enzyme. Dissociation of the C-1/OPP bond of DMAPP in the ternary complex would result in formation of an ion pair or an allylic carbocation stabilized by the countercharge of the still enzyme-bound pyrophosphate. This species would be approached by C-4 of the enzyme-bound tryptophan on the face opposite the pyrophosphate to produce **1** with inversion at C-1 and retention of double bond geometry. If the process does involve some scrambling between the allylic hydrogens, this can be readily accounted for by rotation around the C-1/C-2 bond of the allylic carbocation in some molecules prior to bond formation with the indole. Such a process could be aided by pronounced localization of the positive charge at C-1 by interaction with the pyrophosphate counterion, lowering the barrier to rotation around the C-1/C-2 bond.

Scheme VII. Observed Steric Course and Proposed Mechanism of the DMAT Synthase Reaction



Our data clearly confirm the previously observed[2] partial scrambling of isotope from C-2 of mevalonate between C-7 and C-17 of ergot alkaloids; they also confirm Arigoni's finding that this scrambling does not occur between DMAT and the alkaloids. Together with our results on DMAT synthase this shows that the scrambling, surprisingly, must occur in the formation of DMAPP from mevalonic acid. Of the four enzymes involved in this sequence, the first three seem unlikely to cause such an event, leaving the fourth, isopentenyl pyrophosphate (IPP) isomerase, as the most likely candidate.

Experimental Section

General Methods and Materials. NMR spectra were recorded on Bruker WM-300, AM-500, and IBM AF-300 instruments. GC-mass spectral analyses were carried out on a Hewlett-Packard 5970A gas chromatograph-mass spectrometer as previously described.[7] Radioactivity was measured by liquid scintillation counting in a Beckman LS7500 liquid scintillation counter using internal standards of [^{3}H]- or [^{14}C]-toluene to determine counting efficiencies.

Biochemicals were obtained from Sigma and chemicals from Aldrich; they were of reagent grade and were used without further purification. Stable isotope-labeled compounds were obtained from MSD Isotopes and sodium [^{3}H]borohydride and L-[U-^{14}C]leucine from Amersham.

Feeding Experiments. *Claviceps spec.* SD58[44] was grown for 5 days in shake culture (300 rpm) at 25 °C in 500-mL Erlenmeyer flasks containing 100 mL of medium NL 406.[45] The cultures were then filtered aseptically, and the mycelia were washed with sterile water and resuspended in 100 mL of 67 mM phosphate buffer, pH 7.3, twice and filtered. They were then suspended again in 67 mM phosphate buffer, pH 7.3, and incubated with shaking for 2 h. The mycelia were filtered, washed, and divided in half, and each half was suspended in 50 mL of 67 mM phosphate buffer, pH 7.3, containing the substrates. Typically, these were 5 mg of L-tryptophan, 5 mg of L-methionine, and 10 mg of labeled D,L-mevalonic acid; in one experiment the substrate was 1 mg of (*Z*)-[*methyl*-^{2}H$_3$]-**1**. In each case a control experiment was conducted alongside the experiments with the labeled precursors to be evaluated, in which the labeled substrate was L-[*methyl*-^{13}C,^{2}H$_3$]methionine. After 2 days of incubation with shaking, the alkaloids were extracted from the basified culture filtrate with 3 portions of isopropyl alcohol/$CHCl_3$ 1:3, the combined extracts were evaporated, and the residue was dissolved in 15 mL of 2% succinic acid solution. Following three extractions with 5 mL each of $CHCl_3$ the aqueous solution was made basic with NH_4OH, and the alkaloids were extracted with 3 × 5 mL of chloroform. The combined chloroform extract was dried over Na_2SO_4 and evaporated, and the residue was dissolved in a small volume of methanol and subjected to GC-MS analysis as previously described.[7]

Calculation of Observed and Predicted Isotopic Composition of 3 from (5*R*)- and (5*S*)-[2-^{13}C,5-^{2}H$_1$]Mevalonate. The observed isotope distri-

(42) Plieninger, H.; Mayer, E.; Nassirian, S. F.; Weidmann, E. *Liebigs Ann. Chem.* **1976**, 1475.
(43) Hassam, S. B.; Floss, H. G. *J. Nat. Prod.* **1981**, *44*, 756.
(44) Gröger, D. *Arch. Pharm. (Weinheim)* **1959**, *292*, 389.
(45) Floss, H. G.; Gröger, D. *Z. Naturforsch.* **1963**, *18B*, 519.

bution in the molecular ion and the M–CH_2OH fragment ion of **3** was calculated as described by Biemann.[46] Predicted values for the percentages of unlabeled, single-labeled, and double-labeled species comprising the M–CH_2OH fragment ion F are based on the relationship $x + y = 1$, where x = fraction of **3** derived from endogenous unlabeled precursor and y = fraction of **3** from added labeled precursor. The contribution of y to the fragment ion F and its isotope satellites predicted for independent and correlated scrambling follows from the abundance of the different isotopomers of **3** assuming 10% scrambling each of the ^{13}C and the deuterium label, corrected for 90% ^{13}C enrichment and 89% deuterium enrichment in the labeled positions. Applying the predicted ratios of F:(F + 1):(F + 2) in y and the relationship $x + y = 1$ to the normalized observed value for species of m/z 223 (F = M–CH_2OH) then allows calculation of the expected values for F + 1 and F + 2 for correlated and independent scrambling.

Enzymatic Synthesis of 1. The mycelia from 22 14-day-old stationary cultures of *Claviceps spec.*, strain SD58 grown in 500-mL Roux bottles containing 75 mL of medium NL406 were harvested by filtration and washed with water. They were briefly homogenized in a blender with 400 mL of 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM diethyldithiocarbamate, 20 mM 2-mercaptoethanol, 20 mM thioglycolate, 20 mM $CaCl_2$, and 10% glycerol. The suspension was passed once through a French press, the homogenate was centrifuged for 30 min at 10000×g, and the supernatant was filtered through gauze. The solution was then subjected to ammonium sulfate precipitation, and the 30–45% $(NH_4)_2SO_4$ precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM 2-mercaptoethanol and 10% glycerol. This solution was dialyzed for 30 min against 1 L and then overnight against 4 L of the same buffer. The dialyzed solution was applied to a 3.5 cm × 21 cm column of DEAE cellulose (DEAE-SEPHACEL Sigma) equilibrated with the same buffer. The column was then eluted with a linear gradient (500 mL each) of 0–1 M KCl, collecting fractions of 12–13 mL. DMAT synthase appeared immediately following the main protein peak in fractions 34–36; it was concentrated by ammonium sulfate precipitation (50%) and redissolved in about 2.5 mL of buffer: specific activity[16] 2–4 mU/mg protein.

For the conversion of tritiated DMAPP into **1**, 10.2 μCi and 9.5 μCi, respectively, of (1*R*)- and (1*S*)-[1-^{3}H]DMAPP were each distributed into five reaction mixtures, each containing 440 μL of tritiated DMAPP solution, 1360 μL of DMAT synthase, 600 μL of 5 mM L-tryptophan, and 800 μL of 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$ and 20 mM mercaptoethanol. After incubation for 2 h at 30 °C, **1** was recovered by repeated extraction with 1-butanol and purified by preparative HPLC on a 10 mm × 30 cm Alltech C18 (10 μ) column with $CH_3CN/MeOH/H_2O$ 1:65:35 as solvent at a flow rate of 4 mL/min. **1** was recovered at T_{Ret} 10 min in 52 and 30% radiochemical yield, respectively. UV (EtOH) λ, nm (ε) 296 (3.72), 284 (3.84) sh, 278 (3.89). Similarly, incubation of 9 mg of (*Z*)-[*methyl*-$^2H_3$]DMAPP with 4 mg of L-tryptophan, 2.5 mL of DMAT synthase, 10 mM Tris-HCl, pH 8.0, 17 mM $CaCl_2$, and 20 mM mercaptoethanol in a total volume of 6.9 mL for 5.5 h and workup as above gave about 1 mg of DMAT. ^1H NMR (CD_3OD) δ, ppm (rel signal intensity) 1.748 (97.2), 1.764 (2.8).

Degradation and Configurational Analysis of Chirally Tritiated 1. PtO_2 (1.4 mg) in ethanol (2 mL) was stirred at room temperature for 1 h under H_2 gas. [^{3}H]-**1** (0.32 μCi, from (*R*)-DMAPP) and 1 mg of unlabeled **1** in 3 mL of ethanol was added, and the hydrogenation was continued for 1.5 h. Filtration and evaporation of the filtrate gave 0.30 μCi of dihydro-DMAT. Analogously, 0.47 μCi of [^{3}H]-**1** from (*S*)-DMAPP produced 0.41 μCi of dihydro-DMAT. Unlabeled dihydro-DMAT: ^1H NMR δ ppm (multiplicity; J, Hz; assignment) 7.17 (d, 2.5, H-2), 7.15 (d, 8.1, H-7), 6.94 (dd, 8.1, 7.0, H-6), 6.72 (d, 7.0, H-5), 3.55 (dd, 15.5, 2.9, H-2′), 3.38 (dd, 10.5, 2.9, H-3′), 3.00 (ddd, 13.7, 10.2, 6.0, H-1″), 2.94 (dd, 10.5, 15.7, H-3′), 2.87 (ddd, 13.7, 10.0, 6.3, H-1″), 1.64–1.75 (m, H-3″), 1.40–1.58 (m, H-2″), 0.95 (d, 6.5, methyl), 0.93 (d, 6.5, methyl). Hydrogenation of unlabeled DMAT with D_2 gas under the same conditions as described above gave dihydro-DMAT which, as shown by ^1H NMR and confirmed by MS and degradation, contained deuterium as follows: H-2, 0.95 atom; H-7, 0.30 atom; H-1″, <0.07 atom; H-2″, 1.00 atom; H-3″, 1.00 atom.

Tritiated dihydro-DMAT (0.3 μCi, from (*R*)-DMAPP) dissolved in 3 mL of water was stirred with 100 mg of $NaIO_4$ and 0.8 mg of $RuCl_3 \cdot 3H_2O$ overnight at room temperature. The solution was then filtered through a cotton plug followed by acidification with 2–3 drops of diluted H_2SO_4. The aqueous solution was subjected to bulb-to-bulb lyophilization, the distillate was adjusted to pH 11 with diluted NaOH, and water was removed by lyophilization to give tritiated sodium 4-methylpentanoate (0.13 μCi, 43.6%). Similarly, tritiated dihydro-DMAT (0.41 μCi) from (*S*)-DMAPP gave 0.26 μCi (64.8%) of tritiated sodium 4-methylpentanoate.

Freshly prepared polyphosphoric acid (by stirring 1 g of P_2O_5 and 1 mL of 85% H_3PO_4 overnight) was added to the flask containing 0.13 μCi of tritiated sodium 4-methylpentanoate (from (*R*)-DMAPP), followed by 16.7 mg of NaN_3. The solution was stirred at 50 °C for 67.5 h, and 20 mL of water was added followed by 30% NaOH to an alkaline pH (phenolphthalein indicator). The solution was subjected to bulb-to-bulb lyophilization, the distillate was adjusted to pH 3 with diluted HCl, and water was removed by lyophilization to give tritiated 3-methylbutylamine·HCl (0.096 μCi, 74.2%). Similarly, the material generated from (*S*)-DMAPP gave tritiated 3-methylbutylamine·HCl (0.15 μCi) in 58.2% yield.

3-[U-^{14}C]Methylbutylamine·HCl (21 750 dpm, 70 μL of solution in H_2O) (see below) was mixed with the solution of tritiated 3-methylbutylamine·HCl (0.096 μCi in 0.99 μL of H_2O, from (*R*)-DMAPP). An aliquot of the mixture (200 μL, 19%) was added to a flask containing 11.5 μL of unlabeled 3-methylbutylamine in 20 mL of benzene. After the addition of 260 μL of dimethylaniline and 460 mg of 3,5-dinitrobenzoyl chloride the reaction mixture was stirred for 3 days at room temperature and acidified with diluted HCl, and the product was extracted with ethyl acetate. The extract was washed successively with diluted HCl (2×), saturated $NaHCO_3$ solution (3×), and saturated NaCl solution (once), dried over Na_2SO_4, and evaporated to dryness. The vacuum-dried residue was chromatographed on 15 g of silica gel with benzene, followed by benzene/acetone 99:1 as solvent. The product was crystallized twice from benzene to give 19.5 mg of 3-methylbutyl 3,5-dinitrobenzamide. The $^3H/^{14}C$ ratio was measured after each crystallization: first crystallization, $^3H/^{14}C$ = 11.4; second crystallization, $^3H/^{14}C$ = 11.6. Similarly, 3-[U-^{14}C]methylbutylamine·HCl (30 000 dpm) was added to the tritiated 3-methylbutylamine (0.15 μCi) derived from (*S*)-DMAPP, and an aliquot of the mixture was diluted and derivatized as described above: first crystallization, $^3H/^{14}C$ = 12.1; second crystallization, $^3H/^{14}C$ = 12.2.

About half of the remainder of the 3-[U-^{14}C,2-^{3}H]methylbutylamine·HCl from (*R*)-DMAPP was incubated with 7.5 mg of NADH, 1.0 U of liver alcohol dehydrogenase (LADH), 0.27 U of bovine plasma monoamine oxidase (MAO) (300 μL), and 800 μL of 0.2 M potassium phosphate buffer, pH 7.8, at 25 °C for a total of 93 h. Additional portions of NADH (7.5 mg), LADH (1.0 U), and MAO (0.22 U), were added after 24 and 48 h. Unlabeled 3-methylbutanol (21.9 μL) was then added, followed by 20 mL of benzene, 260 μL of dimethylaniline, and 460 mg of 3,5-dinitrobenzoyl chloride, and the mixture was stirred at room temperature for 3 days. Following the addition of water the product was extracted with ethyl acetate, the extract was washed successively with diluted HCl (3×), saturated $NaHCO_3$ (3×), and saturated NaCl (1×), dried over Na_2SO_4, and evaporated to dryness. The residue was filtered through a small column (1 × 5 cm) of silica gel with benzene as solvent, the filtrate and washings were evaporated to dryness, and the residue was chromatographed on 12 g of silica gel with benzene/*n*-hexane 1:1 as solvent. The product was recrystallized twice from *n*-hexane to give 20 mg of 3-methylbutyl 3,5-dinitrobenzoate: first recrystallization, $^3H/^{14}C$ = 2.21; second recrystallization, $^3H/^{14}C$ = 2.25. Analogously the 3-[U-^{14}C,2-^{3}H]methylbutylamine·HCl derived from (*S*)-DMAPP gave 11.0 mg of 3-methylbutyl 3,5-dinitrobenzoate: first recrystallization, $^3H/^{14}C$ = 9.64; second recrystallization, $^3H/^{14}C$ = 9.53.

Steric Course of Oxidation of 3-Methylbutylamine with Bovine Plasma Monoamine Oxidase. (*R*)- and (*S*)-4-[2-$^2H_1$]methylpentanoic acid[36] (21.2 μL) was subjected to Schmidt degradation and workup as described above for the tritiated material to give 13.6 (64.8%) and 12.5 mg (58.8%) of (*R*)- and (*S*)-3-[1-$^2H_1$]methylbutylamine·HCl, respectively. ^1H NMR (D_2O) 2.89 (tt, 1 H), 1.55 (m, 1 H), 1.42 (m, 2 H), 0.80 (d, 6 H). Each of these samples was then subjected to the combined action of MAO and LADH, and the resulting samples of 3-methylbutanol were converted to the 3,5-dinitrobenzoates without addition of unlabeled carrier. The esters were analyzed by GC-MS, examining two fragment ions at m/z 239 and m/z 70: product from (*S*)-3-[1-$^2H_1$]methylbutylamine, 66% unlabeled and 34% monodeuterated species; product from *R* isomer, 32% unlabeled and 68% monodeuterated species.

Synthesis of 3-[U-^{14}C]Methylbutylamine. L-[U-^{14}C]Leucine (7.75 μCi, 7.6 μmol) was suspended in 5 mL of diphenylmethane and heated under nitrogen to 240–250 °C for 20 min. After cooling off, 5 mL of ethyl acetate was added to the mixture, and the solution was washed twice with 1% aqueous NaOH. 3-Methylbutylamine was then extracted with diluted HCl and obtained as the hydrochloride after evaporation of the aqueous solution: yield 0.70 μCi (10.8%), radiochemical purity 80% (based on dilution with carrier and derivatization to the 3,5-dinitrobenzamide).

Synthesis of (3*RS*,5*R*)- and (3*RS*,5*S*)-[2-^{13}C,5-$^2H_1$]Mevalonate. Anhydrous sodium [1-^{13}C]acetate (90% ^{13}C) was converted into methyl

(46) Biemann, K. *Mass Spectrometry, Organic Chemical Applications*; McGraw-Hill: New York, NY, 1962; pp 224–227.

[2-^{13}C]acetate, bp 57 °C, in 96% yield as described by Ropp.[47] Further conversion into methyl 3-hydroxy-3-methyl-5,5-dimethoxy-[2-^{13}C]pentanoate (bp 90 °C/0.1 Torr) in 80% yield followed the procedure of Lawson et al.[48] Aliquots of this material (0.2 g) were stirred with 1 mL of 1 N NaOH at room temperature for 45 min, cooled to 0 °C, and adjusted to pH 1 with 6 N HCl. The mixture was allowed to warm to room temperature, stirred for 15 min, and cooled again to 0 °C. The pH was adjusted to 7.5 with saturated $NaHCO_3$ solution, and the mixture was lyophilized. The solid residue was triturated with 2 mL of absolute ethanol, and the solution was evaporated to dryness. The residue was then taken up in 1 mL of absolute ethanol and added dropwise from a syringe to a solution of deuterated (+)- or (−)-pinanyl-9-BBN at 0 °C under nitrogen. The reagent was prepared by gently refluxing 1.5 mmol of deuterated 9-BBN[25] (89% ^{2}H) in 2.7 mL of THF and 0.26 mL of (+)- or (−)-α-pinene (100% ee)[30] with stirring for 2 h. The reduction reaction mixture was stirred overnight, and then 40 μL of acetaldehyde was added. The THF and α-pinene were removed by evaporation in a vacuum with gentle warming of the reaction flask, nitrogen was then admitted, the flask was cooled to 0 °C, and 3 mL of 1 N HCl was added. The solution was shaken with 10 mL of pentane, and the pentane solution was discarded. The water layer was evaporated to dryness, keeping the bath temperature below 45 °C, and the residue was triturated with acetone. The acetone solution was dried with $MgSO_4$ and evaporated. The residue was taken up in ether, boiled with charcoal for 5 min, and then subjected first to flash chromatography on silica gel (hexane/ethyl acetate 3:7) and then to HPLC on Porasil (10 μ particle size, column 1.2 mm × 30 cm, hexane/ethyl acetate 4:6). The resulting mevalonolactone was converted to the dibenzylethylenediamine salt of mevalonic acid as described earlier.[10] Overall yield: 10%, mp 115 °C.

Synthesis of (1*R*)- and (1*S*)-[1-^{3}H]Dimethylallyl Pyrophosphate. Tritiated 9-BBN (0.122 mmol), prepared in about 85% yield from 0.5 mCi of NaB[^{3}H]H_4 by an adaptation[28] of the procedure of Brown and co-workers,[29] in 0.5 mL of THF, 1 mL of 0.5 M unlabeled 9-BBN in THF, and 2.5 mL of dry THF was refluxed for 2 h with 160 μL (1.02 mmol) of (+)- or (−)-α-pinene (100% ee).[30] After cooling to room temperature, 84 mg (1 mmol) of 3-methyl-2-butenal, prepared by MnO_2 oxidation of 3-methyl-2-buten-1-ol, was added, and the reaction mixture was stirred for 12 h. THF and α-pinene were then removed by evaporation at 50 °C for 4 h, 2 mL of ether and 65 μL (1 mmol) of 2-aminoethanol were added to the residue, and the mixture was cooled to 0 °C. The resulting precipitate was removed by filtration through Celite, and the filtrate was washed twice with 2 mL of saturated NaCl solution. The ether solution was dried over $MgSO_4$, and the solvent was evaporated carefully at low temperature. The residue, 1.64 g of H_3PO_3 (dried over P_2O_5 in a vacuum), and 10 mL of triethylamine were dissolved in 40 mL of dry acetonitrile. Iodine (7.6 g) was added in portions to the vigorously stirred solution over a period of 1.5 h. Thirty minutes after the last addition the dark solution was poured into a mixture of 20 mL of cyclohexylamine and 600 mL of acetone and kept at −20 °C overnight. The precipitate was collected by filtration, dissolved in 20 mL of *n*-propanol/NH_4OH/H_2O 6:4:1, and applied to a column (4 × 100 cm) of activated silica gel (washed with 6 N HCl, water, dried at 150 °C overnight), which was developed with the same solvent. The elution was followed by TLC, and the fractions containing DMAPP were combined and evaporated. Final purification by paper chromatography[16] gave 23.8 μCi of (1*S*)-[1-^{3}H]DMAPP (from (−)-pinene) and 15.5 μCi of the *R* isomer (from (+)-pinene). The samples were stored in 5% NH_4OH solution at 4 °C.

Synthesis of (*Z*)-[*methyl*-$^2H_3$]Dimethylallyl Pyrophosphate. A mixture of [$^2H_4$]acetic acid (44.06 g, 0.69 mol) and PCl_3 (33.3 g, 0.299 mol) was stirred with protection from moisture for 3 h at room temperature and then 1 h at 45–50 °C. The upper layer was then separated and distilled. The distillate was treated with 2 drops of [$^2H_4$]acetic acid and redistilled to give 31.3 g (55%) of [$^2H_3$]acetyl chloride, bp 49–50 °C.

To a solution of 128 g (0.37 mol) of (ethoxycarbonyl)methylenetriphenylphosphorane[49] in 750 mL of benzene was added dropwise with stirring a solution of 15 g (0.185 mol) of [$^2H_3$]acetyl chloride in 10 mL of benzene at such a rate that the temperature of the reaction mixture stayed below 20 °C. The mixture was stirred at room temperature for another 22 h, the precipitate was removed by filtration and washed with benzene, and the combined filtrate and washings were evaporated to yield a light yellow solid. Crystallization from 90 mL of benzene gave 68 g (94%) 1-(ethoxycarbonyl)-[*methyl*-$^2H_3$]acetonylidenetriphenylphosphorane (**4**), mp 167–169 °C.

4 (68 g, 0.173 mol) was heated for 1 h to 270–280 °C at 8–9 Torr in a distillation apparatus, and the distillate was collected in a receiver cooled in a dry ice–acetone bath. Redistillation gave 10 g (50%) of ethyl [4-$^2H_3$]but-2-ynoate, bp 69 °C (25 Torr). NMR ($CDCl_3$) δ 4.16 (q, 2 H), 1.27 (t, 3 H).

The following reaction was carried out in a dry bag, and all reagents and equipment were rigorously dried and flushed with dry nitrogen before use. Anhydrous cuprous iodide (25.2 g, 0.132 mol) and 1 L of THF were placed in a flask equipped with two dropping funnels. Ethyl [4-$^2H_3$]-but-2-ynoate (6.9 g, 0.06 mol) in 10 mL of THF was transferred into one dropping funnel and methyllithium (0.24 mol, 148 mL of 5.04% solution in ether) into the other. The system was flushed with nitrogen and cooled to −15 °C, and the methyllithium solution was added dropwise with stirring. After stirring at −5 to 0 °C for 5 min the solution was cooled to −69 °C, the ethyl butynoate solution was added, and stirring was continued for 3 h at −69 °C. The reaction mixture was then poured with vigorous stirring into 250 mL of methanol cooled in a dry ice–acetone bath. After warming to −15 °C 100 mL of brine and 100 mL of ether were added, the suspension was filtered through a layer of fiber, and the solid was washed with 500 mL of ether. The filtrate and washings were combined, another 100 mL of brine was added, and the organic layer was separated, washed with brine until neutral, and dried. Evaporation of solvent and fractional distillation gave 4.54 g (58%) of ethyl (*Z*)-3-methyl-2-[4-$^2H_3$]butenoate, bp 76 °C (44 Torr). NMR ($CDCl_3$) δ 5.64 (d, 1 H, = C-*H*), 4.11 (q, 2 H, CH_3CH_2), 1.86 (d, 3 H, *E* CH_3), 1.24 (t, 3 H, CH_3CH_2).

In a three-neck, round-bottom flask was placed 360 mg (10 mmol) of $LiAlH_4$ and 15 mL of dry ether, and to one neck was attached by a piece of Gooch tubing a small flask containing 500 mg (3.7 mmol) of $AlCl_3$. While keeping the solution stirring in an ice bath, the $AlCl_3$ was added slowly to the reaction flask by tapping the container. The slurry was stirred for 5 more min, and then 1.31 g (10 mmol) of ethyl (*Z*)-3-methyl-2-[4-$^2H_3$]butenoate in 15 mL of ether was added dropwise with stirring. After 2 more h of stirring at room temperature, water was added cautiously, the mixture was extracted with ether, and the organic layer was washed with saturated $NaHCO_3$ solution and dried. Evaporation of the ether and distillation gave (*Z*)-3-methyl-[4-$^2H_3$]butenol in 73% yield, bp 47 °C (15 Torr). NMR ($CDCl_3$) δ 5.62 (t, 1 H, =C-*H*), 4.20 (dd, 1 H, CH_2–OH), 4.15 (dd, 1 H, CH_2–OH), 1.77 (s, 3 H, *E* CH_3).

To the above alcohol (178 mg, 2 mmol) was added 1.2 mL (12 mmol) of trichloroacetonitrile. Bis(triethylammonium) phosphate (1.4 g, 4.8 mmol) in 40 mL of acetonitrile was then added slowly with stirring to this solution over a period of 3–4 h. After 2 more h of stirring at room temperature the reaction mixture was concentrated, the residue was transferred to a centrifuge tube, and 20 mL of acetone and 2 mL of concentrated NH_4OH were added. The precipitated ammonium salts, isolated by centrifugation, were washed twice with 10 mL of acetone containing 0.01 N NH_4OH. The crude product was taken up in 30 mL of *n*-propanol/NH_4OH/water 9:4:1, undissolved salts were removed by filtration and washed with the same solvent mixture. The filtrate and washings were concentrated to a few milliliters and chromatographed on a column (2 × 100 cm) of activated silica gel (Brinkmann 70–230 mesh). The silica gel had been activated by stirring in 700 mL of concentrated HCl/water 1:1, washing with 6 × 600 mL water and drying at 150 °C for 48 h. The column was developed with the same solvent at a flow rate of 0.5 mL/min, and 7-mL fractions were collected. DMAPP (fractions 60–75) obtained in 15–20% yield was free of mono- and polyphosphates and of inorganic phosphate as judged by TLC (silica gel, *n*-propanol/NH_4OH/water 6:3:1, R_f 0.13–0.18, monophosphate 0.29–0.32). $C_5H_{18}D_3N_7P_2$ (MW 300) Calcd C, 20.00; H, 6.00; D, 2.00; P, 20.67. Found: C, 20.01; H, 6.03; D, 2.02; P, 20.88. NMR (D_2O), 500 MHz δ, ppm (rel intensity), 1.762 (96.7), 1.717 (3.24).

Acknowledgment. We are indebted to Professor Hans Rilling, University of Utah, for help and advice with his procedure for the purification of DMAT synthase during early stages of this project, to the Purdue University Biological Magnetic Resonance Laboratory and the Ohio State University Campus Chemical Instrument Center for spectral services, and to the National Institutes of Health for financial support.

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