# Chiral Methyl Groups: Small Is Beautiful

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Chemists, particularly those of the organic or biochemical persuasion, tend to be preoccupied with large molecules and complex structures, not with small and simple compounds. Yet, despite this general mindset, chiral (stereogenic) methyl groups, the smallest asymmetric objects ever synthesized, have for some reason managed to capture our imagination. Ever since the initial disclosure[1,2] of methods to synthesize the enantiomers of a methyl group carrying one atom each of $^1H$, $^2H$, and $^3H$ and to distinguish the *R* from the *S* enantiomer of such an entity, there has been considerable fascination with chiral methyl groups and their use to analyze stereochemical problems in chemistry and particularly in biochemistry. In the following we review some applications of chiral methyl groups in work carried out by our laboratory.

Two principal approaches are available to generate the *R* and *S* versions of a chiral methyl group, and these are exemplified by the two approaches taken by the original investigators. In the Arigoni approach[2] an *R*- and an *S*-configured methyl group were independently generated by successive stereospecific introduction of the three isotopes of hydrogen at the same carbon atom. In the Cornforth[1,3] approach a racemate of a molecule C[R*,$^1$H,$^2$H,$^3$H] was generated in which R* contains an auxiliary chiral center whose configuration, by virtue of the synthesis, is correlated with the configuration of the chiral methyl group. Resolution of the enantiomers based on the classical chiral center in R* then also separates the two enantiomers of the chiral methyl group. Numerous implementations of these two principal approaches have been reported. Among these, a few stand out. Without a question the most elegant synthesis is that devised by Townsend, Scholl, and Arigoni[4] which in a beautiful cascade of electrocyclic reactions brings the three hydrogen isotopes together on the same carbon stereospecifically in a single process. The most heroic synthesis, at the time, was published by Altman and co-workers,[5] who in the 1970s prepared multimillicurie quantities of oxidosqualene of specific activity 10 Ci/mmol carrying at C-10 a methyl group of *R* configuration. This material was used in a $^3$H-NMR study of the enzymatic cyclization of oxidosqualene to cycloartenol. Perhaps the most efficient synthesis is the one used in our laboratory,[6] which employs quite ordinary chemistry to generate material of, within the limits of detection, 100% enantiomeric purity (Figure 1). Tritium is introduced late in the synthesis from Super-Tritide which can be prepared at very high specific activities.[7,8] Via this route we have prepared 5–9 Ci quantities of chiral methyl groups at essentially carrier-free levels of tritium.

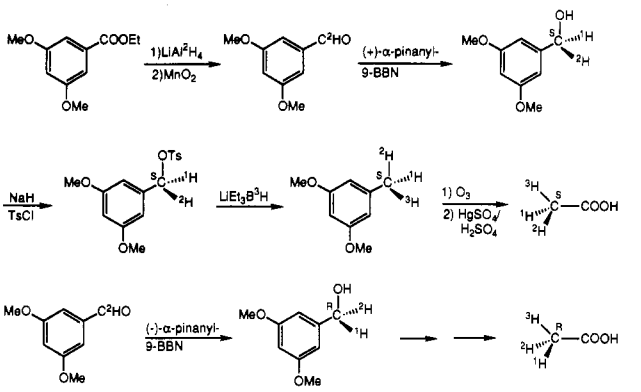


**Figure 1.** Synthesis of chiral acetate modified after Kobayashi et al.[6]

**Analysis.** As the foregoing examples show, the synthesis of enantiomerically pure chiral methyl groups requires essentially only extensions of synthetic methodology that has been widely used to prepare other stereospecifically isotope-labeled compounds. In contrast, the determination of whether an unknown sample contains an excess of *R*- or *S*-configured chiral methyl groups required conceptually new approaches. These can be based either on isotope effects or on the use of $^3$H-NMR spectroscopy. The original solution to this problem developed by Cornforth, Arigoni, and their co-workers relies on a primary kinetic deuterium isotope effect in a reaction removing one hydrogen from the chiral methyl group to generate a methylene group in

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(1) (a) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutschow, C. *Nature* **1969**, *221*, 1212. (b) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutschow, C. *Eur. J. Biochem.* **1970**, *14*, 1.

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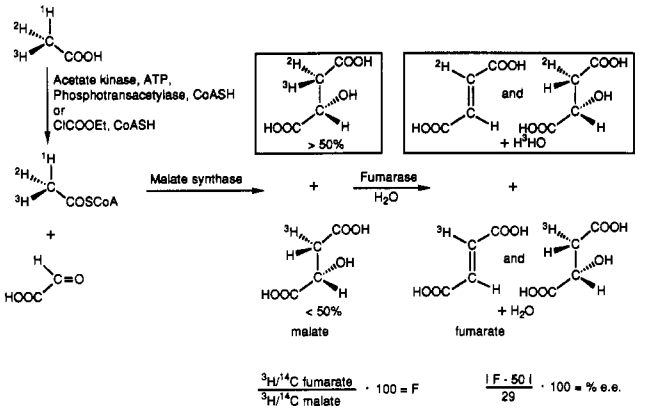


**Figure 2.** Configurational analysis of chiral acetate by the malate synthase/fumarase method.[1,2]

which tritium is now unevenly distributed between the two methylene hydrogens. Analysis of this tritium distribution together with a knowledge of the value of $k_H/k_D$ and the steric course of the reaction reveals the configuration of the original chiral methyl group and provides an estimate of its enantiomeric excess. Both laboratories chose for the implementation of this concept the condensation of acetyl coenzyme A with glyoxylic acid catalyzed by the enzyme malate synthase, which exhibits a primary kinetic deuterium isotope effect $k_H/k_D$ of 3.8.[9] Subsequent incubation of the resulting malate sample with fumarase results in the removal of 79% of its carbon-bound tritium as water if the original acetate was of 100% *S* configuration, whereas 79% of the tritium remains carbon-bound if the chiral methyl group had 100% *R* configuration (Figure 2). The percentage of tritium retention in the fumarase reaction is called the *F* value;[10] from this value the percentage of enantiomeric excess can be calculated as shown in Figure 2. This is by far the most common procedure for the determination of the configuration and optical purity of chiral methyl groups; it requires that the methyl group be converted in a series of stereospecific reactions into the methyl group of acetate for analysis.

Instead of using incubation with fumarase, one could also use $^3$H-NMR to determine the tritium distribution in the malate samples. This has in fact been done in Arigoni's laboratory.[11] The important feature of this approach is that the stereochemical information can still be revealed even if the initial abstraction of a hydrogen from the methyl group does not proceed with a significant kinetic isotope effect.[12] As Figure 2 shows, the malate synthase reaction produces a mixture of two tritiated malate species from each enantiomer of chiral acetate. One carries tritium in one methylene position with deuterium in the other, whereas the second carries tritium in the opposite position with $^1$H as a neighbor. In the malate from the opposite enantiomer of chiral acetate these relationships will be reversed. Whether a given tritium has $^1$H or $^2$H as a neighbor can easily

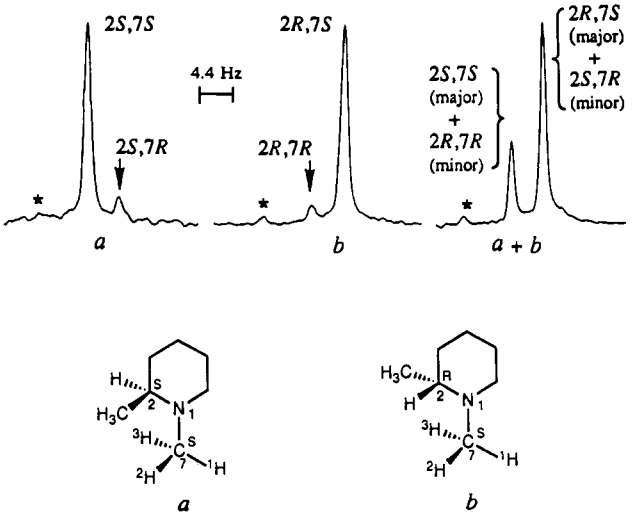


**Figure 3.** Configurational analysis of chiral methyl groups by direct $^3$H NMR observation.

be determined in the tritium NMR spectra from the $^1$H–$^3$H coupling and the $^2$H isotope shift on the $^3$H signal.

$^3$H-NMR should in principle also be able to distinguish the two enantiomers of a chiral methyl group directly when they are observed in an asymmetric environment. However, the need for enantiomeric conformations around the C–R* bond in a system R*–C [$^1$H,$^2$H,$^3$H] to be differentially populated in order to observe nondegenerate $^3$H-NMR signals places severe constraints on the nature of R*. On the basis of extensive NMR studies on the conformational effects of nitrogen lone pairs, Anet and Kopelevich predicted[14] that the 2-methylpiperidine moiety should meet these requirements. This prediction was put to the test by preparing (2*S*,7*S*)- and (2*R*,7*S*)-[7-$^2$H$_1$,$^3$H]-1,2-dimethylpiperidine and recording the $^3$H-NMR spectra of the two samples (Figure 3).[15] The well-resolved $^3$H-NMR signals for the two diastereomers show a chemical shift difference of 0.014 ppm. The NMR method has the advantage that optical purities can be determined very accurately; however, it requires far more material (the spectra in Figure 3 were recorded on samples of 1.1 and 2.1 mCi) and will for that reason alone not replace the enzymatic configurational analysis procedure.

## Applications

**Methyltransferases.** The transfer of methyl groups from the biological methyl donor, *S*-adenosylmethionine (AdoMet), to various biological molecules is a very important biochemical reaction.[16] It was of interest to obtain mechanistic information on this process and, in particular, to probe whether the multitude of methyltransferases operating in different processes have evolved along the same mechanistic blueprint. To study enzymatic methyl-transfer reactions we prepared as substrates the two diastereomers of L-methionine carrying a chiral methyl group on the sulfur (Figure

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(12) Another ingenious way of determining whether a given $^3$H has $^1$H or $^2$H as a neighbor, based on isotope effects in a subsequent reaction, was demonstrated by Rétey et al.[13]

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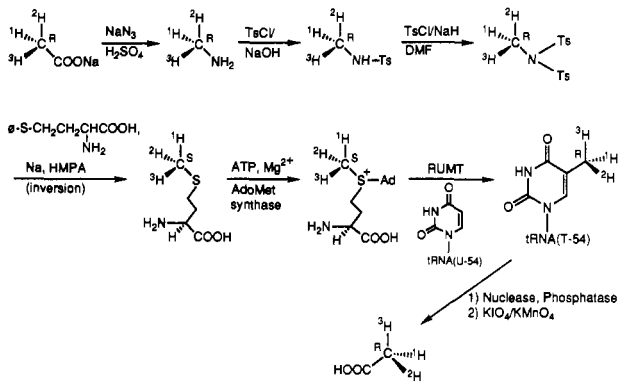

**Figure 4.** Synthesis of chiral methyl labeled AdoMet and stereochemical analysis of the tRNA ($m^5U54$)methyltransferase (RMUT) reaction.

4);[17] these were then enzymatically activated to give the correspondingly labeled samples of AdoMet.[18] The key step in the methionine synthesis is the "umpolung" of the C–N bond in methylamine[19] by attachment of two sulfonate groups to the nitrogen. This makes the product a respectable methylating agent for transfer of the methyl group to suitable nucleophiles with inversion of configuration.

Also illustrated in Figure 4 is an example of the use of AdoMet carrying a chiral methyl group for the stereochemical analysis of a methyltransferase reaction, in this case the methylation of a uracil residue in a tRNA.[20] The recovery of the chiral methyl group from the product as acetic acid for configurational analysis was in this case very simple. In other cases, for example when the methyl group was situated on an oxygen or nitrogen atom, somewhat more elaborate degradation procedures had to be developed to convert the methyl group stereospecifically into the methyl group of acetate. Parallel to our own investigations of methyltransferase stereochemistry, similar studies were carried out in the laboratory of Arigoni using virtually identical methodology.[10] Results from both laboratories are summarized in Table I, showing that a large number of methyltransferases functioning in a wide variety of different metabolic reactions and transferring the methyl group from AdoMet to a variety of different nucleophiles all operate with inversion of methyl group configuration. Although strictly speaking these results only indicate that the methyl group must undergo an odd number of transfers, the most plausible interpretation is that all these enzymes mediate a single direct transfer of the methyl group from the sulfur of AdoMet to the acceptor nucleophile. This rules out ping-pong mechanisms which had been postulated for some of these reactions on the basis of kinetic data. Secondary isotope effect studies on catechol O-methyltransferase (COMT) carried out by Schowen, Borchardt, and their co-workers[21] at the same time as our stereochemical analysis[18] were interpreted to indicate that the enzymatic reaction proceeds through a slightly compressed

**Table I. Enzymatic Methyl Transfer Reactions Proceeding with Inversion of Methyl Group Configuration**

| enzyme or product | methylation on | ref |
|---|---|---|
| vitamin $B_{12}$ (corrin ring) | carbon | 10 |
| loganin | oxygen | 10 |
| homocysteine *S*-methyltransferase | sulfur | 10 |
| indolmycin | carbon | 17 |
| indolmycin | nitrogen | 17 |
| catechol *O*-methyltransferase | oxygen | 18 |
| phenylethanolamine *N*-methyltransferase | nitrogen | 58 |
| pectin | oxygen | 59 |
| histamine *N*-methyltransferase | nitrogen | 60 |
| aplasmomycin | carbon | 61 |
| 4′-*O*-methylnorlaudanosoline 6-*O*-methyltransferase | oxygen | 52 |
| norreticuline *N*-methyltransferase | nitrogen | 50 |
| dimethylallyltryptophan *N*-methyltransferase | nitrogen | 62 |
| *Eco*RI DNA methyltransferase | nitrogen | 63 |
| *Hha*I DNA methyltransferase | carbon | 63 |
| t-RNA-uracil methyltransferase | carbon | 20 |

$S_N2$ transition state.[22] In any event, the stereochemical results suggest that a large number of methyltransferases have evolved around the same mechanistic theme, direct transfer of an activated methyl group from sulfur to a nucleophile. This may signify either that all these enzymes have a common evolutionary ancestor or that this mechanism is sufficiently superior to alternative ones to drive evolution.[24]

**AdoMet-Dependent Methyl Transfer with Retention.** In the biosynthesis of methionine[25] by the $B_{12}$-dependent *Escherichia coli* methionine synthase the operation of a two-stage mechanism for methyl transfer has been demonstrated.[26,27] This raises the question of whether Nature has invented mechanistic alternatives to the prevalent single $S_N2$-type transfer of the methyl group for AdoMet-dependent methyltransferases. The answer to this question is probably yes. Two candidate reactions were discovered in the course of screening methyltransferases for their stereochemistry. The first example was encountered in the biosynthesis of the β-lactam antibiotic thienamycin, which is assembled in *Streptomyces cattleya* from cysteine, glutamate, acetate, and methionine.[28] Notably, methionine donates both carbons of a hydroxyethyl side chain in two successive methyl transfers. When the stereochemistry was probed by feeding methionine carrying a chiral methyl group of *R* or *S* configuration, it was found that this methyl transfer proceeds with net retention of configuration.[29] Hence, the process must involve an even number of "jumps" of the methyl group, most likely two. No further

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(20) Kealey, J. T.; Lee, S.; Floss, H. G.; Santi, D. V. *Nucleic Acids Res.* **1991**, *19*, 6465.
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(22) A recent paper on secondary isotope effects and transition-state structure,[23] however, raises questions about the validity of the earlier[21] interpretation.
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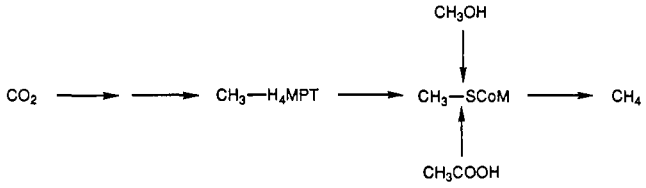


**Figure 5.** Methanogenesis in *M. barkeri.*

mechanistic information is available on this process,[30] although the fact that the thienamycin fermentation has a cobalt requirement[28] suggests the possible involvement of a corrin, as in the case of methionine synthesis.

A second example was discovered in the biosynthesis of the antibiotic thiostrepton.[31] An early step in this process is the methylation of tryptophan at C-2 of the indole ring, and it was found that this reaction, too, proceeds with net retention of methyl group configuration.[32,33] This finding was rather surprising because there is no obvious reason why the transfer of a methyl group from AdoMet to C-2 of an indole ring should be mechanistically unusual. Unfortunately, although the enzyme catalyzing this methylation was detected in a cell-free extract of the thiostrepton producer, *Streptomyces laurentii*, all attempts to purify it have so far been unsuccessful.[33] Thus, the mechanism of this unusual methyltransferase reaction, for the moment, remains cryptic.

**Methanogenesis.** Since an ample body of work, much of it reviewed above, indicates that single intermolecular methyl transfers proceed with inversion of configuration, chiral methyl groups can be used to probe whether a particular sequence of reactions involves an odd or an even number of methyl transfers, i.e., how many intermediates must be involved at minimum. An example is methanogenesis, the reduction of $CO_2$ with hydrogen gas to methane.[34] In this pathway, which operates in methanogenic bacteria, e.g., *Methanosarcina barkeri*, and which involves a number of novel cofactors,[35] $CO_2$ is successively reduced to the level of a methyl group attached to N-5 of tetrahydromethanopterin, the methanogen's equivalent of tetrahydrofolate. In the last two steps of the reaction sequence the methyl group is first transferred from $CH_3$-tetrahydromethanopterin to the SH group of the novel cofactor mercaptoethanesulfonic acid (coenzyme M) to give $CH_3$–SCoM. In the last step, the latter is then reduced to methane by the methyl coenzyme M reductase complex. Some species of methanogens, such as *M. barkeri*, can be adapted to utilize methanol, methylamine, or the methyl group of acetic acid to produce methane (Figure 5). Recent evidence points to a disassembly of acetate on CO dehydrogenase[36] similar to the reverse reaction in acetogenic bacteria.[37,38]

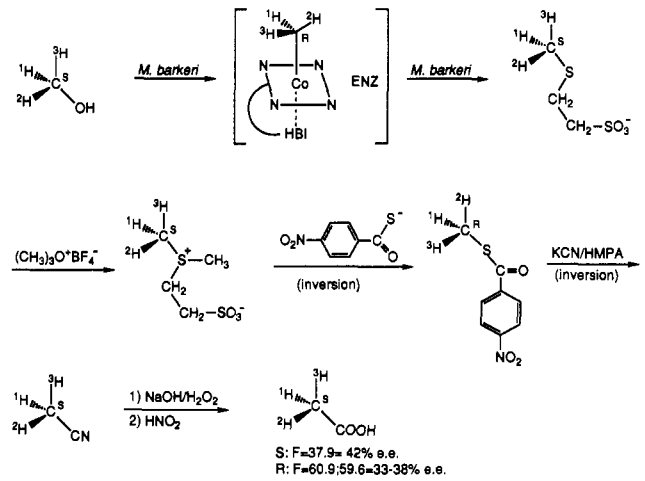


**Figure 6.** Steric course of the enzymatic synthesis of methyl coenzyme M from methanol in *M. barkeri.* HBI = 5-hydroxybenzimidazole.

The methyl transfer from methanol is a rather complex process, apparently involving two methyltransferases, one of which seems to be a corrinoid enzyme requiring reductive activation.[39] This would parallel the mode of operation of $B_{12}$-dependent methionine synthase, discussed above, consistent with the fact that in both cases a relatively inert bond, C–O or C–N, must be cleaved in the transfer of a methyl group.

To probe whether the stereochemistry is consistent with this presumed mechanistic parallel, we synthesized samples of (*R*)- and (*S*)-[$^2H_1$,$^3H$]methanol and incubated them with cell-free extracts of *M. barkeri.* Addition of bromoethanesulfonate to the incubations inhibited methylreductase, leading to the accumulation of methyl coenzyme M, which was then degraded to acetate for configurational analysis as shown in Figure 6.[40] The results clearly indicate that the reaction is stereospecific and proceeds with net retention of configuration. Similar results were obtained for the conversion of the methyl group of acetate into methyl coenzyme M, i.e., again the methyl group in the substrate and the product have the same configuration.[41] Although the simplest explanation of both results would be that the methyl group from methanol or, in the case of acetate, from the nickel site of CO dehydrogenase is first transferred to the cobalt of a corrinoid enzyme and then from the methylcobalamin to coenzyme M, one cannot dismiss the possibility of a more complex process involving four sequential transfers of the methyl group. Such a process could involve transfer of the methyl group first from methanol or the nickel site of CO dehydrogenase via a corrinoid enzyme to $H_4$MPT (two inversions) and then from the resulting $CH_3$–$H_4$MPT again via a corrinoid interme-

(30) Unfortunately, the prospects of studying this reaction further are at present not good. The enzyme catalyzing this reaction has not been isolated, its substrate and product are not known, and since thienamycin is a commercial product, the producing strain is not readily available.

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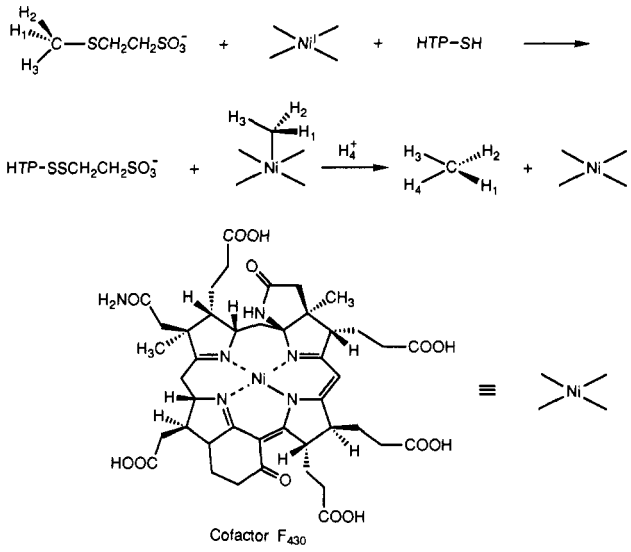


**Figure 7.** Steric course of the methyl coenzyme M reductase reaction. HTP-SH = (7-mercaptoheptanoyl)threonine phosphate.

diate to coenzyme M (also two inversions). Obviously, stereochemistry does not differentiate these two possibilites.

The last reaction in the sequence, which is catalyzed by the methylreductase complex, involves the novel nickel-containing cofactor $F_{430}$ (Figure 7),[42] as well as an additional sulfhydryl compound, (7-mercaptoheptanoyl)threonine phosphate (HTP-SH). It is thought that the methyl group is transferred from methyl-CoM to the reduced nickel of $F_{430}$ in a reaction that is not yet well understood; in the process coenzyme M is oxidized to the mixed disulfide with HTP-SH. If this process involves a direct transfer of the methyl group from sulfur to nickel, one would expect it to proceed with inversion of configuration. Methane would then be generated by a protonolytic cleavage of the methyl–Ni bond, a process which, on the basis of precedent,[43] should proceed with net retention of configuration. The experimental test of these predictions presents an obvious problem: There are only three isotopes of hydrogen, whereas the generation of an isotopically chiral version of methane requires four hydrogen isotopes. Fortunately, although methylreductase is highly substrate specific,[44] the enzyme does reduce ethyl coenzyme M to ethane at about 20% of the rate for the methyl analog.[45] Thus, a viable strategy for the stereochemical analysis of this reaction involved the synthesis of the enantiomeric samples of ethyl coenzyme M carrying deuterium and tritium in the methylene group (Figure 8).[46] These samples were then incubated with a cell-free extract of *M. barkeri* to give samples of ethane carrying one chiral methyl group. These were degraded by halogenation and oxidation to give acetic acid for configurational analysis. The results indicated

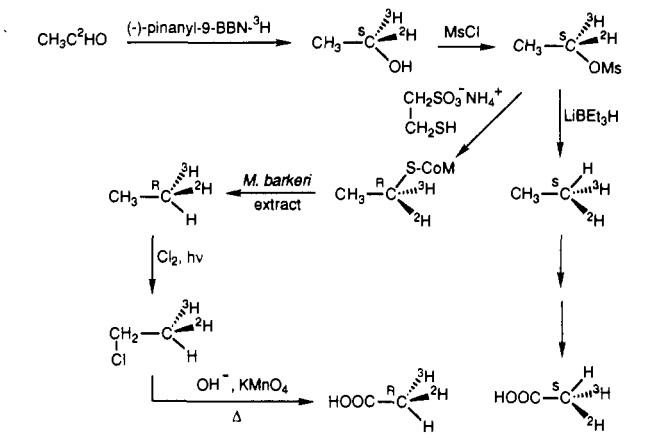


**Figure 8.** Stereochemical analysis of the methyl-CoM reductase reaction.

that in the methylreductase reaction the sulfur of coenzyme M is replaced by hydrogen with net inversion of configuration. This result conforms with the prediction, as outlined in Figure 7, of a process involving a single transfer of the methyl group from sulfur to nickel with inversion of configuration followed by protonolytic cleavage of the nickel–methyl bond with retention of configuration.

**C–P Lyase.** By the same methods we have recently examined the stereochemistry of the cleavage of the carbon–phosphorus bond in ethylphosphonate catalyzed by the bacterial enzyme, C–P lyase.[47] It was found that the replacement of phosphorus by hydrogen proceeds with predominant retention of configuration, accompanied by substantial racemization.[48] This strongly supports the intermediacy of an ethyl radical in the reaction.

## Chiral Methyl Groups and $^3$H-NMR

The pioneering work of Altman and co-workers[5] on the stereochemistry of cycloartenol formation from squalene has clearly demonstrated that utility of tritium NMR spectroscopy for stereochemical studies involving chiral methyl groups. The great advantage of tritium NMR analysis is that the configuration of a methylene group generated from a chiral methyl group can be determined without chemical degradation and independently of whether the removal of one methyl hydrogen proceeded with or without a primary kinetic isotope effect. Also, given enough tritiated product, very accurate quantitative data on the degree of stereospecificity can be obtained and, if racemization occurs, it can be determined whether it resulted from an exchange process or from configurational inversion of an intermediate species. Likewise, if the hydrogen abstraction does involve an isotope effect, the experiment will give its value with good accuracy. The following two examples should serve to illustrate these points.

**Berberine Bridge Formation.** The biosynthesis of the benzylisoquinoline alkaloid berberine involves the transfer of a methyl group to the nitrogen of (*S*)-

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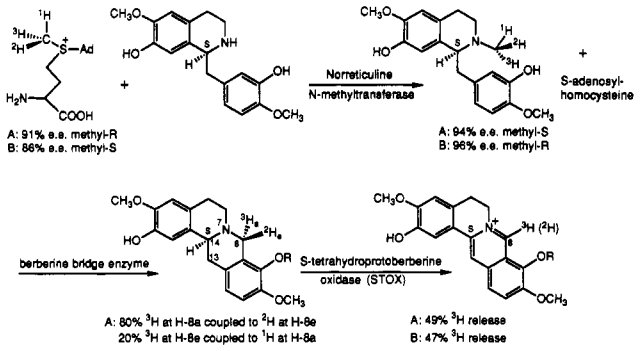


**Figure 9.** Formation of the berberine bridge in benzylisoquinoline alkaloids and its stereochemical course.

norreticuline, followed successively by oxidative cyclization to a methylene group, the berberine bridge, and finally oxidative aromatization to give a methine group, C-8 of berberine (Figure 9).[49] The steric course of this reaction sequence was examined in collaboration with Professor Zenk's group in Munich.[50] The results, summarized in Figure 9, indicate that the initial transfer of the methyl group to (*S*)-norreticuline proceeds cleanly with inversion of configuration. Incubation of the resulting two isomers of reticuline successively with berberine bridge enzyme and (*S*)-tetrahydroberberine oxidase (STOX) resulted in the release of identical amounts of tritium, 49% and 47%, respectively, from the two isomers. Therefore, one of the two reactions or both must proceed nonstereospecifically. A larger amount of reticuline carrying a methyl group of *S* configuration was then cyclized with the berberine bridge enzyme to give 153 $\mu$Ci of tritiated scoulerine, which was analyzed by tritium NMR spectroscopy. The spectra showed the presence of 20% of the tritium in the equatorial position at C-8 ($\delta$ = 4.76 ppm) coupling to a proton, and 80% of the tritium in the axial position at C-8 ($\delta$ = 4.29 ppm), deuterium isotope shifted by 0.05 ppm relative to the corresponding proton resonance.

It follows from these data that the berberine bridge enzyme operates completely stereospecifically, abstracting an *N*-methyl hydrogen with an isotope effect $k_H/k_D$ of 4 and replacing it by the phenolic group in an inversion mode. It also follows that the reaction catalyzed by STOX is nonstereospecific, consistent with earlier notions[51] that this enzyme only catalyzes the dehydrogenation of the substrate to the 7,14-iminium ion, which then air oxidizes spontaneously. The mechanistic interpretation of these results is summarized in Figure 10. It is proposed that the berberine bridge forming enzyme abstracts a hydrogen atom from the methyl group and an electron from the nitrogen in an *anti* fashion. The facial selectivity in the subsequent attack of the phenolic group is dictated by the configuration at C-14.

A related investigation is underway on the stereochemical course of the analogous formation of the methylenedioxy bridge of berberine and related alkaloids.[52]

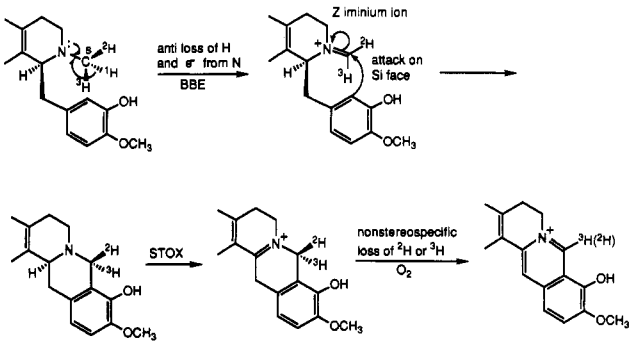


**Figure 10.** Stereochemical mechanisms of the reactions catalyzed by berberine bridge enzyme and STOX.

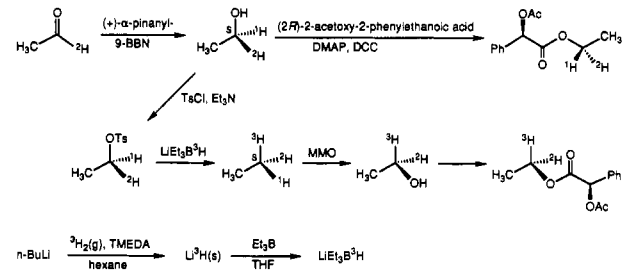


**Figure 11.** Preparation of (*S*)-[1-$^2H_1$,$^3H_1$]ethane and stereochemical analysis of the methane monooxygenase reaction.

**Methane Monooxygenase.** Methane monooxygenase (MMO) catalyzes the first reaction in the assimilation of methane by methanotrophic bacteria, the NAD(P)H and $O_2$ dependent hydroxylation of methane to methanol.[53] The enzyme from *Methylosinus trichosporium* OB3b consists of a 245-kDa hydroxylase component containing a $\mu$-[R/H]-oxo bridged binuclear iron cluster, a 40-kDa NAD(P)H dependent oxidoreductase component, and a 15.8-kDa component B. Mechanistic proposals for MMO invoke both radical[54,55] and carbocation[55] intermediates as well as concerted oxygen insertion into a substrate carbon–hydrogen bond.[56] It was therefore desirable to examine the stereochemistry of this reaction.

For reasons mentioned earlier, the problem cannot be studied with methane itself; however, ethane is a good substrate of the enzyme. Hence, we prepared samples of (*R*)- and (*S*)-[1-$^2H_1$,$^3H_1$]ethane at near carrier-free tritium levels as shown in Figure 11.[57] These were then incubated with the three MMO components and NADPH in the presence of air or with only the

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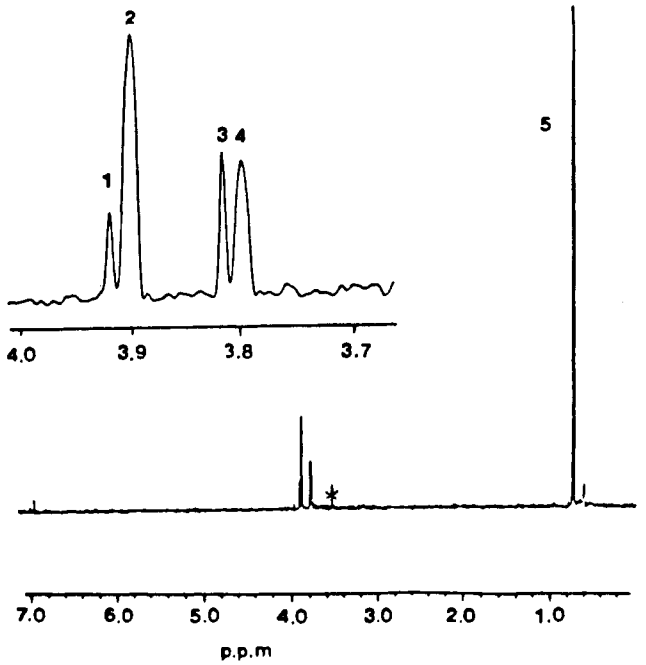
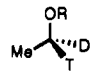
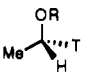
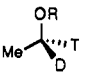
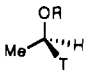



**Figure 12.** $^3$H NMR spectrum of ethyl (2*R*)-*O*-acetylmandelate (2.05 mCi) derived from (*S*)-[1-$^2$H$_1$,$^3$H$_1$]ethane by methane monooxygenase (see Table II for explanation).

hydroxylase component in the presence of $H_2O_2$. Following the addition of unlabeled carrier ethanol, each sample was then lyophilized and the recovered liquid was analyzed by $^3$H-NMR. This analysis revealed the percentage of tritium released into water and the ratio of oxidation at the labeled versus the unlabeled methyl group. Each ethanol sample was then derivatized to the (*R*)-*O*-acetylmandelate ester, which was again analyzed by $^3$H-NMR spectroscopy. As the spectrum in Figure 12 shows, the resonances of the four isotopomers of the methylene-labeled ethyl *O*-acetylmandelate are well enough resolved to allow accurate quantitation.

The results of this study, summarized in Table II, indicate that the MMO-catalyzed hydroxylation of ethane proceeds with predominant retention of configuration, consistent with the close mechanistic similarity to P450-catalyzed reactions. The overall retention of configuration is accompanied by approximately 35% inversion. This stereochemical infidelity does not represent racemization due to an exchange process, but must be due to "flipping" of a planar intermediate. On the basis of the arguments elaborated earlier, this finding strongly implicates an enzyme-bound ethyl radical as an intermediate. The intermediate radical must have a sufficiently long lifetime to undergo configurational inversion with an appreciable frequency. These results thus suppport a radical mechanism as proposed by Lipscomb and co-workers;[54] they argue against mechanisms not involving a free substrate intermediate in the enzyme active site. In cases where it is applicable, the approach employed here is probably a more sensitive indicator for radical intermediates in enzyme reactions than some of the diagnostic substrates traditionally used for this purpose.

**Table II. Distribution of $^3$H Label in the Methylene Group of the (2*R*)-2-Acetoxy-2-phenylethanoate Derivatives of the Ethanol Recovered from the Enzyme Incubations and the Extent of Retention or Inversion of Configuration Calculated from These Data[a]**

| | % $^3$H in methylene group of product | | | |
|---|---|---|---|---|
| |  |  |  |  |
| substrate | 4[b] | 1[b] | 2[b] | 3[b] |
| | 1. Using NADH/$O_2$ | | | |
|  (*R*) | 53 | 12 | 26 | 9 |
| | | retention = 68% | inversion[c] = 32% | |
|  (*S*) | 27 | 7 | 52 | 14 |
| | | inversion[c] = 36% | retention = 64% | |
| | 2. Using $H_2O_2$ | | | |
|  (*R*) | 48 | 12 | 30 | 10 |
| | | retention = 63% | inversion[c] = 37% | |
|  (*S*) | 30 | 7 | 51 | 12 |
| | | inversion[c] = 36% | retention = 64% | |

[a] The inversion/retention data have been corrected for the enantiomeric purity of the substrates. [b] Numbering of products corresponds to the numbering scheme in Figure 12. [c] Due to configurational inversion ("flipping") of the intermediate radical.

## Conclusion

At age 23 chiral methyl groups have made it through their teens, and they still look beautiful. The elegance of the original concept of Cornforth and Arigoni is still as vivid as it was when first revealed in 1969. Many applications of chiral methyl groups have been developed over the years, and in particular the combination with tritium NMR spectroscopy as the analytic tool has expanded the range of problems to which chiral methyl groups can be applied. Hence we can look forward to many more uses of these small but beautiful chiral objects.

*We wish to express our gratitude to the many graduate students and postdoctoral fellows whose dedicated efforts have produced the results reported here and to the numerous academic colleagues in this country and abroad who have collaborated with us on various aspects of this project. Their names are listed in the appropriate references. We also appreciate the long-standing financial support of this project by the National Institutes of Health.*