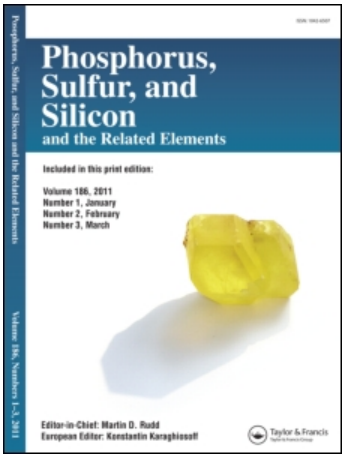

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Sulfur as a Carrier of Methyl Groups in One-Carbon Metabolism - Stereochemical Studies

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SULFUR AS A CARRIER OF METHYL GROUPS IN ONE-CARBON METABOLISM - STEREOCHEMICAL STUDIES

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INTRODUCTION

Sulfur plays a critical role in many biochemical reactions both as a nucleophile and a nucleofuge and, in the form of the sulfhydryl-disulfide pair, as a redox system. It is involved in stablizing protein structures through disulfide bridges (cysteine/cystine), in the catalysis of redox chemistry, e.g., the oxidative decarboxylation of pyruvate (lipoic acid), in the activation of acyl groups (coenzyme A) and it serves as a donor and carrier of methyl groups in biological methylation reactions (methionine and its activated form, S-adenosylmethionine, AdoMet). The latter reactions have been a subject of interest in our laboratory for some time and form the topic of this lecture.

As a means of obtaining mechanistic information we have studied the stereochemical course of a variety of reactions in one-carbon metabolism using the chiral methyl group methodology, i.e., employing methyl groups which are rendered chiral by virtue of the presence of one atom each of the three isotopes of hydrogen on the same carbon. This methodology is based on the pioneering work of Cornforth and Arigoni and their respective coworkers (1). Such a stereogenic (chiral) methyl group can be prepared, for example in the form of the methyl group of acetic acid, independently in either an R or an S configuration by conventional stereospecific labeling techniques. Numerous syntheses of optically active chiral methyl groups have been reported in the literature (2), including a very efficient synthesis used routinely in our laboratory (3). The conceptually difficult problem was: How does one tell whether an unknown sample contains an excess of R or S configurated methyl groups? An elegant

zsolution to this problem was presented by the Cornforth and Arigoni laboratories making use of a substantial primary kinetic isotope effect for the carbon hydrogen bond cleavage involved in the malate synthase-catalyzed condensation of acetyl-coenzyme A and glyoxylate to give L-malate (Figure 1). This reaction converts

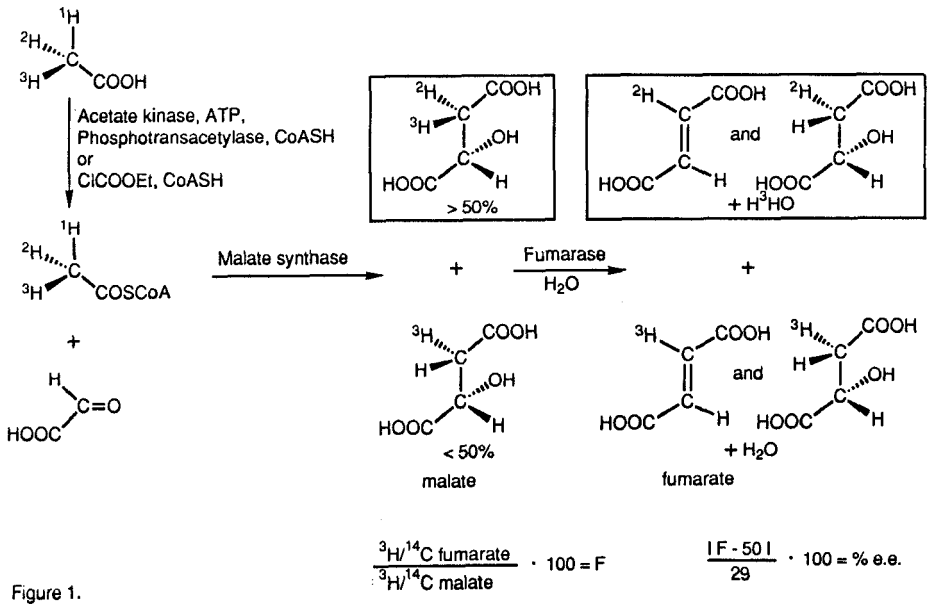


Figure 1.

the methyl group of acetyl-coA into the prochiral methylene group of malate; it results in an uneven tritium distribution between the two methylene hydrogens if the acetyl-CoA contained a chiral methyl group of predominantly of R or S configuration. The tritium distribution between these two methylene hydrogens is analyzed by incubation of the malate with the enzyme fumarase which washes out the pro-3R hydrogen into the solvent; the percentage tritium retention, called the F value (4), is a measure for the configuration and optical purity of the methyl group (1). Calibration of this analysis method has shown that pure R configurated methyl groups give an F value of 79, whereas a pure S methyl group gives an F value of 21. Thus, the amplitude of the assay is a deviation of ± 29 from the value of 50 for a racemic methyl group. This allows an estimation of the optical purity (% e.e.) of the methyl group (Figure 1). This

analytical methodology has allowed the 0analysis of a large number of biochemical reactions involving methyl groups (2).

Both Arigoni's group (4) and our laboratory (5) have examined a substantial number of enzyme reactions in which the methyl group of AdoMet is transferred to various nucleophiles, carbon, oxygen, nitrogen or sulfur, in biological molecules. Samples of AdoMet carrying either an R or an S methyl group were synthesized from the appropriate enantiomers of chiral acetate as shown in Figure 2; the crucial step is the "Umpolung" of the carbon nitrogen bond in

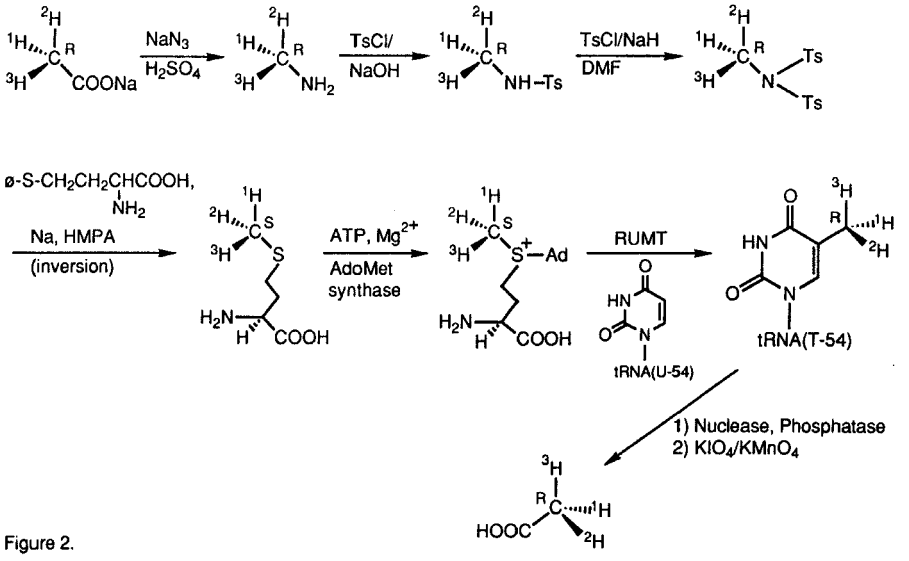


Figure 2.

methylamine to turn this molecule into a suitable methylating agent. These samples of AdoMet were then used as substrates for a variety of methyltransferases followed by degradation of the methylation products to carve out the chiral methyl group in the form of acetate for configurational analysis. This is illustrated in Figure 2 for the enzyme, tRNA-uracil methyltransferase. The analysis of sixteen such enzymes in the two laboratories indicated that in each case the methyl group is transferred with inversion of configuation (5). Thus, these reactions involve a single direct transfer of the methyl group, presumably

through an S_N2 transition state (6). Such a mechanism is plausible for the transfer of the methyl group from the sulfonium group of AdoMet, an excellent methyl donor.

A different mechanism is operating in cases where the methyl group needs to be displaced from a much poorer donor, e.g., a nitrogen or oxygen, and transferred to sulfur. A prime example is the formation of methionine from 5-methyltetrahydrofolate (CH_3-H_4folate) catalyzed by the vitamin B_{12}-dependent methionine synthase. Evidently, the sulfur of homocysteine is not a sufficiently good nucleophile to displace the methyl group from the nitrogen of CH_3-H_4folate directly; hence, the cobalt of B_{12} is interspersed in this reaction sequence. The cobalt is reductively activated to the extremely powerful nucleophile B_{12s} which displaces the methyl group from the nitrogen. In a second step the methyl group of the enzyme-bound methylcobalamin is then displaced by the sulfhydryl group of homocysteine (7). Consistent with such a two-stage process we found that in this case the methyl group is transferred with net retention of configuration, consistent with two transfers, each occuring with inversion (8).

Surprisingly, two cases of methyl transfer from methionine or AdoMet with net retention of configuration have also been observed. One occurs in the biosynthesis of the antibiotic, thienamycin, where both carbons of the hydroxyethyl group are derived from the methyl group of methionine (Figure 3)(9). The second case involves a methylation at C-2 of the indole ring of

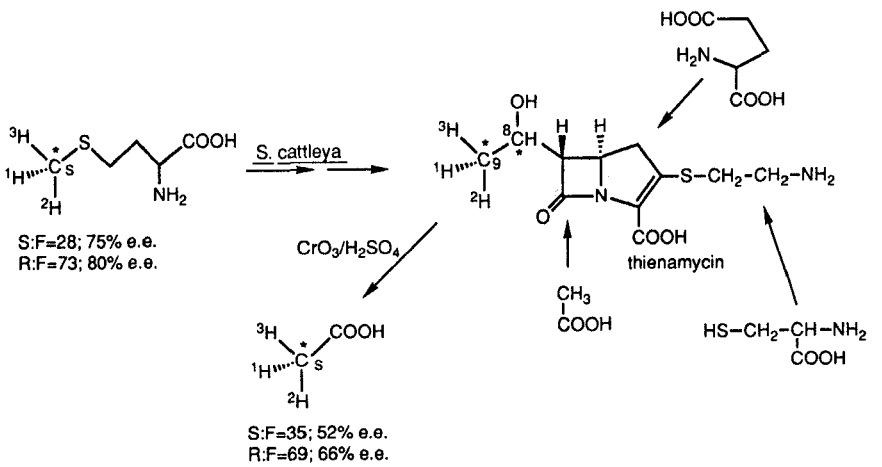


Figure 3.

tryptophan (10) the opening step in the biosynthesis of a quinolinic acid moiety which is part of the thiopeptide antibiotic, thiostrepton (Figure 4)(11). The

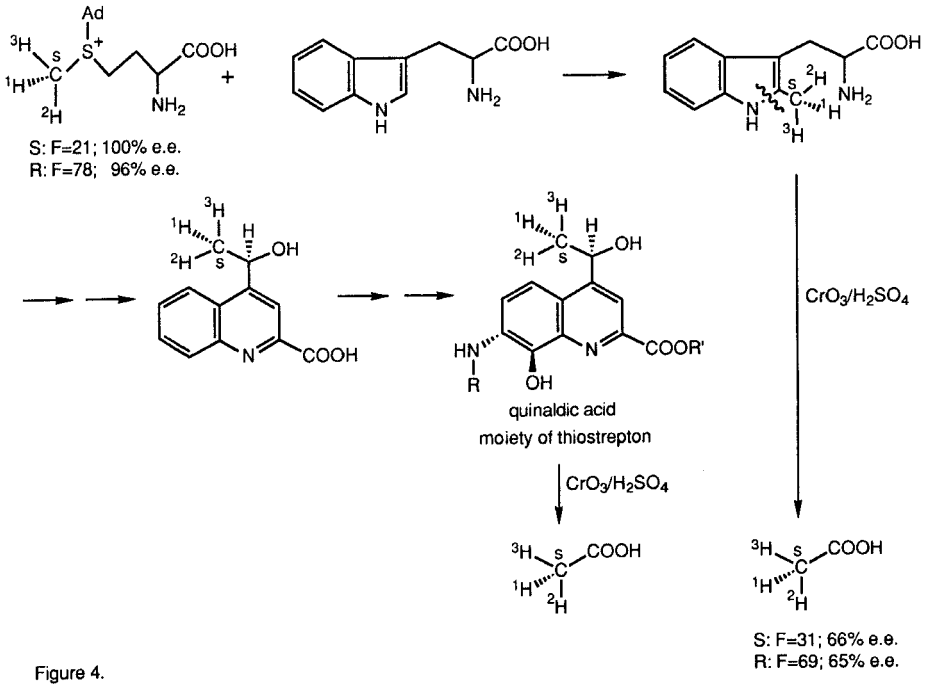


Figure 4.

mechanistic significance of this finding is at the moment entirely obscure. In the thienamycin case a mechanistic switch may be required because the carbon serving as acceptor of the methyl group may not be a good nucleophile. However, no such reason is evident for the methylation of the 2-position of an indole ring. Further work will be necessary to shed light on these atypical methylation reactions.

An important process involving the manipulation of one-carbon units is the formation of methane gas by anaerobic methanogenic bacteria (12). In this process, which involves a number of novel cofactors (13), CO_2 is successively reduced with hydrogen gas to the level of a methyl group attached to N-5 of tetrahydro-methanopterin, an analog of H_4 folate. The methyl group is then transferred to the SH group of the cofactor, mercaptomethanesulfonic acid

(coenzyme M) followed by reduction to methane by methyl-coenzyme M reductase. The bacteria can also be adapted to generate methane from other sources of methyl groups, e.g., methanol, methylamine or the methyl group of acetate (Figure 5). As in the case of methionine formation, discussed above,

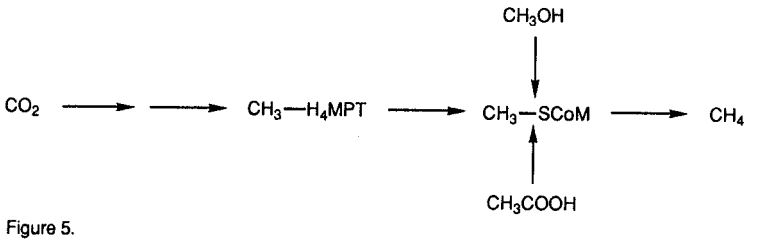


Figure 5.

a rather stable bond must be broken to transfer the methyl group from nitrogen, oxygen or carbon to the sulfur of coenzyme M. Thus, not surprisingly, there is evidence for the involvement of corrinoid enzymes requiring reductive activation (14). Consistent with a two-step mechanism we found that both the methyl group of methanol (15) and the methyl group of acetate (16) are transferred to the sulfur of coenzyme M with net retention of configuration (Figure 6). The is

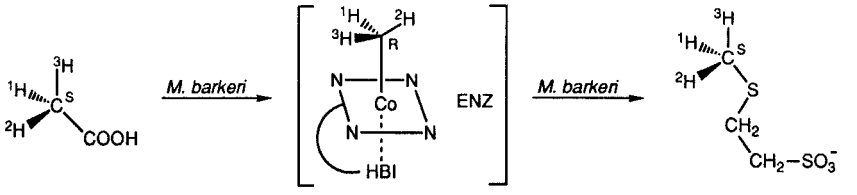
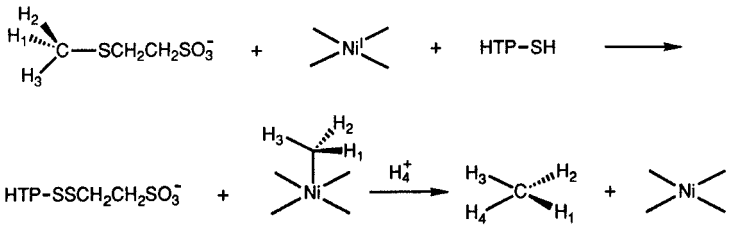


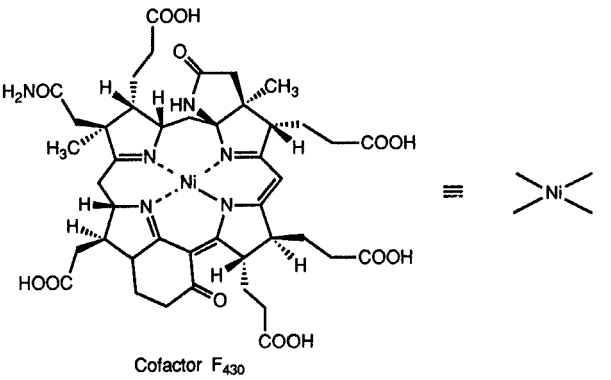
Figure 6.

assembly of acetate probably occurs on the enzyme CO-dehydragenase (17) in analogy to the reverse process, the production of acetate from CO and CH_3-H_4folate in acetogenic bacteria, a process which also occurs with net retention of methyl group configuration (18).

The last reaction, the reduction of CH_3-SCoM to methane by methylreductase involves two unique cofactors, 7-mercaptoheptanoylthreonine phosphate (HTP-SH) and cofactor F_{430} (Figure 7). Based on model studies (19) it is thought



HTP-SH = 7-mercaptoheptanoylthreonine phosphate



Cofactor F_{430}

Figure 7.

that attack of reduced $(Ni^I)F_{430}$ on the methyl-sulfur bond of CH_3-SCoM leads to transfer of the methyl group to the nickel of the cofactor, leaving behind a heterodisulfide of coenzyme M with HTP from which coenzymeM is reductively regenerated. Methane is then generated by protonolysis of the methylated cofactor. The initial transfer of the methyl group should occur with inversion whereas the protonolytic cleavage of the carbon-nickel bond, based on precedent (20) should proceed with retention. Thus, the overall prediction for this mechanism would be replacement of the sulfur of methyl-CoM by hydrogen with net inversion of configuration. The stereochemical analysis of this reaction presents an obvious problem. To generate a chiral version of methane would

require the use of four isotopes of hydrogen but Nature only provides three. Fortunately, although methylreductase is highly substrate specific, it does reduce ethyl-CoM to ethane gas at about 20% of the rate of the methyl compound. Thus, we synthesized R- and S-[1-$^2H_1$,^3H]ethyl-CoM as shown in Figure 8.

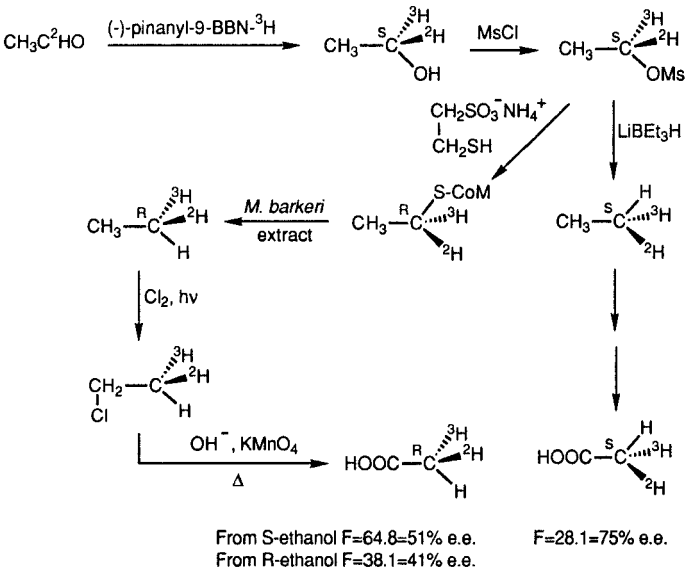


Figure 8.

Incubation of these substrates with cell-free extracts of *Methanosarcina barkerii* gave ethane gas which was converted into acetate for configurational analysis by photochemical halogenation followed by hydrolysis and permanganate oxidation. The configurational analysis of the acetate indicated overall inversion of configuration consistent with the proposed mechanism (21). As a control, a sample of the intermediate stereospecifically labeled ethyl mesylate was converted chemically into ethane and degraded in the same way. As shown in Figure 8, the enantiomeric purity of this acetate sample was substantially higher than that of the enzymatic generated samples, suggesting the possibility of some racemization during the enzyme reaction. Thus, while the overall stereochemistry is consistent with that predicted for a nucleophilic displacement mechanism, the possible racemization during the enzyme reaction suggests

that an alternative mechanism via a methyl radical (22) should also be considered.

The question of a radical intermediate also arises in a reaction which can be viewed as the formal reverse of the methylreductase reaction, the oxidative functionalization of methane to methanol catalyzed by methane monooxygenase (MMO)(23). The enzyme from *Methylosinus trichosporium* consists of three protein components with a nonheme diiron cluster at the active site. Mechanistic proposals for this reaction, which superficially resembles P_{450}-catalyzed hydroxylations, invoke both radial (24,25) and carbocation (25) intermediates as well as concerted oxygen insertion into a substrate carbon-hydrogen bond (26). In the example of methylreductase the weight of the stereochemical evidence for an alkyl radical intermediate is diminished somewhat by the fact that the stereochemical analysis involves a multitude of reactions which leave room for partial racemization to occur, i.e., the observed decrease in the enantiomeric purity of the methyl group could be an artifact of the analytical methodology. We therefore sought more stringent methodology for the stereochemical analysis of the MMO reaction. Since ethane is a good substrate of the enzyme but the product, ethanol, is an inhibitor, we prepared samples of R- and S-[1-$^2H_1$,$^3H_1$]ethane at near carrier-free tritium levels as shown in Figure 9 (27). Samples of the tritiated ethane gas were then converted with MMO into ethanol samples, which were derivatized with O-acetyl-S-mandelic acid and analyzed by tritium NMR spectroscopy. The tritium NMR spectra (Figure 10) cleanly resolve all the tritiated species present in the samples. Integration of the four peaks in the methylene region indicated overall retention of configuration accompanied by 35% inversion. The net retention is consistent with the mechanistic parallel to P_{450} reactions. The high degree of inversion, which is not due to an exchange process, rules out the concerted insertion mechanism and points to the involvement of a planar substrate intermediate with a sufficiently long lifetime to undergo configurational conversion with an appreciable frequency. Since ionic species, i.e., carbocation or carbaniion

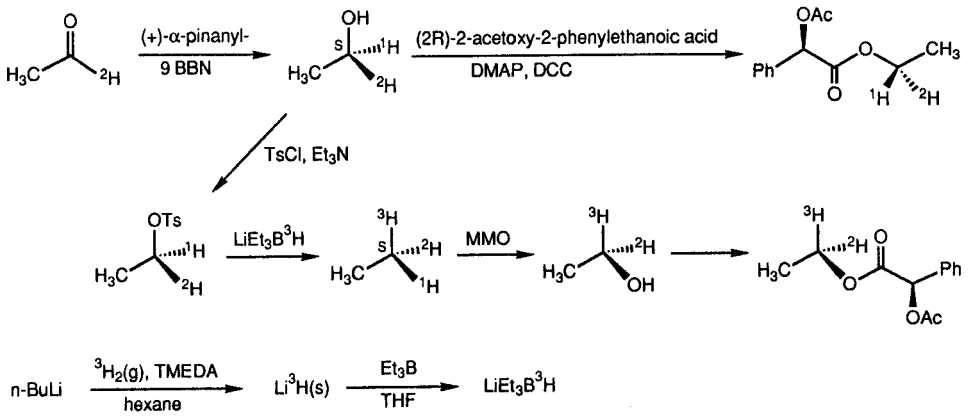


Figure 9.

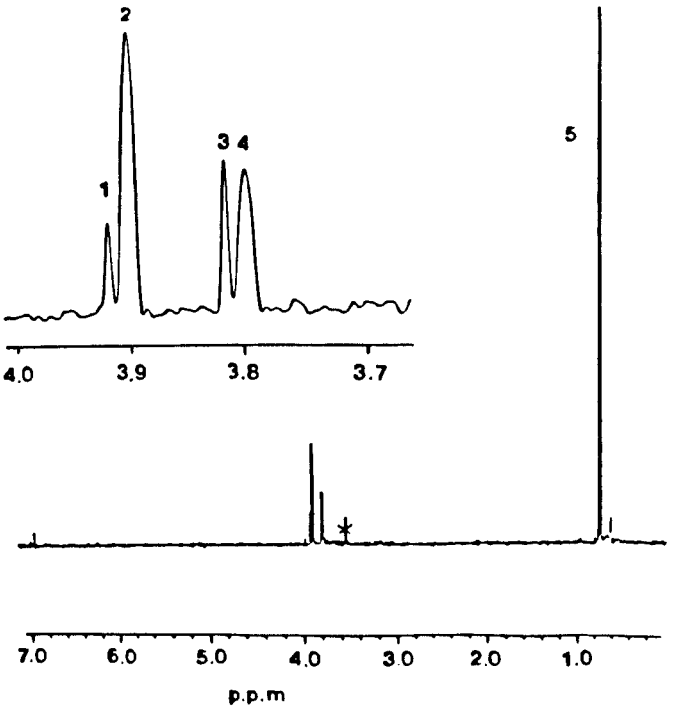


Figure 10. : 320 MHz ^{3}H-NMR spectrum of ethyl (2R)-O-acetylmandelate.
1. R-[1-^{3}H]ethyl; 2. R-[1-^{2}H$_1$, 1-^{3}H]ethyl; 3. S-[1-^{3}H]ethyl; 4. S-[1-^{2}H$_1$, 1-^{3}H]ethyl;
5. S-[2-^{2}H$_1$, 2-^{3}H]ethyl O-acetylmandelate.

intermediates, would have a high barrier to such inversion due to ion pair interactions with charged groups in the enzyme active site, this finding strongly supports a substrate radical intermediate as the only species that would have a very low barrier to configurational inversion. This methodology, in cases where it is applicable, is probably a far more sensitive indicator for radical intermediates in enzyme reactions than some of the diagnostic substrates traditionally used for this purpose.

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