Table IV. Molar Enhancement Factors ($X10^{-2}$)[a] for Selected Raman Bands of the Aromatic Amino Acids[b] at the Indicated Excitation Wavelengths

	tryptophan	tyrosine	phenylalanine	histidine
selected band (cm^{-1})	1016	1617	1000	1575
200 nm	29	202	36	8
218 nm	506	126	96	32
240 nm	53	30	8	4

[a] Measured relative to the 3400 cm^{-1} H_2O Raman band. See ref 5a for details of the calculation. [b] Values for tryptophan, tyrosine, and phenylalanine from ref 7.

tyrosine, and tryptophan at concentrations which are the same as those of the amino acid constituents of the stellacyanin solution. Stellacyanin contains five phenylalanine, seven tyrosine, three tryptophan, and four histidine residues;[27] two of the histidine imidazoles are believed to be coordinated to the single Cu atom in the protein.[28] At this wavelength the spectrum is dominated by tryptophan bands. It is possible to discern histidine bands at 1165 and 1324 cm^{-1} in the amino acid mixture (Figure 13; see inset) because they are eliminated at low pH, as expected from the spectrum of $HisH_2^+$ (see Figure 1). They are extremely weak, however, and the remaining HisH bands are covered up by bands from the other amino acids, as are all the $HisH_2^+$ bands at low pH. The stellacyanin spectra (Figure 12) show no pH-associated changes that can be attributed to histidine. (Minor changes can be seen in tryptophan bands at 877 and 1350 cm^{-1}, which may be due to altered solvent exposure).[6b] Even the 1165- and 1324-cm^{-1} HisH bands detectable in the spectrum of the amino acid mixture are lost in the noise for stellacyanin. We conclude that the prospects are not good for monitoring histidine in proteins via RR spectroscopy at the currently available UV wavelengths.

Acknowledgment. We thank Professor Harvey Schugar for helpful discussions. This work was supported by NIH Grant GM13498.

Registry No. ImH, 288-32-4; ImH_2^+, 17009-90-4; 4-MeImH, 822-36-6; HisH, 71-00-1; $HisH_2^+$, 70805-60-6; $Cu(ImH)_4^{2+}$, 24349-51-7.

(27) Bergman, C.; Candrik, E. K.; Nyman, P. O.; Strid, L. *Biochem. Biophys. Res. Commun.* **1977**, *77*, 1052.

(28) Peisach, J.; Powers, L.; Blumberg, W. E.; Chance, B. *Biophys. J.* **1982**, *38*, 277.

Measurement of Deuterium Kinetic Isotope Effects in Organic and Biochemical Reactions by Natural Abundance Deuterium NMR Spectroscopy

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Abstract: Natural abundance deuterium NMR spectroscopy is a powerful and convenient tool for the estimation of deuterium kinetic isotope effects in organic reactions, obviating in many cases the preparation of isotopically enriched reactants for such measurements. Determinations of the primary or secondary kinetic isotope effects for a broad variety of reaction types are described to illustrate this technique.

Molecules enriched with isotopes of hydrogen are often employed in the diagnosis of reaction pathways in organic chemistry and biochemistry.[2] The isotopic label must be present in quantities great enough to permit easy detection, and, in most circumstances, a knowledge of the location of the isotope in the starting material and product(s) is essential for the correct interpretation of the experiments performed. Unfortunately, the most commonly used methods for detection of deuterium (mass spectrometry) and tritium (liquid scintillation counting), while very sensitive, do not usually permit the unambiguous assignment of the precise sites of isotopic substitution. For this reason, the synthesis of organic compounds specifically labeled with deuterium or tritium has become a frequently necessary if laborious task for the mechanistic chemist or biochemist. However, deuterium is present at low concentrations (~0.015%)[3] in all ordinary hydrogen-containing compounds. A satisfactory method for quantitation of this isotope at each site in a molecule would eliminate, in many cases, the requirement for specific isotopic enrichment.

In fact the great sensitivity and chemical shift dispersion achievable with modern high field NMR instrumentation permit the relatively easy assessment of the amount of deuterium at specific molecular sites, and it is now possible to employ deuterium at natural abundance as an isotopic tracer, with detection of the tracer by ^2H NMR spectroscopy. By using this method Martin et al.[4] have shown that there can be dramatic variations in the deuterium distributions within identical compounds from different sources. These variations must reflect the differing chemical histories of these molecules as illustrated for anethole samples[5a] and for camphor samples[5b] of natural and synthetic origins. Martin's group has further demonstrated the feasibility of using these variations from the statistical abundance of deuterium for determination of the origin of alkyl groups in organic reactions.[6] In this paper we explore the use of natural abundance deuterium NMR for the measurement of kinetic isotope effects (KIE's) in organic reactions.[7]

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(2) Several excellent reviews are found in: *Isotopes in Hydrogen Transfer Processes*; Buncel, E., Lee, C. C., Eds.; Elsevier: New York, 1976.

(3) Garson, M. J.; Staunton, J. *Chem. Soc. Rev.* **1979**, *8*, 539-561.

(4) Martin, G. J.; Martin, M. L. *Tetrahedron Lett.* **1981**, *22*, 3525-3528.

(5) (a) Martin, G. J.; Martin, M. L.; Mabon, F.; Brieout, J. *J. Am. Chem. Soc.* **1982**, *104*, 2658-2659. (b) Grant, D. M.; Curtis, J.; Croasmun, W. R.; Dalling, D. K.; Wehrli, F. W.; Wehrli, S. *J. Am. Chem. Soc.* **1982**, 104, 4492-4494.

(6) Martin, G. J.; Martin, M. L.; Mabon, F.; Michon, M.-J. *J. Chem. Soc., Chem. Commun.* **1982**, 616-617.

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Theory

When deuterium is employed at natural abundance for the measurement of KIE's, only a small fraction of the substrate molecules contain any deuterium at the site(s) of interest, and it is impossible to measure directly the absolute rate of reaction of the deuterated species. This situation is entirely analogous to that for the measurement of KIE's by using tritium as a tracer; either (a) there is only one possible site of reaction in a given molecule, and therefore only intermolecular competititon between isotopic sites occurs, or (b) there are two or more possible sites of reaction in the substrate molecule, and both intermolecular and intramolecular competition between isotopic sites occurs. In all of the cases which are examined here, the second condition obtains.

This situation has been analyzed in detail by Melander and Saunders[8,9a] for the general hydrogen-transfer reaction scheme below in which secondary isotope effects are ignored.[9b] These

$$X^1H_{n-1}{}^2H \begin{cases} \xrightarrow{(n-1)k_H} Y^1H_{n-2}{}^2H + Z^1H \\ \xrightarrow{k_D} Y^1H_{n-1} + Z^2H \end{cases} \quad (1)$$

$$X^1H_n \xrightarrow{nk_H} Y^1H_{n-1} + Z^1H$$

authors define a series of parameters R which are the "ratios between the numbers of isotopic molecules", in this case, the number of molecules containing a deuterium at a site of interest divided by the number of molecules containing only protium. In principle, one may determine the relative rates of reaction of the two kinds of isotopic sites (k_H/k_D) by comparison of the deuterium content of the product which received the transferred hydrogen (R_{ZH}) with the initial deuterium content of the reactant (R_0) or by comparison of the deuterium content of the product which retained the hydrogens not transferred ($R_{YH_{n-1}}$) with R_0. For tracer amounts of deuterium ($R_0 \ll 1$) the isotopic composition of the products is given by[8]

$$\frac{R_{YH_{n-1}}}{R_0} = \frac{(n-1)\beta}{(n-1)\beta+1}\frac{1}{F}[1-(1-F)^{1(n-1)\beta+1]/n\beta}] \quad (2)$$

$$\frac{R_{ZH}}{R_0} = \frac{1}{(n-1)\beta+1}\frac{1}{F}[1-(1-F)^{1(n-1)\beta+1]/n\beta}] \quad (3)$$

where $\beta = k_H/k_D$, and F is the fractional amount of reaction of the isotopic species. Unfortunately, it is impractical to determine R_{ZH} when, as in many cases, hydrogen has been transferred to a solvent molecule. At complete consumption of the isotopic substrate ($F = 1$), eq 2 reduces to

$$\frac{R_{YH_{n-1}}}{R_0} = \frac{(n-1)\beta}{(n-1)\beta+1} \quad (4)$$

The ratio $R_{YH_{n-1}}/R_0$ is the value obtained from deuterium NMR measurements, and it is greatest when $F = 1$, so we attempt to make most of our determinations at this point. However, it must be remembered that $R_{YH_{n-1}}/R_0$ varies most rapidly in the region near $F = 1$,[8] so that care must be taken to ensure that the reaction is driven to completion.

In a minority of cases, it is possible to determine both R_{ZH} and $R_{YH_{n-1}}$. Combining eq 2 and 3 we have

$$\frac{R_{YH_{n-1}}}{R_{ZH}} = (n-1)\beta = (n-1)k_H/k_D \quad (5)$$

(7) A part of this work has been reported previously in a preliminary communication: Pascal, R. A., Jr.; Baum, M. W.; Wagner, C. K.; Rodgers, L. R. *J. Am. Chem. Soc.* **1984**, *106*, 5377-5378.

(8) Melander, L.; Saunders W. H. *Reaction Rates of Isotopic Molecules*; John Wiley & Sons: New York, 1980; pp 91-111.

(9) (a) Equations 1, 2, and 3 are taken directly from ref 8. Those authors used the general variables $A_{(1)}$ and $A_{(2)}$ to indicate the isotopic species; however, they have been changed to ^1H and ^2H in this work to reflect the fact that hydrogen isotopes are involved in the present discussion. (b) As will be seen, most secondary isotope effects are smaller than the experimental uncertainties in the method discussed herein; however, in some cases secondary effects may introduce a small but significant systematic error in the primary isotope effect determinations.

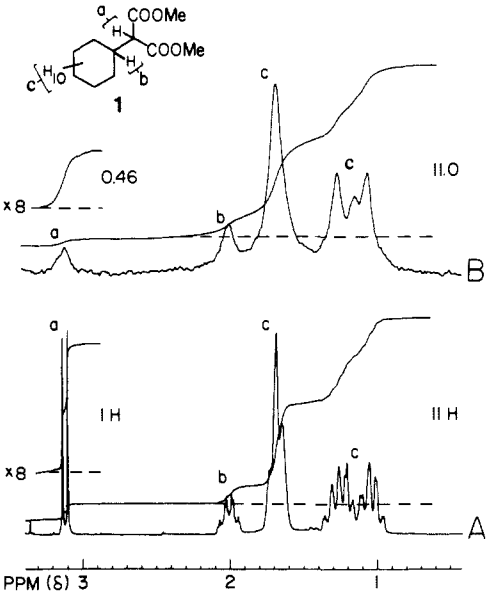


Figure 1. 250-MHz ^1H NMR spectrum (A) and 38.4-MHz natural abundance ^2H NMR spectrum (B) of dimethyl 2-cyclohexylmalonate produced by photolysis of dimethyl diazomalonate in excess cyclohexane.

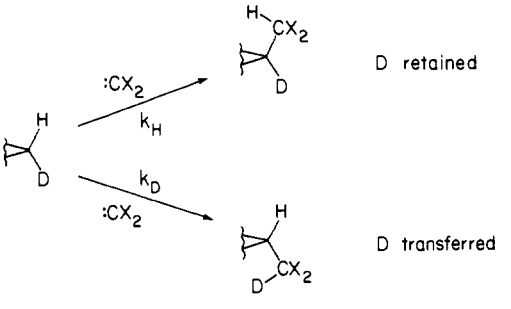
The value of F is irrelevant, and direct determination of the KIE is very simple.

The preceding analysis is applicable only when there can be intramolecular competition between hydrogen isotopes, but tracer methodology may also be used where there is only intermolecular competition between isotopic sites. The latter situation also has been analyzed by Melander and Saunders,[8] and in most cases a determination of the change in the deuterium content of recovered unreacted isotopic substrate at a known fractional conversion would be required, a task for which ^2H NMR is certainly also suitable. Unfortunately, in this situation the isotopic composition of the recovered substrate is relatively insensitive to k_H/k_D when the isotope effect is large, so that this method would be appropriate only for measurement of secondary or small primary effects.[8]

Results and Discussion

A. Use of Natural Abundance ^2H NMR for the Measurement of Primary KIE's in Specific Organic Reactions. For reactions which may be carried out conventiently on a multigram scale, natural abundance ^2H NMR provides a convenient method for the measurement of kinetic isotope effects without the need to prepare isotopically enriched compounds. The following three reactions, for which KIE's have also been determined by using conventional techniques, demonstrate the ease with which such measurements may be made.

Primary Deuterium KIE for the Insertion of Dicarbomethoxycarbene into a C–H Bond of Cyclohexane. The concerted reactions of singlet carbenes with C–H bonds are particularly well-suited for analysis by our ^2H NMR method because both the transferred and retained hydrogens are found in the same product. The ^1H



and ^2H NMR spectra of dimethyl 2-cyclohexylmalonate (**1**) prepared by photolysis of dimethyl diazomalonate in the presence of excess cylohexane are illustrated in Figure 1. The deuterium resonances are readily assigned by comparison with the proton

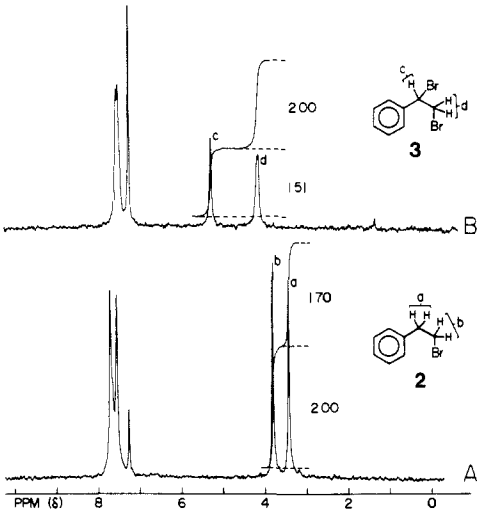


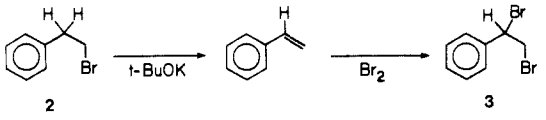
Figure 2. 38.4-MHz natural abundance ^2H NMR spectra of (2-bromoethyl)benzene (spectrum A) and styrene dibromide prepared by elimination and bromination of (2-bromoethyl)benzene (spectrum B). The peaks at δ 7.26 are due to chloroform.

spectrum: the signals at δ 0.9–2.1 are due to the cyclohexyl group, while the peaks at δ 3.1 are those of the transferred hydrogen. Comparison of the peak areas in both spectra indicates that the rate of deuterium transfer has been roughly half as great as that of protium. If the integral of the retained cyclohexyl deuterium is set to 11.0 in arbitrary units, the integral of the transferred deuterium is 0.46, and the ratio $R_{YH_{n-1}}/R_{ZH}$ is simply 11.0/0.46. Cyclohexane has 12 chemically equivalent sites, so, from Eq 5

$$k_H/k_D = \frac{R_{YH_{n-1}}/R_{ZH}}{n-1} = \frac{11.0/0.46}{12-1} = 2.2$$

Data from three separate experiments gave an average value of k_H/k_D of 2.1 ± 0.1. In a control experiment, dimethyl diazomalonate was photolyzed in the presence of 1:1 cyclohexane:cyclohexane-d_{12}; analysis of the reaction mixture by GC–MS showed the ratio of protic and deuterated products to be 2.2, in good agreement with the ^2H NMR experiments.

Primary Deuterium KIE for the Dehydrobromination of (2-Bromoethyl)benzene. The ^2H NMR spectra of (2-bromoethyl)-benzene (**2**) and the dibromide derivative of styrene **3** generated by potassium *tert*-butoxide treatment of **2** are displayed in Figure



2

3

2. The initial reaction conditions were very similar to those used by Saunders and Edison,[10] and the reaction was carried to completion. The C-2 deuterium resonance is employed as an integration standard in both spectra, and its integral is set to 2.00 arbitrary units. The two C-1 hydrogens of the starting material **2** contain a total of 1.70 units of deuterium, but the single remaining C-1 hydrogen in the product **3** contains almost as much, 1.51 units. Clearly there has been a strong selection against deuterium transfer in this reaction. For the determination of k_H/k_D, eq 4 must be used since the transferred hydrogen has been lost to the solvent. We note that $n = 2$, and the key ratio $R_{YH_{n-1}}/R_0$ is simply 1.51/1.70; there is no need to calculate the absolute deuterium concentrations. Rearranging eq 4 we have

$$k_H/k_D = \frac{R_{YH_{n-1}}/R_0}{(1 - R_{YH_{n-1}}/R_0)(n-1)} = \frac{1.51/1.70}{(1 - 1.51/1.70)(2-1)} = 7.9$$

(10) Saunders, W. H., Jr.; Edison, D. H. *J. Am. Chem. Soc.* **1960**, *82*, 138–142.

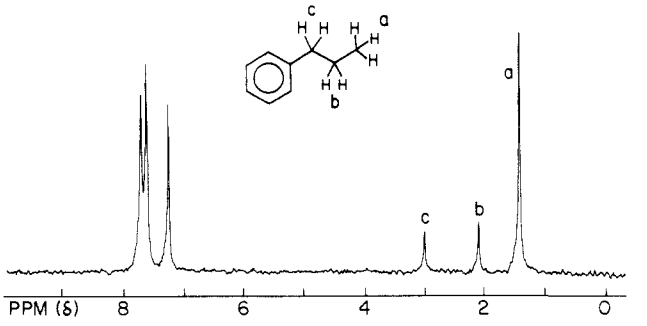
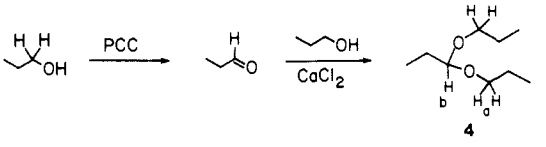


Figure 3. 38.4-MHz natural abundance ^2H NMR spectra of *n*-propylbenzene prepared by the catalytic hydrogenation of 1-phenylpropyne.

Triplicate measurements (on the same sample) gave $k_H/k_D = 7.9 \pm 0.8$, in excellent agreement with the literature value of 7.9 ± 0.5[10] obtained under similar conditions.

Primary KIE for the Oxidation of 1-Propanol to Propanal by Pyridinium Chlorochromate. In the preceding example, the deuterium contents of the starting material and one product were compared in order to obtain the KIE, and so it was necessary (a) to record two separate ^2H NMR spectra, (b) to find a suitable peak in each spectrum to act as an integration standard, and (c) to measure and compare the intensities of the two isotopically important resonances as well as the standards. The opportunities for error are greatly reduced if all the required data are obtainable from a single spectrum. The simple approach of adding equal amounts of the two materials to the same sample tube is not the best solution because of possible measurement errors, selective decomposition or evaporation of one of the compounds during the experiment, or reaction of the two components with each other. However, it is often possible to prepare a derivative which contains the isotopically interesting fragments of both molecules, and the preceding potential diffuculties may be avoided. Of course, one must also be careful that the derivatization procedure does not result in further isotopic fractionation at the sites of interest!



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Thus, when we measured the KIE for the oxidation of 1-propanol with pyridinium chlorochromate, the product propanal was treated with 1-propanol[11] in the presence of a dehydrating agent to give propanal dipropyl acetal (**4**). In this derivative, comparison of the deuterium contents of hydrogens a (R_0) and b ($R_{YH_{n-1}}$) gave the KIE for the reaction: $k_H/k_D = 6.7$. The KIE for the oxidation of 1-propanol by pyridinium chlorochromate has not been reported, but k_H/k_D for the oxidation of ethanol with the same reagent is 5.71,[12] which agrees reasonably well with our data.

B. Use of Natural Abundance ^2H NMR to Search for Unusual Fractionation of Hydrogen Isotopes. Natural abundance ^2H NMR is perhaps best used to identify unusual or unexpected kinetic isotope effects by screening readily available compounds for unusual deuterium distributions. Such site-specific variations in deuterium content can be quite dramatic and may provide unexpected information concerning the origins of the substances in question. The following examples illustrate the use of ^2H NMR for the identification, if not necessarily the precise quantitation, of KIE's in a few organic and biological reactions.

Multiple KIE's in the Preparation of *n*-Propylbenzene. The ^2H NMR spectrum of *n*-propylbenzene, prepared by catalytic hy-

(11) The same bottle of *n*-propanol was used for the oxidation and acetal formation. Materials from the same lots must always be used for such comparisons, since the deuterium content of a given lot of some chemical is dependent on its method of synthesis and the origin of the starting materials for that synthesis.

(12) Banerji, K. K. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 2732–2734.

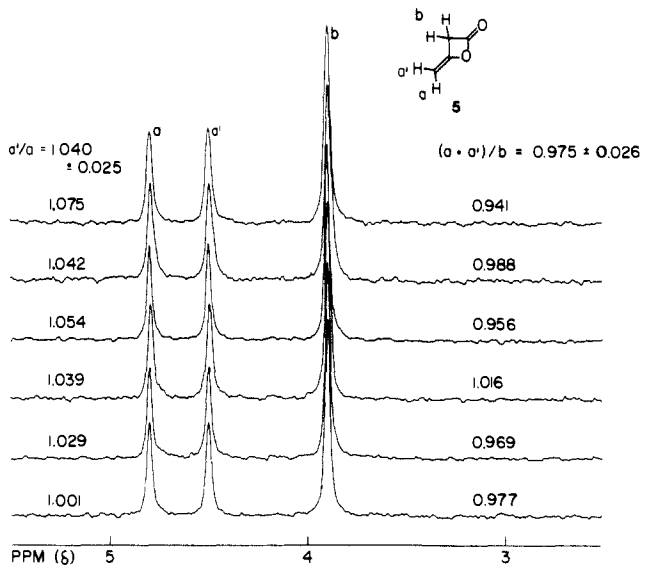
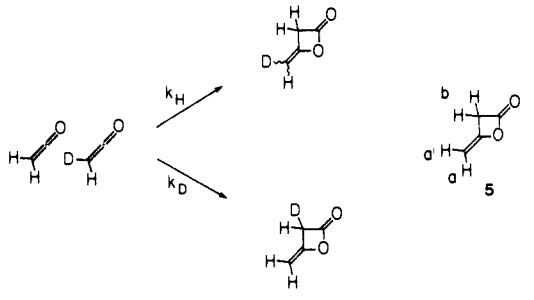


Figure 4. 38.4-MHz natural abundance ^2H NMR spectra of diketene. The ratios of the integrals of the resonances are indicated in the figure.

drogenation of 1-phenylpropyne, is illustrated in Figure 3. In the the absence of any KIE's, one would expect the three propyl resonances (a, b, and c) to integrate in the ratio 3:2:2, but in fact the newly formed methylene groups are remarkably depleted in deuterium, and the ratio of integrals is approximately 3:0.6:0.6. This results from descrimination against deuterium in at least two steps in the chemical history of the methylene hydrogens, i.e., (1) during the electrolysis which generated the hydrogen gas used in the hydrogenation and (2) during the hydrogenation process itself.

Secondary Isotope Effects on the Dimerization of Ketene. The sensitivity of natural abundance ^2H NMR is clearly insufficient to permit the measurement of secondary KIE's in the presence of primary KIE's. Even when there is no interference from a primary effect, the circumstances must be quite favorable to obtain a significant result. Diketene (**5**) is perhaps an ideal compound



for such a study; the molecule is very small, and the three kinds of hydrogens are well resolved in the ^2H NMR. Figure 4 contains six 2000-scan ^2H NMR spectra of the same sample of purified diketene. The ring methylene hydrogens (b) are consistently enriched in deuterium compared to the exo methylene hydrogens (a plus a'), suggesting the presence of an inverse secondary KIE of 0.975 ± 0.026 for the dimerization of ketene. This value is a reasonable one for the cycloaddition, but there appears to be no literature value for comparison. More surprising was the finding that exo methylene hydrogens show unequal deuterium concentrations; the ratio of the integrals of resonances a' and a is 1.040 ± 0.025.[13] The origin of this larger secondary isotope effect is unclear, but this observation highlights one of the main advantages of the ^2H NMR method: the ability to measure simultaneously the deuterium concentration at a variety of positions in a molecule enhances the chance of discovery of unanticipated isotope effects.

(13) We have tentatively assigned resonance a' to the hydrogen cis to C-2.

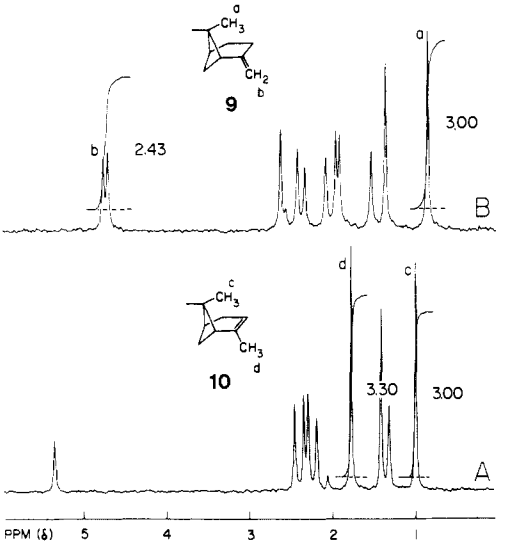
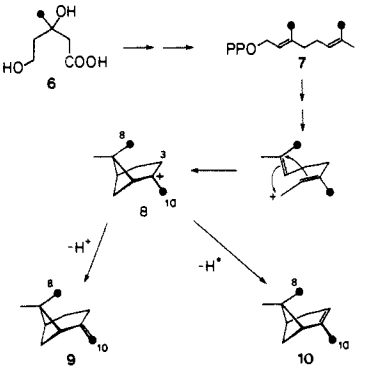


Figure 5. 38.4-MHz natural abundance ^2H NMR spectra of (−)-α-pinene (spectrum A) and (−)-β-pinene (spectrum B).

Kinetic Isotope Effects in the Biosynthesis of the Pinenes. Consider the biosynthesis of β-pinene (**9**) from mevalonic acid (**6**), which is illustrated in an abbreviated form below.[14] The last



step in this process is the deprotonation of the pinane cation (**8**) to give the exo methylene group (C-10) of β-pinene. Both C-8 and C-10 of the intermediate **8** are derived from the methyl group of mevalonic acid and therefore should have identical deuterium contents.[15] The vast majority of these molecules will have no deuterium at C-10, of course, but about 0.045% will have exactly one deuterium on this methyl group. If there is a primary deuterium KIE for the deprotonation of **8**, then the deuterium at C-10 will be eliminated less frequently than the statistical expectation, so the two hydrogens of the exo methylene of β-pinene should be enriched with deuterium with respect to the hydrogens of the methyl group C-8. The natural abundance ^2H NMR spectrum of (−)-β-pinene is shown in Figure 5. Peak a is due to the deuterium on C-8, while b is due to the exo methylene (C-10) deuterium.[16] Were there no isotope effect on the deprotonation of the pinane cation, the ratio of the integrals would be 3:2, but in fact a ratio of 3.00:2.43 is observed. Recalling that the C-8 integral gives the initial deuterium concentration for the C-10

(14) Croteau, R. *Biosynthesis of Monoterpenes* in *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; John Wiley & Sons: New York, 1981.

(15) The biosynthetic origin and geometry of the methyl groups of geranyl-PP (**7**), the immediate precursor of β-pinene, has been established: Cornforth, J. W. *Tetrahedron* **1974**, *30*, 1515–1524 and references cited therein.

(16) The ^1H NMR spectra of α- and β-pinene have been completely assigned, and ^1H and ^2H NMR chemical shifts are identical except for a small isotope shift of ~0.02 ppm. (a) Nakagawa, N.; Saito, S. *Tetrahedron Lett.* **1967**, 1003–1007. (b) Wenzel, T. J.; Sievers, R. E. *J. Am. Chem. Soc.* **1982**, *104*, 382–388. (c) Brevard, C.; Kintzinger, J. P. *Deuterium and Tritium*. In *NMR and the Periodic Table*; Harris, R. K., Mann, B. E., Eds.; Academic Press: New York, 1978.

hydrogens, $R_{YH_{n-1}}/R_0 = 2.43/3.00$, $n = 3$, and from eq 4 the apparent k_H/k_D is 2.1.

We emphasize that this value is only a lower limit for the isotope effect on this step, since the cyclization is an enzyme-catalyzed process with a number of reversible steps preceding the isotopically sensitive step. If tracer amounts of a single isotope are employed to determine a KIE in an enzymatic reaction, it is only possible to measure the isotope effect on V_{max}/K_m,[17] a value which is always less than or equal to the intrinsic KIE on the isotopically sensitive step. Our data are no different from that which would be derived from the use of tritium-labeled precursors in feeding experiments, but our result was obtained without the need for the synthesis of specifically labeled compounds, administration of the compounds to a growing plant, or isolation, purification, and degradation of the labeled product. It was only necessary to acquire a sample of the pure natural product and record its ^2H NMR spectrum.

β-Pinene is derived from cation **8** by elimination of one of the C-10 hydrogens, but α-pinene (**10**) is formed from the same intermediate by elimination of one of the C-3 hydrogens. Interestingly, Gambliel and Croteau have presented data suggesting that a single enzyme in sage catalyzes the formation of both (−)-α-pinene and (−)-β-pinene.[18] If this is also true in pine trees (from which commercial samples of (−)-α- and (−)-β-pinene are obtained) then the presence and location of the deuterium in cation **8** should influence the partitioning of **8** into the two pinenes. Specifically, if there is a single deuterium bound to C-10 of **8**, and the ratio of the rates of reaction of the protic and deuterated sites is 2.1, then β-pinene formation should be slowed by about 17% relative to its rate of formation from all-protic **8**.[19] We therefore expect the C-10 hydrogens of α-pinene to be enriched in deuterium relative to those at C-8 since the latter are not involved in this fractionation. Figure 5 shows the natural abundance ^2H NMR spectrum of (−)-α-pinene, and the deuterium content at C-10 is about 10% greater than at C-8, suggesting that such a fractionation does indeed occur. It should be noted that a similar intramolecular isotope effect apparently has been observed previously during studies on the biosynthesis of (−)-longifolene and (−)-sativene from tritiated mevalonates in *Helminthosporium* sp.[20]

Conclusion

Natural abundance ^2H NMR may be employed for the measurement of primary kinetic isotope effects in a wide variety of chemical reactions without recourse to the synthesis of isotopically enriched reactants. One need only perform the reaction of interest, isolate the appropriate product(s), and examine the product(s) (and often also a reactant) by ^2H NMR. The main practical limitations are imposed by the low sensitivity and chemical shift dispersion of ^2H NMR. With a spectrometer operating at 38.4 MHz (250 MHz for ^1H NMR), 5 mmol of sample are desirable for a good signal-to-noise ratio, and the resonances of interest should be separated from intefering signals by about 0.2 ppm for accurate integration. Of course, both requirements are greatly relaxed if one can record spectra at 76.8 MHz (500 MHz for ^1H). This method should be useful for the study of the very large number of ordinary organic reactions which can be carried out conveniently on a sufficiently large scale. For the study of enzymatic processes the requirement for large samples is a more serious limitation, but for natural products which can be obtained in gram quantities, natural abundance ^2H NMR can provide a window through which to examine isotopic fractionation in complex metabolic pathways.[21]

Experimental Section

General Methods. Melting points were recorded on an Electrothermal apparatus and are uncorrected. Proton NMR spectra were recorded on Bruker WM-250 or IBM NR-80 spectrometers. Chemical shifts are reported as parts per million downfield (δ) from tetramethylsilane. Coupled gas-liquid chromatography–mass spectrometry (GC–MS) was performed on a Hewlett-Packard 5992 system; an electron beam energy of 70 eV was used.

Materials. (2-Bromoethyl)benzene, β-pinene, α-pinene, cyclohexane (spectrophotometric grade), cyclohexane-d_{12}, 1-propanol (spectrophotometric grade), 1-phenyl-1-propyne, and pyridinium chlorochromate were purchased from Aldrich and used without further purification. Potassium and bromine were obtained from Baker, *tert*-butyl alcohol from Mallinckrodt, and benzene, methylene chloride, chloroform, and carbon tetrachloride from Fisher. Diketene (Aldrich) was purified by distillation and crystallization by partial freezing as described previously.[22] Dimethyl diazomalonate was prepared as described by Hendrickson and Wolf.[23]

Deuterium NMR Spectroscopy. The 38.4-MHz ^2H NMR spectra were recorded on a Bruker WM-250 Fourier transform spectrometer. Samples were prepared as 60–95% solutions in chloroform or carbon tetrachloride in 10 mm (o.d.) tubes. Spectra requiring 2000–16000 scans were obtained by using a 90° pulse and a 4-s data acquisition of 4096 points with broad-band ^1H decoupling at 5 W of power. Free induction decays were weighted by using a 0.3 Hz line broadening and, in some cases, zero-filled to 8192 points prior to Fourier transformation. The spectrometer was operated in the unlocked mode, so to compensate for the slight drift in the magnetic field, FID's were acquired in blocks of 1000 scans. The blocks were individually transformed, and the resulting spectra were aligned and added. The line widths ($\Delta\nu_{1/2}$) for isolated singlets in the finished spectra were usually in the range of 0.8–2.0 Hz; the lone exception was the spectrum of compound **1**, where $\nu_{1/2} = 5$ Hz. Peak areas were measured by digital integration and by the cut-and-weigh method. There was no significant difference in the areas obtained by the two methods. Furthermore, neither exponential weighting nor zero-filling of the FID's had a significant effect on the relative peak areas, nor did insertion of a delay between scans. For most samples, chemical shifts were reported relative to the chloroform solvent peak, which was taken as δ 7.26.

Dimethyl Cyclohexylmalonate (1) from Dimethyl Diazomalonate and Cyclohexane. The following modification of the procedure of Ledon et al.[24] was used for the preparation of **1**. A solution of dimethyl diazomalonate (20 g), cyclohexane (1000 mL), and benzene (200 mL) at reflux (80 °C) in a Pyrex vessel was irradiated with a General Electric 275-W mercury vapor sun lamp for 72 h. Dimethyl cyclohexylmalonate (**1**), the major product (>90% by GC analysis on 3% OV-17, 120–200 °C), was isolated by careful fractional distillation: ^1H NMR ($CDCl_3$) δ 1.16 (m, 5 H, ring CH_2's), 1.69 (m, 5 H, ring CH_2's), 2.09 (m, 1 H, ring CH), 3.18 (d, 1 H, $J = 9$ Hz, $CH(COOMe)_2$), 3.72 (s, 6 H, ester CH_3's).

The control KIE determination in which dicarbomethoxycarbene was added to a 1:1 mixture of protic and deuterated cyclohexane was carried out in the following way. Dimethyl diazomalonate (40 mg), cyclohexane (1.00 mL), cyclohexane-d_{12} (1.00 mL, 99.5 atom % D), and benzene (0.40 mL) were irradiated as described above. The reaction mixture was analyzed directly by GC–MS (Hewlett-Packard 5992 GC–MS system; 3% OV-17 on Chromosorb W-HP, 150 °C; 70 eV electron beam energy). Mass spectra were recorded repeatedly at close intervals across the entire peak of the emerging product dimethyl cyclohexylmalonate (there is a slight fractionation of the protic and deuterated species on the GC column), and the isotopic composition of the mixture was determined from a summation of these spectra.

Styrene Dibromide (3) from (2-Bromoethyl)benzene (2). Potassium (2.5 g, 0.063 mol) was dissolved in *tert*-butyl alcohol (470 mL). Phenethyl bromide (9.0 g, 0.049 mol) was added, and the solution was left at room temperature for 144 h. Saturated aqueous sodium chloride (100

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(19) The ratio of the rates of the C-10 monodeuterated cation and the normal cation (k_{CH_2D}/k_{CH_3}) is given by

$$\frac{k_{CH_2D}}{k_{CH_3}} = \frac{2k_H + k_D}{3k_H} = \frac{2 + k_D/k_H}{3} = \frac{2 + 1/2.1}{3} = 0.83$$

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(21) It must also be remembered that, except for reactions at methyl groups, intramolecular competition between isotopic sites is usually not possible in enzymatic reactions. For example, there is no possibility of intramolecular competition at the methylene group of ethanol in a reaction catalyzed by alcohol dehydrogenase, since this enzyme acts on only one of the two enantiopic hydrogens.

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mL) was added, and the mixture was filtered. Hexane was added to the filtrate, and after shaking the organic layer was separated and dried over magnesium sulfate. The resulting solution was treated with bromine (6 g) in methylene chloride. Evaporation of the solvent left a white crystalline residue. Recrystallization from hexane gave styrene dibromide (4.7 g), mp 70.5-73.5 °C (lit.[25] 72-73 °C).

Propanal Dipropyl Acetal (4) from 1-Propanol. To a stirred mixture of pyridinium chlorochromate (48 g, 0.22 mol), Celite (15 g), and methylene chloride (200 mL) cooled in a water bath at room temperature (21 °C) was added 1-propanol (9.0 g, 0.15 mol). After 1.5 h, anhydrous ether (200 mL) was added, and the supernatant solution was decanted from the residual black gum. The black gum was extracted twice more with 100-mL portions of ether, and the combined organic phases were filtered through a column of Florisil. Anhydrous calcium chloride (60 g) and 1-propanol (30 mL) were added, and the mixture was left to stand for 24 h with occasional shaking. The mixture was filtered, and the filtrate was washed 5 times with dilute aqueous sodium bicarbonate. After drying over anhydrous sodium sulfate, the solvent was evaporated, and the residual oil was fractionally distilled under reduced pressure to yield pure compound **4** (3.2 g): ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3 H, J = 7 Hz, $CHCH_2CH_3$), 0.94 (t, 6 H, J = 7 Hz, $CH_2CH_2CH_3$), 1.60 (m, 6 H, CH_2CH_3), 3.46 (m, 4 H, OCH_2), 4.40 (t, 1 H, J = 6 Hz, OOCH).

***n*-Propylbenzene from 1-Phenyl-1-propyne.** A mixture of 1-phenyl-1-propyne (5 g), methanol (25 mL), and 5% palladium on carbon (0.2 g) was hydrogenated at 40 psi at room temperature for 12 h. The catalyst was filtered off, and the product *n*-propylbenzene (bp 159 °C) was isolated by distillation.

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Registry No. :$C(CO_2Me)_2$, 15274-66-5; $C_6H_5(CH_2)_2Br$, 103-63-9; $Me(CH_2)_2OH$, 71-23-8; $C_6H_5C{\equiv}CMe$, 673-32-5; cyclohexane, 110-82-7.

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Multiple-Quantum-Filtered Two-Dimensional Correlated NMR Spectroscopy of Proteins

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Abstract: The use of multiple-quantum-filtered two-dimensional correlated spectroscopy (MQF-COSY) is investigated with regard to the identification of amino acid spin systems in proteins. In addition to a simplification of the spectra by the use of multiple-quantum filters, the multiplet structures and symmetry properties of the cross-peaks in MQF-COSY can give new information, which is complementary to that obtained from normal COSY. A catalogue of cross-peak patterns and cross-peak fine structures expected for the common amino acids is presented, and the practical consequences of modified selection rules for MQF-COSY with macromolecules are investigated. Optimized experimental procedures to minimize spectral artifacts and maximize sensitivity in MQF-COSY are described and applied to the protein basic pancreatic trypsin inhibitor (BPTI).

1. Introduction

Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR[1])[2] is now an established tool for detailed structural studies of small proteins and DNA fragments.[3] Indentification of the individual ^{1}H-spin systems corresponding to the building blocks in these biopolymers is an important step in the spectral analysis. The first 2D experiments for structure determinations with proteins used absolute value displays.[4] In the meantime the power of 2D spectroscopy has been enhanced by recording pure phase spectra[5] and by two-quantum filtering.[6] These modifications improve the presentation of the 2D spectra with respect to multiplet resolution and phase properties without loss of relevant information. In spite of these advances it has become desirable to develop means for editing the complex ^{1}H NMR spectra of biological macromolecules into simpler subspectra. This can, for example, be achieved by selective excitation of certain spin systems[7] or by application of multiple-quantum filter techniques.[6,8,9] In contrast to 2D multiple-quantum spectra, where one frequency coordinate represents sums or differences of several chemical shift values, the representation of multiple-quantum filtered (MQF) COSY spectra does not differ from that of ordinary COSY. The spectra can therefore be analyzed with the same approach, and spectra obtained with p-quantum filters (pQF's) of different order p can be compared by simple superposition.

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(1) Abbreviations used: 2D NMR, two-dimensional nuclear magnetic resonance spectroscopy; rf, radio frequency; FID, free induction decay; COSY, 2D correlated spectroscopy; MQF, multiple-quantum filter(ed); *p*QF, multiple-quantum filter of order *p*, with p = 2, 3, 4, ...; MQC, multiple-quantum coherence; *p*QC, multiple-quantum coherence of order *p*; BPTI, basic pancreatic trypsin inhibitor (Trasylol, Bayer AG, Leverkusen); TSP, trimethylsilylpropionic acid.

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