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## STRUCTURAL BIOLOGY DIVISION

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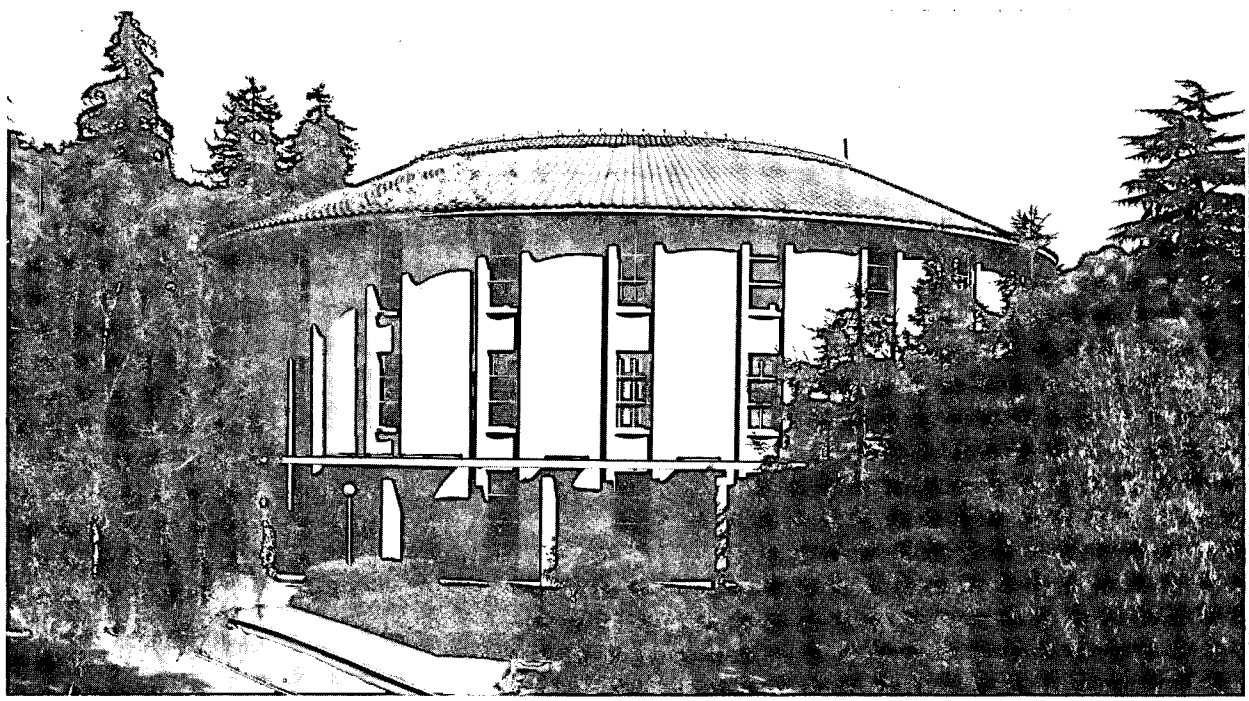



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# Tritium NMR

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Contribution for the Encyclopedia of NMR

1. Article Title

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TRITIUM NMR §

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## 1. Background

The fundamental magnetic properties of tritium ($^3H$) were first reported in 1947[1-3] in a series of NMR measurements which demonstrated the triton to be a spin 1/2 nucleus, with a positive magnetic moment about 6.66 % larger than that of the proton. Despite the fact that these measurements established tritium as the most sensitive NMR active nucleus, very little research was conducted over the next two decades. In short, we have found only 24 reports of tritium spectra in the first 30 years, and 14 of those were published in the period 1974-1976. The lack of activity in the early years of tritium NMR development is readily rationalized – tritium is a radioactive nucleus, and with the limited sensitivity of early spectrometers, the quantities required to make the 1947 measurements were enormous.¶ Hence, applications were limited to U.S. National Laboratories, where facilities existed for obtaining and handling large quantities of tritium.

The first liquids $^3H$ NMR spectrum other than HTO was described in 1964,[4] and set several important precedents for the newly developing field: i). samples required large amounts of tritium (*ca.* 370 GBq in this instance), ii). instrumental modification for $^3H$ detection was simple, iii). proton-tritium couplings were obvious and readily measured, and iv). the labelling pattern (or percentage of $^3H$ in various positions) was accessible from the spectrum. The sample used in this experiment was ethylbenzene prepared by metal-catalyzed reduction of phenylacetylene with tritium gas, and interestingly, the integrated ratio of methylene to methyl tritium was 1:1.5, not 1:1 as predicted. This result established high resolution $^3H$ NMR as the tool of choice for analysis of tritiated products. The 'non textbook' labelling pattern would have been difficult to detect and almost certainly overlooked using the conventional methods of the time, and incorrect assumptions about the labelling pattern would have affected downstream uses

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¶ No specific details of sample sizes were given, but 50 μL of HTO with 50 % abundance of $^3H$ contains *ca.* 3,000 GBq of radioactivity, and we estimate that the samples must have been of this order *i.e.* 1,850-18,500 GBq (50-500 Ci).

of the product. Indeed, biochemical applications of tritium have been slow to recover from the poorly characterized materials used in the 1950's and 1960's, when $^3H$ NMR was not available for product analysis.

Such demonstrations of the power of $^3H$ NMR spectroscopy led to a renewed interest in technical development in the late 1960's and early 1970's. Fortunately, the resurgence coincided with advances in instrumentation that allowed many other low abundance and/or low sensitivity nuclei to be routinely observed. This revolution in NMR spectrometer sensitivity was crucial. Since tritium sensitivity is >75 times that of $^{13}C$, the ability to collect $^{13}C$ spectra at natural abundance clearly implies that tritium spectra could be obtained at the very low $^3H$ concentrations necessary to ensure the technique is safe and accessible to laboratories around the world. During this period, the sample requirement of 370-3,700 GBq was drastically reduced to the range of 3.7-3,700 MBq, depending on the complexity of the signals and the S/N level required. Of course, tritium detection by NMR still suffers from the inherent insensitivity of the technique, and some perspective can be gained from the fact that liquid scintillation counting (LSC) techniques can detect tritium at a level $10^{-7}$ lower than the highest field spectrometers of today. Yet, the detailed information which NMR yields about the chemical and magnetic environment of the nuclei makes this loss of sensitivity easy to justify.

The most influential force in the promotion of $^3H$ NMR spectroscopy as a routine research tool came from a fruitful collaboration between the University of Surrey and Amersham International. The early published work (1971-76) concentrated on methodology and proof of principle, and in the late 1970's a host of applications were reported. A number of other groups adopted the $^3H$ NMR approach, and by 1985 the Surrey group was able to publish an excellent compilation of techniques and results, describing a mature field.[5] In this article, we will discuss the major features of $^3H$ NMR spectroscopy, and address the more recent, popular and significant chemical applications.

## 2. Safety

Tritium is the very rare, but naturally occurring, radioactive isotope of hydrogen. It is a pure β emitter and its 12.4 year half-life is convenient for most chemical or biological experiments. In Table 1 we list a variety of chemical and physical properties of tritium and elementary tritiated compounds.

As mentioned above, the large amounts of tritium necessary for early experiments made containment of tritiated NMR samples a point of major concern, and a number of convenient containment systems have evolved.[5] Since tritium is a 'soft' β emitter, *i.e.* the energy of the $\beta^-$ particle associated with its radioactive decay has very little penetrating power (Table 1, $E_{max}$ = 18.6 keV, 6 μm range in water), it is readily contained by a thin surface of plastic, Teflon, or glass. The major hazard is from ingestion, so care should be taken to keep samples sealed and clean on the exterior. We favour a double encapsulation approach using either a 3 mm glass tube inside a regular NMR tube, or a capped Teflon liner inside a sealed glass tube. These components are all commercially available.

Since the number of tritium nuclei needed for NMR detection exceeds that required for liquid scintillation counting by a factor of *ca.* $10^7$, the monitoring of equipment or personnel for contamination is readily accomplished by standard radiochemical techniques. At the minimum level readily detectable by LSC (37 Bq $L^{-1}$) in a conventional urine analysis, the radiation exposure for an individual is less than 0.001 mSv per year. The sensitivity of LSC thus provides a level of detection well below the average exposure from background, medical and terrestrial sources (3 mSv per year) or the current recommendation for a U.S. Department of Energy radiation worker (< 20 mSv per year). In addition, tritium uptake in the body is cleared as tritiated water (HTO) with a 10 day biological half-life, and this period can be drastically reduced by increasing fluid intake or by diuretics (*e.g.* water, beer or coffee).

The hazards associated with analyzing $^3H$ samples are real, but have been grossly over-emphasized in the past. A rational assessment of the risks involved is in order. Tritium is readily contained and is physiologically benign at the levels used in most high resolution NMR

experiments. When making a benefit/hazard judgement of conducting research on a 2220 MBq (60 mCi) sample contained as described above, it is reasonable to remember that safety lights in aircraft contain up to 370 GBq (10 Ci) of tritium, a marine compass may have as much as 28 GBq (0.75 Ci), and commonly available night sights for rifles contain 2 GBq (50 mCi) of tritium. The spectroscopist may be at greater risk from the lock solvent or other chemical entity in the sample than from the radioactivity, *e.g.* in a 0.25 mL sample containing 2220 MBq of a tritiated compound in $C_6D_6$, there are 2 μmoles $^3H$ *cf.* 2.75 mmoles benzene. It is clearly less hazardous to handle a well-contained tritium sample than milligram quantities of neurotoxins and other biologically active compounds routinely analyzed in normal glass NMR tubes in many laboratories.

In summary, the handling and health physics of analyzing tritiated samples is an important but trivially solved consideration, allowing $^3H$ NMR spectroscopy to be readily executed in most laboratories having a pulsed Fourier transform NMR spectrometer.

## 3. Features and Parameters

The characteristics of $^3H$ are compared with other common NMR active nuclei in Table 2. Tritium enjoys all the spectral advantages normally associated with spin 1/2 nuclei of high gamma: narrow lines, high sensitivity and good dispersion. A brief perusal of Table 2 indicates the strong similarity between triton and proton magnetic properties, with tritium having slightly higher sensitivity and dispersion, and similar relaxation characteristics. The similarity between nuclei extends to chemical shifts, allowing one to use the extensive library of proton chemical shifts when making tritium resonance assignments. Since tritium also has extremely low natural abundance, spectra are free of background signals and this, combined with the *a priori* knowledge of chemical shifts from analogous protons, makes assignment of tritium spectra particularly straightforward.

PLACE FIGURE 1 HERE

Many of the favorable properties of tritium are illustrated in the spectra in Figure 1, where a comparison of proton, deuterium and tritium NMR spectra of labelled glucose is shown. The superior resolution and dispersion of tritium over deuterium, a spin 1 nucleus, is obvious. Note that this labelled molecule is low molecular weight, and the line broadening of deuterium signals for larger molecules is even more striking, hence eliminating deuterium NMR as a useful technique for structural analysis of large molecules in solution. In addition since the dominant relaxation mechanism for deuterium is quadrupolar, NOE determinations are impossible, and sensitivity in saturation transfer is reduced due to rapid spin-lattice relaxation. In contrast, tritium analysis greatly simplifies the proton spectrum in Fig. 1A, particularly around the solvent where a less fortuitous proton chemical shift (or sample temperature) would have completely obscured one of the glucose signals under the HDO peak. Even in $H_2O$, tritium spectra require no solvent suppression and are much more easily assigned than the generally more crowded proton analog.

To understand the quantities involved in a tritium NMR experiment, one must be aware of the common units and conversion factors, which are listed in Table 1. One of the most important benchmarks is that complete replacement of hydrogen by tritium in one position of a molecule gives a specific activity (S.A.) of 1064 GBq $mmol^{-1}$ (28.76 Ci $mmol^{-1}$). Obviously, for lower percentages of tritium replacement the S.A. is proportionately reduced and this relationship, combined with a knowledge of the total number of millimoles of labelled substrate, readily yields the amount of radioactivity involved in the experiment.

## 3.1 Chemical Shifts and Referencing

Early measurements established that the ratios of nuclear screenings among the hydrogen isotopes was very close to unity. This implies that the chemical shift for a triton is essentially identical to that of a proton or deuteron in the analogous molecular environment. Thus tritium spectra can be referenced to say, monotritiated TMS and chemical shifts will be nearly identical

to proton chemical shifts. Since the tritium/proton Larmor frequency ratio is very well determined for TMS ($1.066639739 \pm 2 \times 10^{-9}$),[6] in practice it is not necessary to add a tritiated reference material to a sample to achieve accurate referencing. Instead, a simple measurement of the *proton* frequency of a reference material and multiplication by the figure above gives the tritium resonance frequency for the reference. This procedure is known as ghost referencing. Some care must be taken in very precise work when using ghost referencing because more careful studies of the Larmor frequency ratio for a variety of tritium labelled materials[6,7] have shown that nuclear screenings are not identical, but vary slightly with carbon-hydrogen (tritium) bond hybridization. Thus, when referencing tritiated methyl groups a TMS ghost may be appropriate, but a tritiated phenyl position is more accurately referenced by a phenyl proton and a slightly different Larmor frequency ratio. These differences are usually so small that they do not interfere with routine analysis, and are not discerned in a wide display (0-6 ppm), such as that in Figure 1.

## 3.2 Coupling Constants and Isotope Effects

Coupling constants between tritium and other nuclei may be calculated from the analogous proton coupling constants. In particular, $J_{TT}$ and $J_{HT}$ are given by the relationships in Table 2, *i.e.* tritium-proton spin-spin coupling constants may be calculated as *ca.* 6.664% larger than the analogous proton constant. There is a small isotope effect on the coupling constant,[8] and measurements have been reported for tritium coupled to a number of other nuclei besides protons including carbon, tin, silicon and boron.

Although there are isotope effects, measurement of a $J_{TH}$ is still an accurate way of predicting a proton-proton coupling constant, since the calculation involves division by 1.06664, rather than multiplication by 6.5144, as required by prediction from $J_{DH}$. For isochronous protons (*e.g.* methyl protons, having degenerate chemical shifts), measurement of the $J_{TH}$ readily yields a usually 'invisible' coupling constant (see Fig. 2, below).

Besides the small isotope effects on nuclear screening and on coupling constants discussed above, there are also more commonly observed isotope effects on tritium chemical shifts caused by substitution of a heavy isotope in place of a proton. These are largest and therefore most easily observed when a geminal proton is replaced by a triton. This effect was first reported in analyses of highly tritiated methyl groups,[9] where the R-$CT_3$ singlet was resolved from the R-$CT_2H$ doublet in the proton-coupled tritium spectrum. The proton decoupled spectrum was used to show that this primary isotope effect is *ca.* 0.025 ppm (*i.e.* 2.4 Hz at 96 MHz).

**PLACE FIGURE 2 HERE**

Figure 2 illustrates tritium isotope effects on chemical shifts, and demonstrates how the methyl $J_{HH}$ may be calculated from measured $J_{HT}$ values, as discussed above. A sample of m-xylene was tritium labelled by Raney Nickel catalyzed exchange with $T_2$, which is known to give almost exclusive methyl tritiation. All three tritio-methyl species are produced, as shown in the proton-decoupled tritium spectrum in Fig. 2A, where these species are separated by the $1^o$ isotope effect ($\Delta\delta$) on the tritium chemical shift. At 320 MHz, tritium signals from methyl groups have a proton-tritium coupling constant which is double $\Delta\delta$, so that the proton coupled tritium spectrum in Fig. 2B has a deceptively simple appearance. The splitting patterns are shown on the Figure, and our assignments are readily verified by integration of the signals in each of the spectra. The methyl $J_{HH}$ for m-xylene methyl groups may be calculated from the observed $J_{HT}$.

Secondary tritium isotope effects on chemical shifts have also been well quantified,[10] and the primary deuterium isotope effect on tritium chemical shifts is also easily measured (Table 2).

## 3.3 Tritium NMR Intensity and the NOE

One of the major advantages of tritium NMR spectroscopy is that the labelling pattern in a sample may be determined without the time-consuming and laborious degradative determinations previously required.[11] Indeed, a tritium spectrum gives nondestructive,

quantitative information on relative tritium abundance at different sites in a molecule. Of course, care must be exercised in any measurement of NMR peak intensities so that accumulation of the data does not *differentially* affect the various peaks. When proton decoupling is used, for example, the tritium intensities will be affected by the Nuclear Overhauser Effect (NOE). Because of the frequent use of proton decoupling in tritium experiments, the importance of this effect has been the subject of several studies and discussions.[12] While the maximum theoretical NOE enhancement to tritons from proton irradiation is 47%, the observed enhancements vary from 10-35%. More importantly, there are situations where differential NOEs may prejudice tritium spectral intensities, such as a labelled molecule with high abundance of tritium in a position without neighboring protons and a second tritiated position which has many relaxation partners, and integrals should only be compared when these issues have been considered.

## 3.4 Relaxation

The similarity of tritium and proton nuclei means that their pathways for relaxation are nearly identical. A feature of tritium NMR is that the simple spectra allow straightforward relaxation measurements. The analogous measurements in proton spectra can be difficult due to spectral crowding, solvent suppression, or the complexity of the physical system. Hence tritium relaxation can be the more useful probe of molecular interactions. A classic example is the use of proton and tritium relaxation measurements combined with homo- and heteronuclear saturation transfer experiments to study the relaxation of water molecules in the presence of a macromolecular suspension.[13] A rather simple suspension was created by the formation of egg phosphatidylcholine/cholesterol lipid bi-layers in solution. In a lipid suspension the water can exist in two distinct environments, a 'free' state exhibiting bulk water properties, and a motionally hindered or 'bound' state that is associated with the lipid in a hydration shell. Nuclei in these populations relax differently. The free water has the familiar characteristics of small rapidly tumbling molecules in solution, while the bound water has an additional slow component of tumbling due to its association with the lipid. This lipid-induced hydrodynamic effect causes

a more rapid relaxation in the bound state and the presence of two water populations with different relaxation rates makes the overall relaxation behaviour of the water signal complex. In general, it will depend on a combination of processes including the hydrodynamic effect, chemical exchange between free and bound environments, and the dipolar interaction (cross relaxation).

In the investigation of these processes, tritium doped water was used to separate the exchange contribution from other relaxation processes in the heteronuclear tritium proton experiments. The measurements made on this rather complicated system were thus simplified to analysis of several single line spectra using inversion recovery and saturation transfer experiments. The results indicated that the dipolar interaction (in the spin diffusion limit) dominates the relaxation of the water, and two models of this limit were proposed which accurately predicted the results, including a rigid and dynamic description of the lattice in the lipid solution. A complete model of the relaxation behaviour of water is very desirable since the difference in relaxation properties of free and bound water populations is the basis for contrast and tissue characterization techniques in magnetic resonance imaging.

## 4. Applications

We will limit the discussion here to chemical applications: i). analysis of labelled materials for chemical and radiochemical purity, ii). reaction mechanisms and tritium NMR techniques, iii). specific activity measurements, iv). solution conformation, stereochemistry and optical purity, v). proton exchange studies, and vi). miscellaneous tritium NMR studies. Biological applications of $^3H$ NMR, *e.g.* ligand binding, metabolism studies, *etc.* are discussed elsewhere (see 'Tritium NMR in Biology').

### 4.1 Analysis of Labelled Materials

The vast majority of reports involving $^3H$ NMR spectroscopy describe the products of tritium labelling reactions, and in this application the technique is invaluable. Tritium NMR has

been used extensively for investigation of many tritium exchange techniques, including radiation-induced and acid-, base-, metal- or zeolite-catalyzed reactions.[14] In general, these techniques rely on the replacement of a hydrogen by a tritium (*i.e.* exchange) in the native structure, and give low specific activity products, which may contain tritium in one of several distinct sites.

In contrast, high level tritium labelling usually involves a synthetic transformation such as dehalogenation, hydrogenation or hydride reduction of a suitable precursor. Dehalogenation reactions are an attractive method of introducing tritium into a compound, but surprisingly these reactions rarely give quantitative replacement of halogen by tritium, even when 100% $T_2$ gas is used. Our experience is that 10-30% of product molecules will incorporate adventitious hydrogen ($^1H$) rather than $^3H$. In general, the shorter the reaction time, the less hydrogen incorporated (*i.e.* the higher the S.A. obtained), and it is advisable to use an iodo derivative as substrate since the facility of halogen removal is I > Br > Cl >> F. Also, an equivalent of organic base (*e.g.* triethylamine) enhances the reaction rate and neutralizes the acid formed.

**PLACE FIGURE 3 HERE**

The power of tritium NMR in analysis of a tritiodehalogenation reaction product is best illustrated by example. Figures 3A-E show a full proton and $^3H$ NMR analysis of a peptide labelled by catalytic dehalogenation of the analogous 3,5-di-iodo-tyrosyl-peptide. The aromatic portion of the proton spectrum of the tritiated material (Fig. 3A) shows *ca.* 15% proton intensity at the tyrosine 3/5 chemical shift, with the usual tyrosine 2/6 and Phe aromatic signals. Note that the splitting of the tyrosine 2/6 doublet reflects $J_{TH}$, <u>not</u> coupling to the 3/5 protons. The proton coupled tritium spectrum in Fig. 3B shows a clean doublet at the tyrosine 3/5 chemical shift for the incorporated tritium. Broadband decoupling of the protons yields the singlet tritium signal in Fig. 3C, as expected. Selective irradiation at the tritium tyrosine 3/5 frequency causes the tyrosine 2/6 doublet to collapse, as shown in the proton spectrum in Fig. 3D (*cf.* Fig. 3A). Similarly, selective $^1H$ decoupling at the tyrosine 2/6 frequency yields a singlet tritium signal at

the tyrosine 3/5 chemical shift (Fig. 3E). These experiments are readily performed, and characterize the product beyond any doubt.

The other most common method for introducing high levels of tritium is catalytic hydrogenation of an unsaturated site on a suitable precursor. Interestingly, heterogeneous metal-catalyzed hydrogenation reactions often do not yield 'textbook' results, and it is remarkable that so few researchers are aware of these details, despite their repeated demonstration.[5] Two effects contribute to the complicated mixture of isotopomers obtained from these reactions:[10] (i). there is an inventory of hydrogen ($^1H_1$) in the catalyst, solvent, substrate and reaction vessel which serves to **decrease** the maximum level of tritium which may be incorporated into the labelled material, and (ii). vinyllic and allylic protons may undergo metal-catalyzed exchange prior to saturation of the multiple bond, thus leading to **increased** specific activity.[15] As a result, products of a hydrogenation reaction often have (i). lower than theoretical incorporation of tritium, (ii). tritium in positions remote from the original site of unsaturation, and (iii) unequal amounts of tritium on each side of the original multiple bond.

**PLACE FIGURE 4 HERE**

When $^3H$ NMR is used to analyze the products of both heterogeneous and homogeneous catalysis of an identical reduction, the complexities of heterogeneous catalysis are clear. Such a comparison of tritiation techniques for the labelling of an unsaturated phospholipid molecule is shown in Fig. 4. All the problems described above for heterogeneous catalysis complicate the spectrum shown in Fig. 4A, including small amounts of tritium incorporated at the positions adjacent to the double bond. In contrast, the product of the homogeneous tritiation using Wilkinson's catalyst (Fig. 4B) shows clean addition of tritium, with a 1:1 ratio at the C7 and C8 positions. A $^3H$-$^3H$ COSY analysis of this product (Fig. 4C) showed that the two expected diastereotopic products were obtained, in a 1:1 ratio.[16] Although this tritium analysis shows that Wilkinson's catalyst gives a very clean product, it is not applicable in all circumstances. Such mundane issues as sample solubility and recovery of the labelled material from the reaction mixture often affect the decision of which catalyst is used. Heterogeneous catalysts have the

advantages that they are tolerant of a variety of solvent systems, and are easily filtered away after the reaction. Wilkinson's catalyst is usually employed as a benzene solution, and may not perform well in a binary mixture if the substrate is insoluble in benzene. In addition, Wilkinson's catalyst is designed to form an intimate mixture with the substrate, and post-reaction separation steps need to be optimized for each new substrate.

## 4.2 Reaction Mechanisms and Tritium NMR Techniques

Tritium spectra are almost always simple, hence there has been no strong drive to apply more sophisticated NMR techniques to tritium analyses. However, the subtleties of exchange effects in heterogeneous catalysis, and the complex products from enzymatic reactions often require careful interpretation, and such studies may be greatly enhanced by the use of modern multi-pulse approaches.

The following techniques have been applied to tritium analyses over the past decade: double quantum filtering, DEPT, 2D homonuclear COSY, 2D tritium/proton correlation, 2D J-resolved, and 2D NOESY and EXSY experiments. The NOESY and EXSY approaches have been used for macromolecular structure studies, and are discussed elsewhere (see 'Tritium NMR in Biology'). The pulse sequences we regard as most useful for analytical applications are the DQF and 2D J-resolved experiments. These have been used for detailed investigation of the 'non textbook' results typically encountered in tritiation reactions, which are usually invisible in the corresponding reaction with $^1H_2$. Hence tritiation and tritium NMR spectroscopy provide an excellent approach for investigation of these imperfectly understood reaction mechanisms, as discussed below.

**PLACE FIGURE 5 HERE**

The research staff at Berkeley routinely use a mixture of 5-15% $T_2$ in $H_2$ for exploratory labelling reactions, and in a training program. In hydrogenation of 3-indoleacrylic acid using a 10% Pd/C catalyst and 15% $T_2$ in $H_2$ gas, simple statistics predict that 2.25% of labelled 3-indolepropionic acid product molecules will contain two $^3H$ atoms, 25.5% will have a tritium at

one carbon or the other, and 72.25% will contain no tritium. The $^{1}H$ decoupled tritium NMR spectrum in Fig. 5A shows several features: (i). the large singlet signals arise from molecules containing one tritium atom, and it is obvious that unequal amounts of $^{3}H$ are incorporated across the double bond, and (ii). one signal from a small doublet can be discerned at the base of each singlet (asterisked), presumably arising from the doubly tritiated species. The unequal substitution pattern in the singly tritiated species is thought to arise from exchange at those carbons prior to saturation of the double bond, and tritium NMR represents the best method for the further study of the mechanism of such exchange.[15] The doubly tritiated species can be observed simply and directly by application of a familiar multiple pulse technique, a double quantum filtered (DQF) experiment. The results of the DQF experiment on this molecule are remarkably clean, as shown in Fig. 5B. The spectrum is simple because the doubly tritiated species is the only one present with tritium homonuclear double quantum transitions, and is further simplified because all couplings to protons are heteronuclear and have been removed by broadband decoupling. This result has tremendous potential for the study of the mechanism of this type of addition and would not have been possible without the use of tritium NMR. Clearly this unique ability to separate labelled species and perform careful analysis of complex mixtures of isotopomers resulting from a theoretically simple tritiation reaction is essential for understanding the mechanism of these reactions.

2D J-resolved $^{3}H$ NMR[10] can be an even more powerful tool for analysis of complex isotopic mixtures. β-Methylstyrene was tritiated by catalytic reduction with 100% $T_2$ over 5% Pd/C catalyst, to give highly tritiated n-propylbenzene with *ca.* 40% of the tritium on the α-methylene carbon, 50% on the β-methylene, and 10% in the methyl group. In addition, all of the multiplets for these tritiated positions showed complex structure, not at all like the simple doublet of doublets which might have been proposed assuming clean addition of a tritium atom to each end of the double bond.

PLACE FIGURE 6 HERE

The methyl region of the proton-decoupled tritium J-resolved analysis of this sample (Fig. 6) provided most insight into the species giving rise to the various signals. There is a repeating and slightly overlapped singlet-doublet-triplet pattern, with each repetition moved successively to higher field by $^3H$ isotope effects. These first of these patterns (group A, Fig. 6) is due to the species X-$CH_2$-$CH_2$T, singlet; X-CHT-$CH_2$T, doublet; and X-$CT_2$-$CH_2$T, triplet (species 1, 2 and 3), and the small change in chemical shift between these signals is the 2° tritium isotope effect induced by addition of a T at the β-$CH_2$. There are similar patterns for the -$CHT_2$ (B) and -$CT_3$ (C) methyl species, successively moved to higher field by the 1° tritium isotope effect on the methyl chemical shift. Inspection of several slices along the $\omega_2$ dimension (submatrix, or .smx plots shown in Fig. 6) show the relative abundance of each species in this mixture, and analysis of $\omega_1$ slices allows us to extract all the $^3H$-$^3H$ coupling constants. Similar detailed analysis of the α-$CH_2$ and β-$CH_2$ regions of the J-resolved is also possible, and evidence was found for almost all of the 35 statistically possible, tritium-containing isotopomers. While the theoretical product, Ph-CHT-CHT-$CH_3$, was the most abundant species, the presence of another 34 tritiated isotopomers makes it clear that the mechanism of heterogeneous metal-catalyzed hydrogenation reactions is not as simple as commonly thought.

## 4.3 Specific Activity Measurements

The molar specific activity (*i.e.* radioactivity per unit weight) of tritium labelled molecules is an important property, and tritium NMR may be used to determine this quantity.[5] This approach is essential when the labelled material has no UV spectrum, or the high specific activity precludes weighing to determine sample concentration. An example of the latter was the determination of the S.A. for a series of $NaBT_4$ preparations.[17] Boron has two NMR active isotopes ($^{10}B$, 20%, I=3 and $^{11}B$, 80%, I=3/2), so the proton spectrum of sodium borohydride in basic $CD_3OD$ shows a large quartet ($Na^{11}BH_4$, δ = -0.168, $J$ = 80.6 Hz) and a small septet ($Na^{10}BH_4$, δ = -0.166, $J$ = 26.98 Hz). The proton-decoupled tritium spectrum of exchange

labelled $NaBH(T)_4$ shows a similar spectrum for each isotopomer, *i.e.* $NaBH_3T$, $NaBH_2T_2$, $NaBHT_3$, $NaBT_4$, shifted to higher field for each additional T. The multiplicity (redundancy) of the peaks is useful since peak overlap does not occur for all the species, and the isotopomer ratios may be compared between large quartet signals and the smaller septets. The amount of unlabelled ($NaBH_4$) material present was estimated by comparison with $NaBH_3T$ signals in the proton spectrum. Knowing the mole ratio of all the isotopomers yields the S.A., and this property was compared for several production 'lots' of tritiated borohydride.

Even when determination of S.A. by UV spectroscopy and LSC counting, or by mass spectrometry is possible, the NMR technique provides a useful cross-check. In comparison to the sodium borohydride case above, a very simple example of using $^3H$ and $^1H$ NMR to determine specific activity is given in Fig. 7. Pinoline (6-methoxy-1,2,3,4-tetrahydro-9H-pyrido-[3,4-b]-indole) was labelled by catalytic hydrogenation of the unsaturated 3,4 dihydro analog. The product consisted of a mixture of three isotopomers, containing zero, one or two tritium atoms per molecule. Obviously, at the chemical shift of the C1-methylene, one of these species has only a proton spectrum, one has a tritium spectrum, and one (R-CHT, $\delta = 4.39$ ppm, Fig. 7A and 7B) has both a tritium and a proton NMR spectrum. In this case, the use of selective tritium (Fig. 7A) or proton (Fig. 7B) decoupling allows the observation of single lines for each of the isotopomers in the proton or tritium spectrum. Since the R-CHT signal occurs in both spectra, the molar ratio of the three species may be extracted from these spectra, and the specific activity calculated.

**PLACE FIGURE 7 HERE**

4.4 Solution Conformation, Stereochemistry and Optical Purity

$^3H$ NMR has been used to obtain the axial-equatorial conformational preference of tritium in [$^3H$]-cyclohexane.[18] Tritium has the advantage of giving sharp and well separated peaks that are well suited for high-accuracy integration at low temperatures, *i.e.* the peak separation in these experiments was *ca.* 150 Hz at 7.1 Tesla, whereas the analogous deuterium

experiment at 11.7 T gave a separation of only 36 Hz. [$^3$H]-Cyclohexane was made from cyclohexylmagnesium chloride in ether by the addition of HTO (5% tritium) followed by distillation of the tritiated product. A sample (*ca.* 0.9 GBq, 33 mCi) in $CS_2$ containing some $CD_2Cl_2$ for lock purposes was transferred into a standard thin-wall glass 5 mm NMR tube, and $^3$H {$^1$H} NMR spectra were obtained at 320 MHz (300 MHz $^1$H frequency). Narrow lines (*ca.* 2 Hz at half-height) with good line shapes were obtained at –88°C by using the tritium FID as well as the $^2$H lock signal for shimming. For accurate integration it is essential that the base of the peaks be narrow and symmetrical, and that the baseline is flat. A number of data sets, each consisting of the sum of 512 FID's were acquired, several with the carrier at a higher frequency than the sample signals, some with the carrier at a lower frequency, and others with the carrier centered between the signals. The $^{13}$C satellites of both the axial and equatorial $^3$H signals ($\Delta\nu$ = 0.471 ppm) were visible in each set of spectra and gave $J(^{13}C\text{-}^3H_{ax})$ = 131.1 Hz and $J(^{13}C\text{-}^3H_{eq})$ = 135.2 Hz. Integration of the spectra showed that tritium prefers the equatorial over the axial site by about 11.2 cal mol$^{-1}$ and a $^3$H EXSY experiment showed that the axial and equatorial tritium atoms are undergoing (slow) exchange at –88°C. Conformational analysis by $^3$H NMR relaxation has also been used in enzyme ligand studies (see 'Tritium NMR in Biology').

**PLACE FIGURE 8 HERE**

The absolute stereochemistry of a tritium atom on a carbon may be determined by tritium NMR if there is another chiral center in the molecule. If this is not the case on the parent molecule, it can often be created by esterification or some other derivatization. This feature was exploited in analysis of tritiated ethanol produced by enzymatic oxidation of 'chiral ethane', *i.e.* $CH_3$-CHDT, as part of a study of the mechanism of methane monooxygenase.[19] Tritium NMR analysis of the methylene region of the ethanol product showed signals from $CH_3$-CHT-OH and $CH_3$-CDT-OH, separated by the $^2$H isotope effect on the tritium chemical shift (Fig. 8A). Whereas this spectrum gives information on the relative rate of removal of an H or D atom in the oxidation process, it gives no stereochemical data. A simple derivatization with (2*R*)-2-acetoxy-

2-phenylethanoic (mandelic) acid yields ethyl mandelate, and the four possible species can be readily separated and quantitated by $^{3}H$ $\{^{1}H\}$ NMR, as shown in Fig. 8B. The absolute stereochemistry was previously established by a known synthesis of the deuterium substituted molecules, and proton NMR analysis.

Tritium NMR has been used to determine the optical purity of labelled materials. The use of a Lanthanide shift reagent[20] or Pirkle's alcohol[21] is a more general technique than the careful stereochemical determinations discussed above, and does not require the presence of an alcohol or amine functionality for derivatization to the appropriate ester or amide. This general approach is especially important for materials labelled at low tritium abundance because the optical properties of the bulk material may not be the same as the small sub-population of labelled molecules.

## 4.5 Proton Exchange Studies

The NMR properties of tritium make it particularly well suited for *in situ* measurements of acid- and base-catalyzed proton-exchange kinetics using tritium NMR spectroscopy.[22] Despite the great sensitivity and simplicity of this approach,[5] previous tritium NMR mechanistic studies of proton exchange have been confined to a static approach, *i.e.* the analysis of aliquots of a kinetic experiment.[23] In most circumstances, it is desirable to perform one-tube, accurate, kinetic experiments, especially for compounds which are only available in small amounts, or have several exchanging positions with similar rates. Use of deuterium NMR spectroscopy, hampered by its low sensitivity and resolution, is inherently limited to initial rates,[24] and if several positions are measured simultaneously, relative rates for the positions must lie within a small range. In contrast, tritium NMR spectroscopy allows one to measure the proton exchange kinetics of several compounds which may have similar rates, or vary by a factor of 1000. Since tritium is a highly sensitive nucleus, the sample need not contain high levels of tritium for detection (especially compared with deuterium) and tritium therefore functions as a true tracer in the reaction kinetics. Only 20-180 MBq (0.5-5 mCi) per position ($1.5 \times 10^{-5}$% incorporation) is

necessary for an adequate signal-to-noise ratio. This low tritium concentration also ensures the absence of tritium-tritium coupling, and the high $\gamma$ and excellent relaxation characteristics of the nucleus lead to sharp resonance lines. The integrity of the reactant mixture may be monitored by proton NMR spectroscopy at any stage of the experiment. An inverse-gated proton decoupling scheme may be used to ensure that single peaks are observed for each magnetic environment and that the relative peak integrals are not affected by NOE build-up. The quality of the kinetic data obtained by this technique suggests that it may be readily applied to other chemical proton transfer systems.

As an example, this technique has been applied to the measurement of detritiation rates, for the characterization of isotope effects. The Swain-Schaad relationship predicts that the deuterium and tritium isotope effects on the rate of a reaction are related as follows, $(k_H/k_D)^x = k_H/k_T$. Theoretical calculations put $x$ in the range 1.33 to 1.58, and a simple zero-point energy formulation of isotope effects gives $x = 1.44$. Deviations from this range have recently been used as evidence of tunnelling in enzyme-catalyzed reactions, so the accuracy of experimental results in simple chemical systems is important. Such a simple system is the hydroxide-ion-catalyzed enolization of acetone (a prototype ketone ionization reaction), but the originally published measurements give an anomalously low exponent, $x = 1.08$.[25] This exponent was based upon rates of detritiation of labelled acetone, which were determined by a technique that involved a difficult separation of acetone from water.

This reaction was reinvestigated[26] using tritium NMR to monitor the exchange of tritium between acetone and water *in situ*,[22] thus avoiding the troublesome acetone-water separation. In NMR experiments using a range of base concentrations, the tritium signal from the acetone at $\delta$ = 2.1 ppm was seen to decay while another resonance at $\delta$ = 4.7 ppm, attributable to HTO, appeared (Fig. 9). Least squares fitting of the observed exponential rise and decay gave first order rate constants that agreed well with each other.

PLACE FIGURE 9 HERE

When the NMR result was combined with $k_H$ and a literature value of $k_D$ determined by mass spectrometric measurements, it yielded $k_H/k_D = 7.2$ and $k_H/k_T = 19.2$. These isotope effects provide the Swain-Schaad exponent, $x = 1.49 \pm 0.07$, which is in complete agreement with theory. Thus, tritium NMR provided a simple method for measurement of an important exchange rate, which was essentially inaccessible by chemical techniques.

## 4.6 Miscellaneous

Tritium NMR spectroscopy has been used in a number of other very specific chemical and physical investigations, most of which have been comprehensively reviewed elsewhere.[5] These applications have included: (i). the observation of hydrogen bonding effects on the chemical shift of proton vs tritium NMR signals, (ii). determination of solvent isotope effects and fractionation factors, (iii). aspects of radiation chemistry including sample radiolysis, studies of DNA hydration, and *in situ* production of labelled carbocations, (iv). investigations of labelled molecules in a liquid crystal environment, (v). solids tritium NMR and characterization of trapped gases in irradiated lithium hydride (tritide) samples,[27] and (vi). study of the origin of an anomalously large proton-proton coupling in transition metal polyhydrides.[28]

## 5. Conclusion

We have discussed a wide range of applications where the use of tritium NMR has been useful, and some instances where it was essential. The similarity in NMR properties between protons and tritons means that many systems may be studied using either nucleus. Moreover, when proton systems become too complex, the spectral simplification of tritium spectroscopy suggests that tritium NMR should be considered. Radioactivity is the only issue hindering the universal application of tritium in many more general NMR studies.

## 6. Related Articles

Biochemical Applications of Isotopes; Multidimensional NMR: An Overview; Tritium NMR in Biology.

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TABLE 1:

General Physical and Chemical Data for Tritium, and Common Radioactivity Units.

| | | | | |
|---|---|---|---|---|
| Production | | $^6Li\ (n, \alpha)\ ^3H$ | | |
| Radiation | | β (100%); range = 4.5-6 mm in air, 6 μm in $H_2O$ | | |
| β energy | | $E_{max}$ = 18.6 keV, $E_{mean}$ = 5.7 keV | | |
| Radioactive Half-life | | 12.43 years (4540 days) | | |
| Biological Half-life | | *ca.* 10 days | | |
| Decay product | | $^3He$ | | |
| Maximum specific activity | | 1064 GBq milliatom$^{-1}$ (28.76 Ci milliatom$^{-1}$) | | |
| Detection Limit (in 24 hours): | LSC | 0.037 Bq = 2.09 x $10^7$ atoms (*ca.* 0.1 fmole) | | |
| | NMR | 1064 kBq = 6.02 x $10^{14}$ atoms (*ca.* 1 nmole) | | |
| Diameter of tritium atom (approx.) | | 1.1 Å | | |
| Volume of 37 GBq (1 Ci) of tritium gas at standard temperature and pressure | | 0.385 mL | | |
| Radiation produced by 37 MBq (1 mCi) in a man (70 kg = 40 L, *i.e.* 25 μCi/L) | | 0.044 mSv/day (= 4.4 mrem/day, or 1.6 rem/yr) | | |
| Density, triple point and boiling point of isotopic water molecules | | $H_2O$: | 1.000 g mL$^{-1}$ | 0.01 °C 100.00 °C |
| | | $D_2O$: | 1.106 g mL$^{-1}$ | 3.82 °C 101.42 °C |
| | | $T_2O$: | 1.214 g mL$^{-1}$ | 4.49 °C 101.51 °C |
| Tritium content of pure $T_2O$ @ STP | | 117.4 TBq mL$^{-1}$ (3,173 Ci mL$^{-1}$) | | |
| | | 96.7 TBq g$^{-1}$ (2,614 Ci g$^{-1}$) | | |

37 GigaBecquerels (GBq) = 1 Curie (Ci) = 3.7 x $10^{10}$ disintegrations per second.

1 Gray (Gy) = 10,000 erg gm$^{-1}$ = 100 rad (units of absorbed dose).

1 Sievert (Sv) = 100 rem (units of radiation exposure).

TABLE 2:

NMR Properties of $^{3}H$, and Common NMR Nuclei [a]

| Nucleus | Natural Abundance | Spin | $\gamma$ | Resonant Frequency | Relative [b] Sensitivity |
|---|---|---|---|---|---|
| $^{1}H$ | 99.984 | 1/2 | 26.7510 | 300.13 | 1.00 |
| $^{2}H$ | 0.0156 | 1 | 4.1064 | 46.07 | $9.65 \times 10^{-3}$ |
| **$^{3}H$** | **$<10^{-16}$** | **1/2** | **28.5335** | **320.13** | **1.21** |
| $^{13}C$ | 1.10 | 1/2 | 6.7263 | 75.46 | $1.59 \times 10^{-2}$ |
| $^{15}N$ | 0.37 | 1/2 | -2.7116 | 30.40 | $1.04 \times 10^{-3}$ |
| $^{19}F$ | 100 | 1/2 | 25.1665 | 282.23 | 0.83 |
| $^{31}P$ | 100 | 1/2 | 10.8289 | 121.43 | $6.63 \times 10^{-2}$ |

| | |
|---|---|
| Chemical Shift ($\delta$) range: | 0-20 ppm |
| Isotope effects: | $^{3}H$ Primary, $1^{o} \approx 0.028$ ppm; 9.0 Hz @ 7.1 T |
| | $^{3}H$ Secondary, $2^{o} \approx 0.013$ ppm; 4.2 Hz @ 7.1 T |
| | $^{2}H$ Primary, $1^{o} \approx 0.020$ ppm; 6.7 Hz @ 7.1 T |
| Coupling Constant ($J$) range: | 0-20 Hz |
| | $J_{HT}=J_{HH} \times \gamma_T/\gamma_H$ |
| | $J_{TT}=J_{HH} \times (\gamma_T)^2 \times (1/\gamma_H)^2$ |
| $T_1$ | 0-10 sec |
| $T_2$ | 0-10 sec |

a — 7.1 Tesla field.

b — Sensitivity given for equal numbers of nuclei in the same field.

**Figure Legends:**

Figure 1. Hydrogen NMR spectra of D-glucose in water. A) Proton spectrum of C-1 tritiated D-glucose; the large peak is due to HDO. B) Proton-decoupled deuterium NMR spectrum of C-1 deuteriated glucose; DMSO is an integration marker. C) Proton-coupled tritium spectrum of C-1 tritiated glucose, labelled by the same catalytic technique as in (B).

Figure 2. Deceptively simple tritium NMR spectra in $C_6D_6$ of m-xylene labelled by $T_2$ gas exchange over Raney Nickel catalyst. A) Proton-decoupled tritium spectrum showing peaks for R-$CH_2T$, R-$CHT_2$ and R-$CT_3$ species separated by $\Delta\delta = 0.025 \pm 0.001$ ppm ($7.89 \pm 0.21$ Hz @ 7.1 T). B) Proton-coupled spectrum showing overlapped tritium multiplets separated by $J_{HT} = 15.58 \pm 0.21$ Hz.

Figure 3. Proton and tritium NMR spectra in $D_2O$ of a peptide labelled by catalytic tritio-dehalogenation of the appropriate di-iodo-tyrosyl-peptide. A) Aromatic region of the proton spectrum, showing signals from the phenylalanine residue, and a clean doublet from the tyrosine 2 and 6 protons. Residual proton signals are obvious from the tyrosine 3 and 5 position. B) Tritium spectrum showing the doublet of the tyrosine 3 and 5 tritons coupled to the 2,6 protons. C) Proton-decoupled tritium spectrum showing the tritium singlet from the 3/5 tritium atoms. D) Selectively tritium-decoupled proton spectrum. Comparison with the spectrum in (A) shows the collapse of the tyrosine 2/6 doublet. E) Selectively proton-decoupled tritium spectrum, with irradiation only at the tyrosine 2/6 doublet.

Figure 4. Proton-decoupled tritium NMR spectra of a labelled phospholipid in $D_2O$. A) Product of a heterogeneous catalytic hydrogenation reaction. B) Product from

catalytic hydrogenation in the presence of Wilkinson's catalyst. C) $\{^1H\}$ $^3H$-$^3H$ COSY of the product in (B).

Figure 5. Tritium NMR spectra of tritiated 3-indolepropionic acid in $CD_3OD$. A) Proton-decoupled tritium spectrum of the product of a heterogeneous catalytic hydrogenation reaction, using 15% T/H. B) Double quantum filtered proton-decoupled tritium spectrum of the same product as shown in (A). The observed $J_{TT} = 7.32 \pm 0.12$ Hz, suggesting a $J_{HH}$ of *ca.* 6.4 Hz.

Figure 6. The methyl section (0.68-0.93 ppm) of the contour plot of the proton-decoupled tritium J-resolved spectrum of n-propylbenzene, labelled by catalytic tritiation of β-methylstyrene. Sine-bell window functions were applied in both dimensions, 2K x 512W transform, with magnitude calculation of peak intensities. The data were 'tilted' and 'symmetrized'.

Figure 7. Proton and tritium NMR spectra of tritiated Pinoline in $CD_3OD$. A) Selectively tritium-decoupled proton spectrum. B) Selectively proton-decoupled tritium spectrum.

Figure 8. Proton-decoupled tritium NMR spectra of the products from enzymatic oxidation of chiral ethane. (A) Ethanol in $H_2O$. (B) Derivatized ethanol in $C_6D_6$, where R = (2*R*)-2-acetoxy-2-phenylethanoic acid.

Figure 9. A stacked plot of the proton-decoupled tritium NMR spectra of the base-catalyzed detritiation of acetone ($\delta = 2.1$ ppm), with exchange into the solvent (HTO, 4.7 ppm). The experimental parameters were: 500 μL 0.04M NaOH; 5 μL $^3H$-acetone; T = 298 K; 120 transients; 100 experiments over 10.5 hours (*ca.* 4 minute interval for the first

50, then 8 minute interval); spectrometer unlocked; t = 0 when the acetone was added, and the first spectrum was finished at t = 20 minutes. 77 Spectra are plotted.

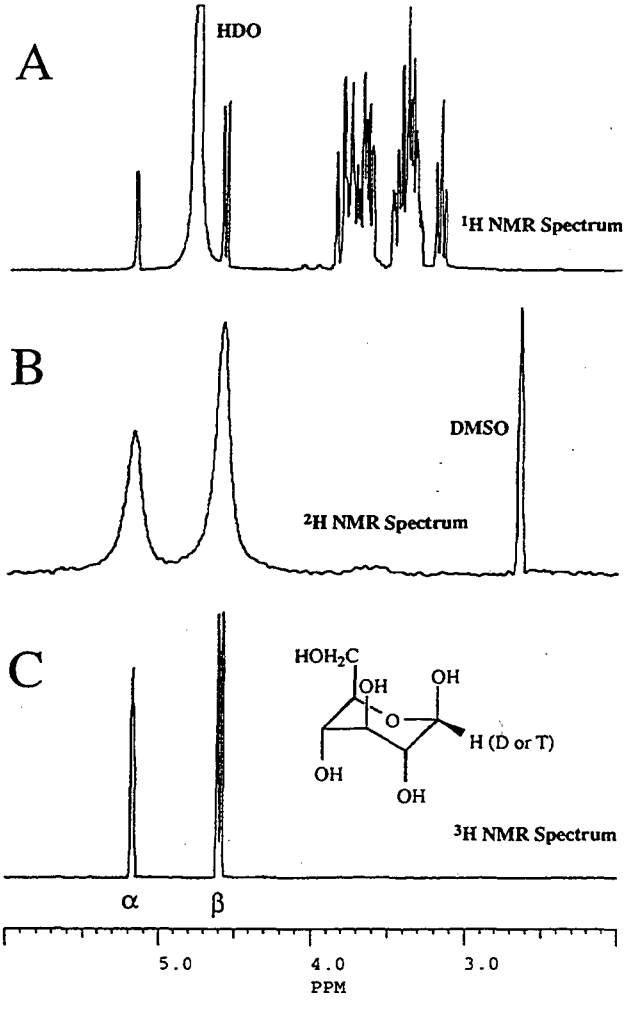

A
HDO
$^1$H NMR Spectrum
B
DMSO
$^2$H NMR Spectrum
C
HOH$_2$C
OH
OH
O
H (D or T)
OH
OH
$^3$H NMR Spectrum
α
β
5.0
4.0
3.0
PPM

Figure 1

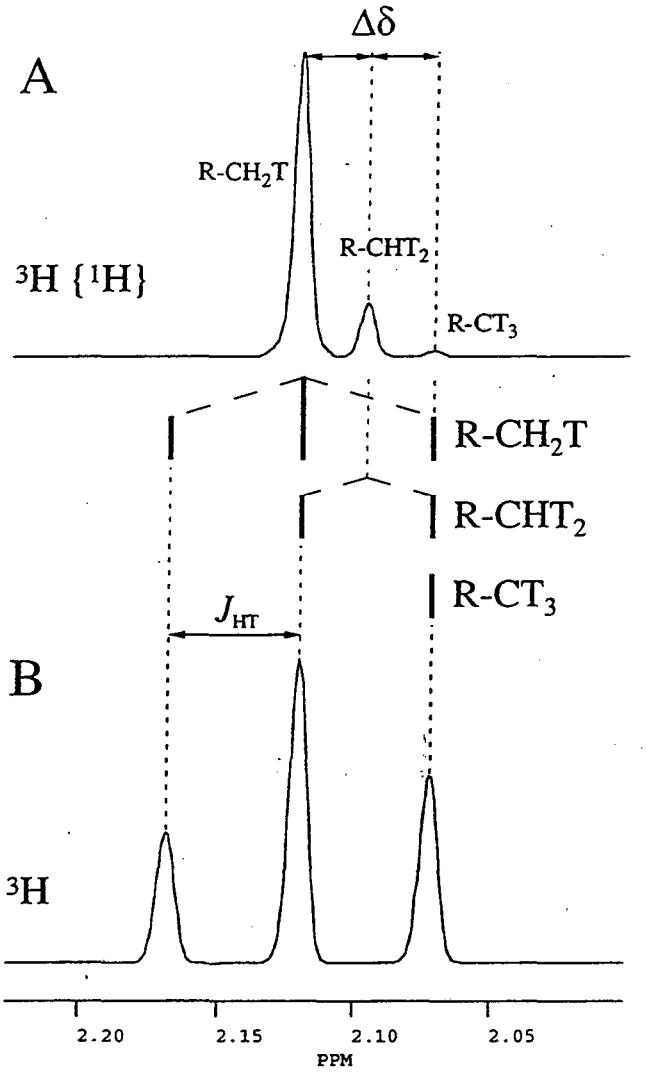
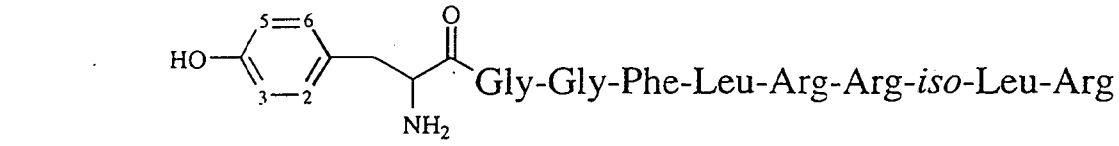

Δδ
A
R-CH2T
R-CHT2
3H {1H}
R-CT3
R-CH2T
R-CHT2
R-CT3
JHT
B
3H
2.20
2.15
2.10
2.05
PPM

Figure 2


HO
5
6
3
2
O
NH2
Gly-Gly-Phe-Leu-Arg-Arg-iso-Leu-Arg

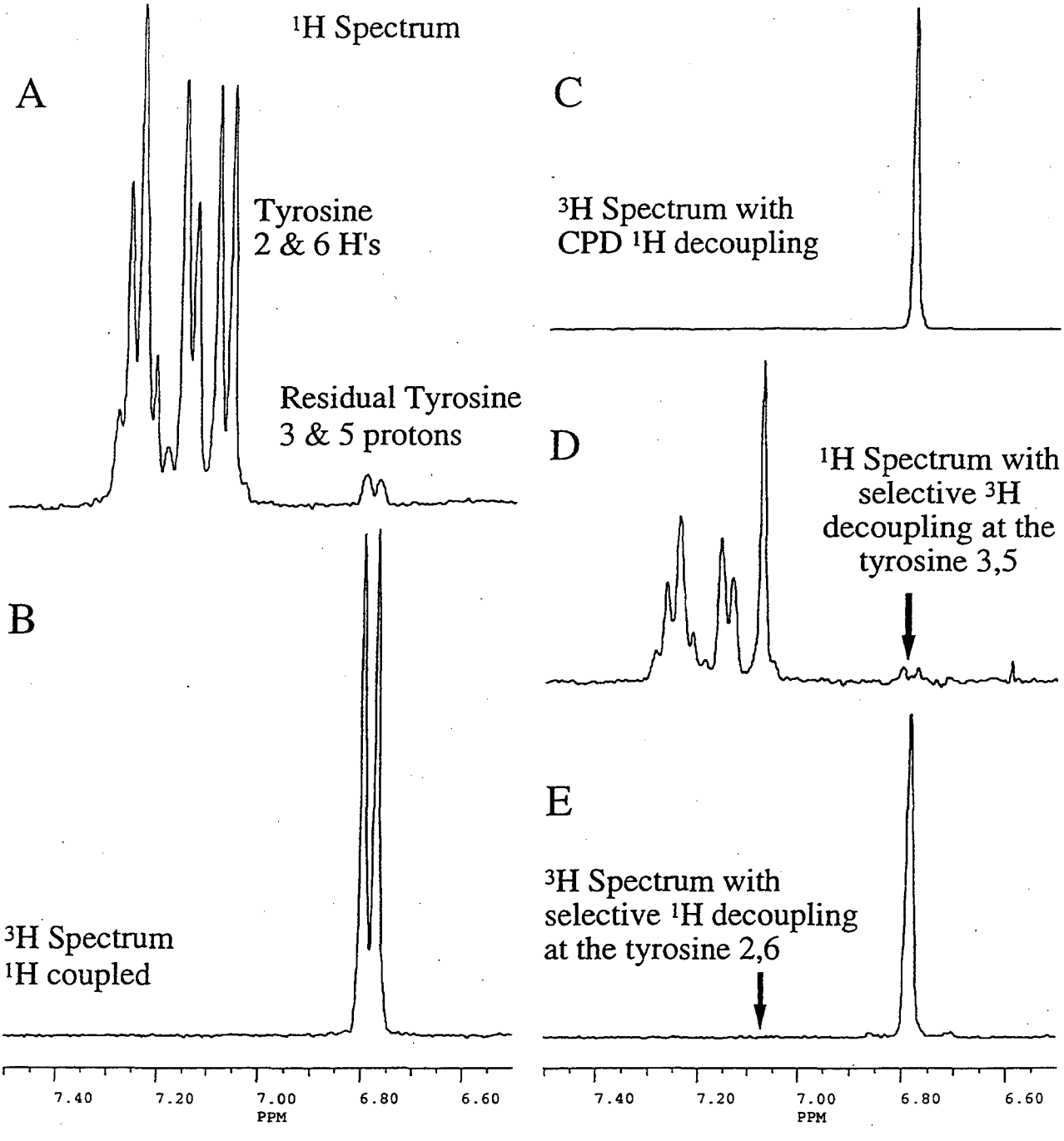

A
1H Spectrum
Tyrosine
2 & 6 H's
Residual Tyrosine
3 & 5 protons
B
3H Spectrum
1H coupled
C
3H Spectrum with
CPD 1H decoupling
D
1H Spectrum with
selective 3H
decoupling at the
tyrosine 3,5
E
3H Spectrum with
selective 1H decoupling
at the tyrosine 2,6
7.40
7.20
7.00
6.80
6.60
PPM

Figure 3

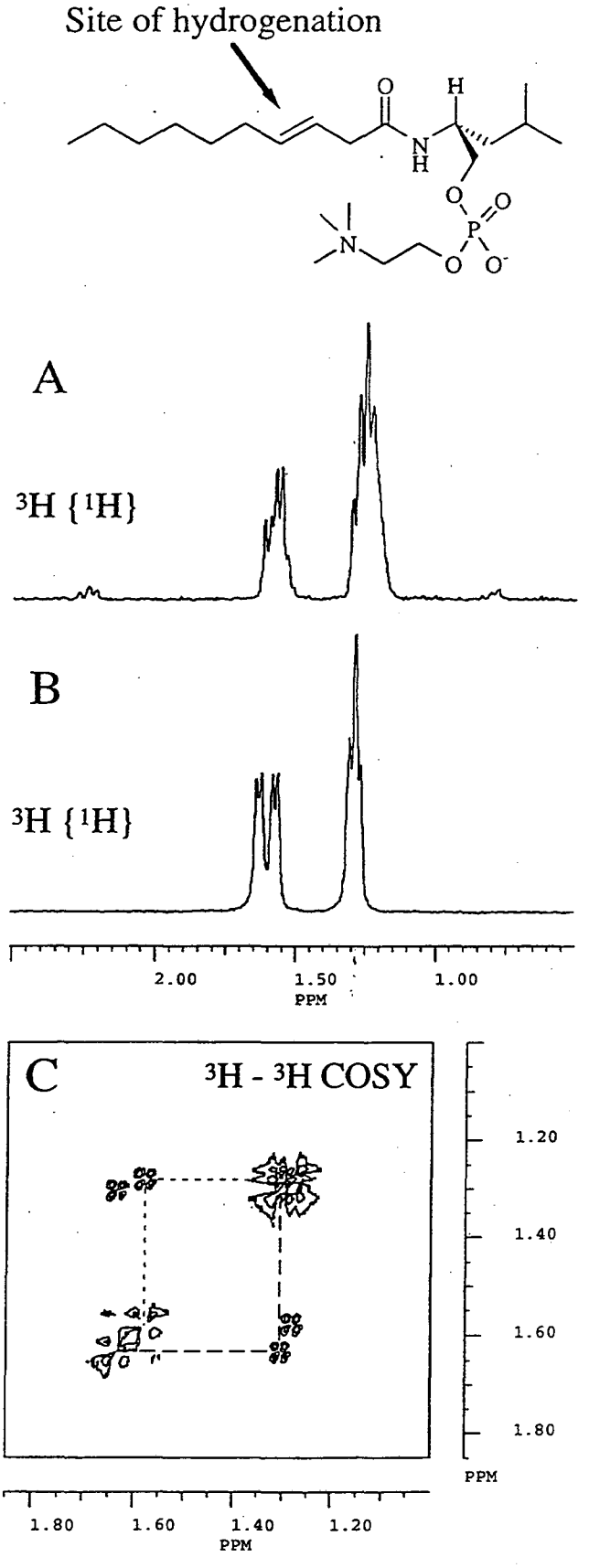

Site of hydrogenation
O
H
N
H
O
O
P
O
O
N
O
A
$^3$H {$^1$H}
B
$^3$H {$^1$H}
2.00
1.50
1.00
PPM
C
$^3$H - $^3$H COSY
1.20
1.40
1.60
1.80
PPM
1.80
1.60
1.40
1.20
PPM

Figure 4

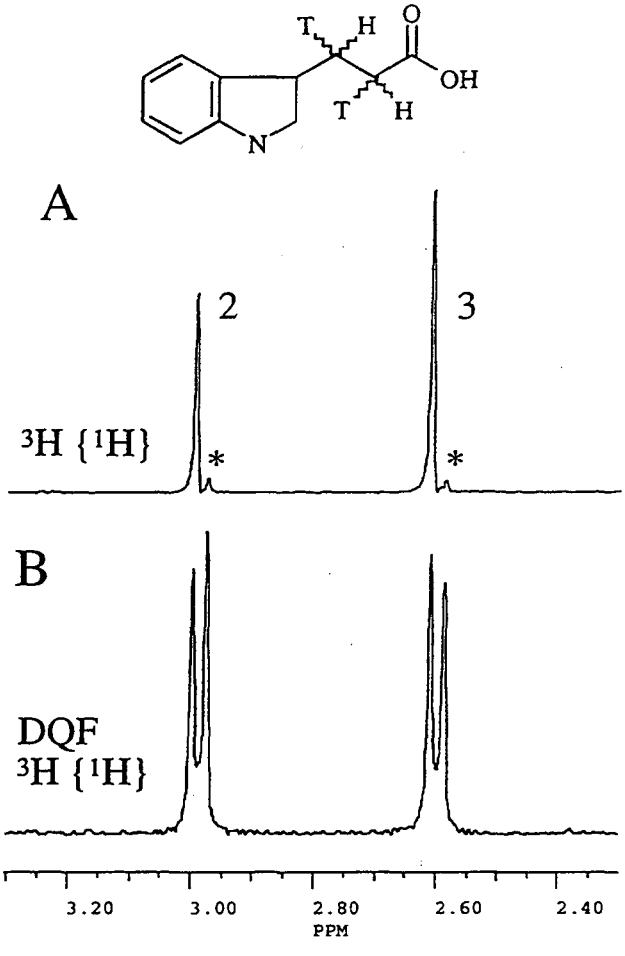



Figure 5

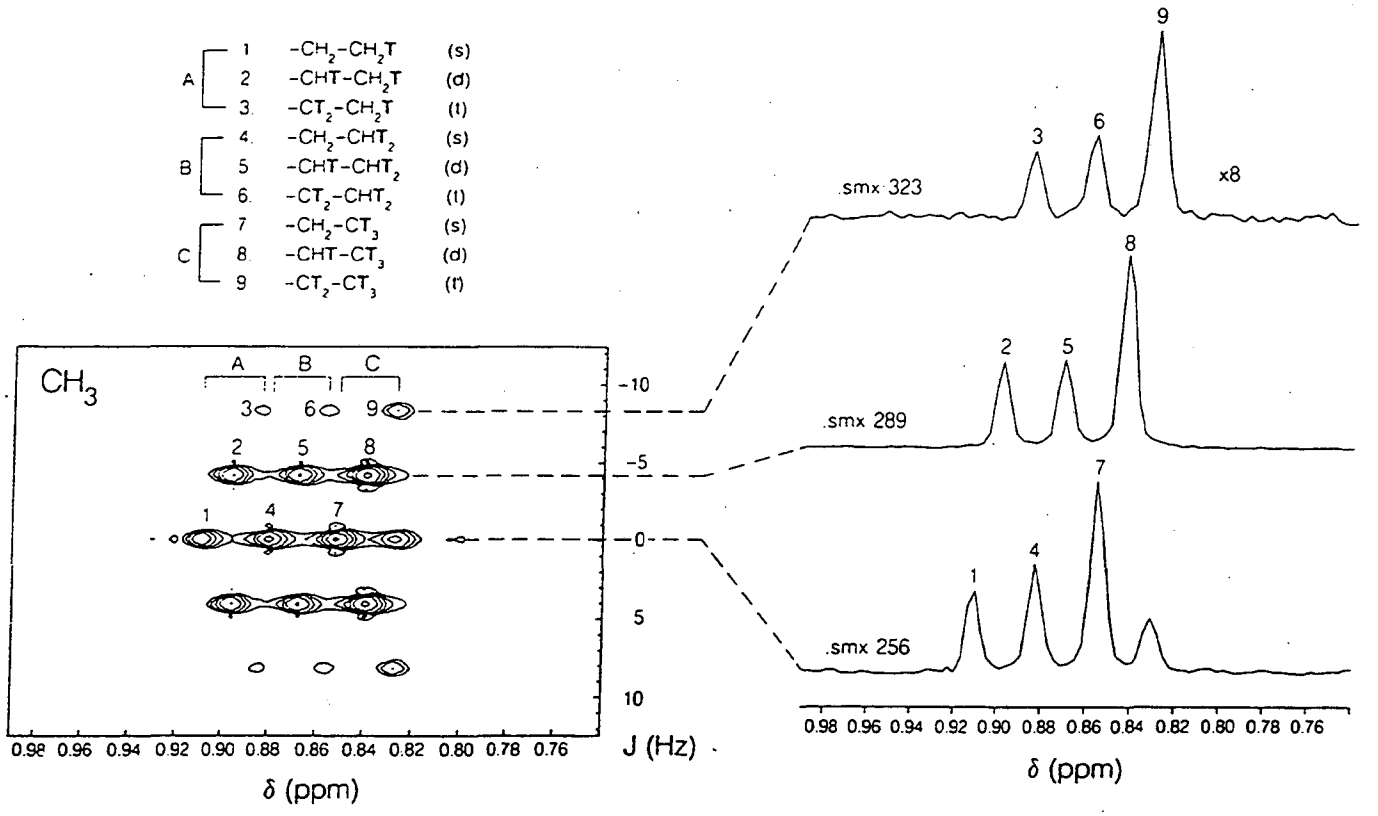

A
1 -CH2-CH2T (s)
2 -CHT-CH2T (d)
3 -CT2-CH2T (t)
B
4 -CH2-CHT2 (s)
5 -CHT-CHT2 (d)
6 -CT2-CHT2 (t)
C
7 -CH2-CT3 (s)
8 -CHT-CT3 (d)
9 -CT2-CT3 (t)
CH3
A B C
3 6 9
2 5 8
1 4 7
-10
-5
0
5
10
J (Hz)
0.98 0.96 0.94 0.92 0.90 0.88 0.86 0.84 0.82 0.80 0.78 0.76
δ (ppm)
.smx 323
.smx 289
.smx 256
x8
0.98 0.96 0.94 0.92 0.90 0.88 0.86 0.84 0.82 0.80 0.78 0.76
δ (ppm)

Figure 6

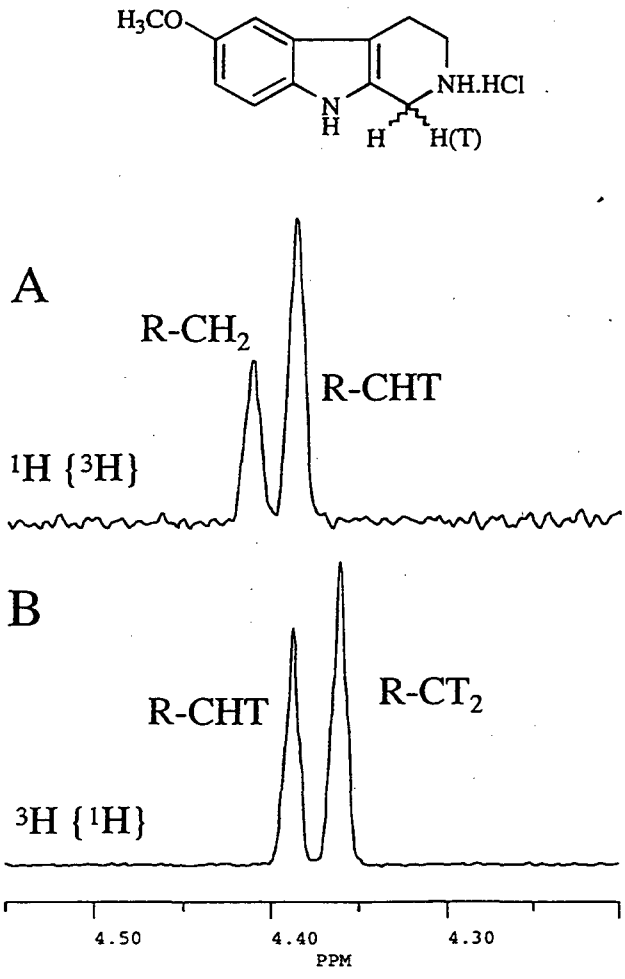

H3CO
NH.HCl
N
H
H
H(T)
A
R-CH2
R-CHT
1H {3H}
B
R-CHT
R-CT2
3H {1H}
4.50
4.40
4.30
PPM

Figure 7

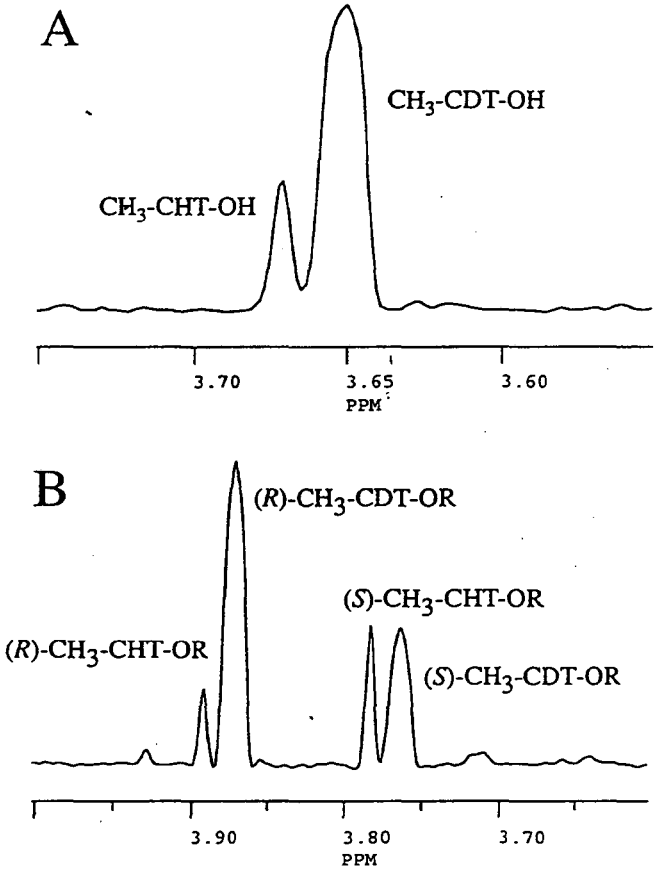

A
CH3-CDT-OH
CH3-CHT-OH
3.70
3.65
3.60
PPM
B
(R)-CH3-CDT-OR
(S)-CH3-CHT-OR
(R)-CH3-CHT-OR
(S)-CH3-CDT-OR
3.90
3.80
3.70
PPM

Figure 8

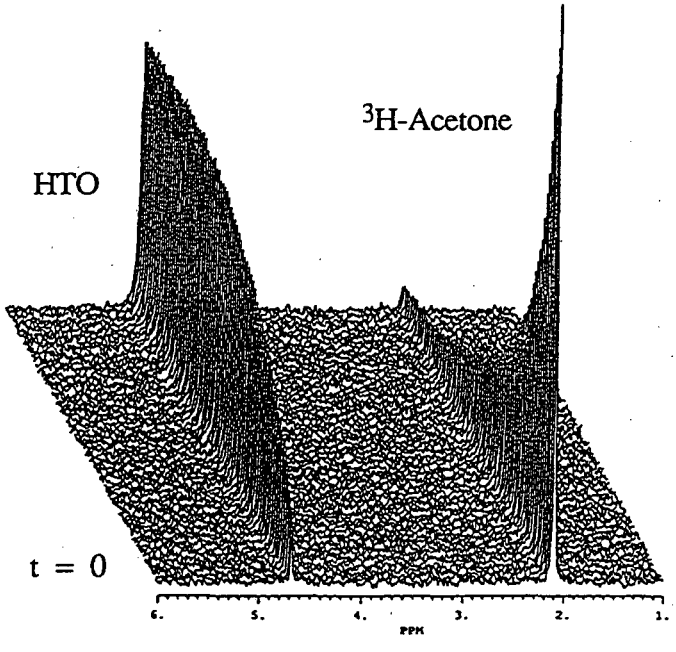



Figure 9

Subject Index of Tritium NMR

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