The ^{18}O Isotope Shift in ^{13}C Nuclear Magnetic Resonance Spectroscopy. 15. Studies of [^{18}O,1,4-$^{13}C_2$]Succinic Acid and [^{18}O]Dimethylmaleic Anhydride[1]

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Abstract: The complex ^{13}C NMR spectra arising from the various isotopomers in solutions of ^{18}O-labeled [1,4-$^{13}C_2$]-succinic acid and dimethylmaleic anhydride are investigated. These studies reveal a way to measure the coupling constant between the carboxyl carbons of succinic acid by utilizing isotopic substitution to remove the chemical shift degeneracy. The pH dependence of this coupling constant and the isotope shift give rise to AB systems of varying strengths that dramatically influence spectral appearance. The eight signals observed in the carbonyl and olefinic regions of 53% ^{18}O-labeled dimethylmaleic anhydride are interpreted in terms of isotope shifts over one, two, and even three bonds.

Introduction

Extensive studies of the ^{18}O isotope shift in ^{13}C NMR spectroscopy[2] consistently show that an ^{18}O atom directly attached to a carbon atom causes a small (0.010–0.050 ppm) upfield shift in its ^{13}C NMR resonance position relative to that of the ^{16}O isotopomer.[3,4] The magnitude of this isotope shift depends primarily on the structure of the carbon–oxygen functional group with minor influences exerted by conjugation, substituent inductive effects, and the hybridization of the carbon atom.[4] The isotope shift is also additive. That is, separate, equally spaced resonances are generally seen for each chemically equivalent isotopic replacement.[4b] Ketones, for example, may exhibit two and carboxylic acids three possible peaks, while carbonic acid may exhibit four possible peaks.

In the course of studying the oxygen exchange kinetics of some succinic acid derivatives, we observed that the ^{13}C NMR spectra of ^{18}O-labeled [1,4-$^{13}C_2$]succinic acid exhibited features that seemed at first to be at variance with expectations based upon previous studies. In particular, it was initially surprising to observe 15 peaks in the ^{13}C NMR spectrum of 50% ^{18}O-labeled [1,4-$^{13}C_2$]succinic acid.[5] The basis for this phenomenon was investigated, and the results were found to provide a direct method for measuring the three-bond coupling constant, $^3J_{CC}$, between the carboxyl carbons of succinic acid as well as a straightforward method for probing the pH dependence of its conformer populations. In addition, we report spectra for the succinic acid derivative, dimethylmaleic anhydride (3,4-dimethyl-2,5-furandione). These two systems illustrate how the ^{18}O-isotope shift may be employed to distinguish between isotopomers that have identical ^{18}O enrichment but different ^{18}O-labeling patterns.

Experimental Section

Materials. [1,4-$^{13}C_2$]Succinic acid (99 atom % ^{13}C), sodium [^{13}C]-cyanide, and [^{18}O]water (98 atom % ^{18}O) were purchased from Merck. Dioxane (spectral grade) and benzyl alcohol were purchased from Boehringer. Deuterium oxide (99.8 atom % D), dioxane-d_8 (98.5 atom % D), dimethylmaleic anhydride (98%), citraconic anhydride, and succinic acid were purchased from Aldrich.

Sodium [1-^{13}C]succinate was prepared by hydrolysis of ethyl 3-[^{13}C]-cyanopropionate which was prepared as described.[6] Succinic acid was ^{18}O labeled by heating overnight at 60 °C in [^{18}O]water acidified with gaseous HCl. Dimethylmaleic anhydride was placed in [^{18}O]water containing sufficient dioxane to achieve solubility of the anhydride at 60 °C. After 3 days at 60 °C, the ^{18}O-labeled anhydride was recrystallized. Mass spectral analysis indicated the following ^{18}O-labeling pattern—$^{18}O_0$, 10.4%; $^{18}O_1$, 32.3%; $^{18}O_2$, 40.2%; $^{18}O_3$, 17.2%—giving an overall ^{18}O incorporation of 53%.

NMR Measurements. Spectra were obtained using either an NTC-200 spectrometer fitted with a 12-mm probe operating at 50.3 MHz, a Varian 200 spectrometer (5-mm probe, 50.3 MHz), a QE-300 spectrometer (5-mm probe, 75.6 MHz), or an NTC-470 spectrometer (8-mm probe, 118.1 MHz). All spectra were broad-band ^1H decoupled using either a Waltz-16 scheme or an MLEV sequence.[7] Small sweep widths (40–200 Hz) were centered about the signals of interest and 50–200 scans were collected using a 90° pulse angle and a sufficiently large block size (4K to 32K) to give relatively long acquisition times (30–50 s) to ensure excellent digital resolution. The FIDs were zero filled and a moderate resolution enhancement function (DM = 2 on the GENT and CHARM software) was applied prior to Fourier transformation.

Results

Figure 1E shows the ^{13}C NMR spectrum of ^{18}O-labeled [1,4-$^{13}C_2$]succinic acid in D_2O. In this spectrum, nine peaks are readily discerned within a chemical shift range of 0.054 ppm. This is in sharp contrast to the three peaks that are generally observed for an ^{18}O-labeled carboxylic acid within the same chemical shift range (see Introduction).[3a] Furthermore, as the solution pH is varied near the pK_a's of succinic acid, significant changes in the spectrum occur. The chemical shift range of the spectra shown in spectra A–D in Figure 1 is nearly 50% larger than that of Figure 1E, and it encompasses 0.077 ppm. Even more interestingly, during this expansion, some of the peaks actually shift their relative position with respect to neighboring peaks.

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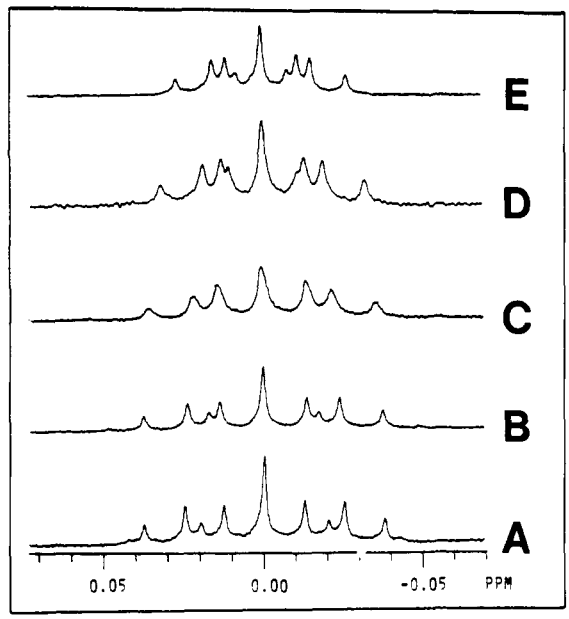

Figure 1. ^{13}C NMR spectral changes of ^{13}C, ^{18}O-labeled succinic acid with solution acidity. Expansions of the carboxyl regions of the 50.3-MHz ^{13}C NMR spectra obtained using solutions of 52% ^{18}O-labeled, 99% ^{13}C-enriched [1,4-$^{13}C_2$]succinic acid prepared in D_2O. Solutions gave (uncorrected) pH meter readings of (A) 5.0, (B) 5.3, (C) 5.6, (D) 6.1, and (E) 7.4. The spectra were plotted using the same horizontal scale with the central peak referenced to 0 ppm. Note that some peaks actually shift positions with respect to one another as the solution acidity is varied.

The spectrum shown in Figure 2A was obtained from the same sample after the spectrometer was very carefully shimmed and more scans were acquired in order to increase the signal-to-noise ratio. The line width at half height of the central resonance in the spectrum was measured at 0.10 Hz. Fifteen peaks can readily be discerned under these conditions.

Several experiments were performed in order to elucidate the origin of these signals. The possibility of sample impurities was ruled out by the following observations. Only one peak was observed in the ^{13}C NMR spectrum of the non-^{18}O-labeled [1,4-$^{13}C_2$]succinic acid. Addition of this non-^{18}O-labeled material to the NMR sample resulted in an increase of the peak referenced to 0.0 ppm in Figure 2A. Furthermore, when the non-^{18}O-labeled material was placed in ^{18}O-enriched water under acidic conditions, the several peaks shown in Figure 2A began gradually to appear, presumably as a result of in-exchange of the ^{18}O label. Finally, the reverse of this exchange process could also be observed, wherein the several peaks of Figure 2A collapsed into a single peak as the ^{18}O label was exchanged out into (unlabeled) bulk solvent.

In order to assess the importance of two sites of ^{13}C enrichment in the ^{13}C NMR spectrum of the diacid, ^{18}O-labeled [1-^{13}C]-succinic acid was then prepared. The removal of ^{13}C enrichment at one of the carboxyl groups resulted in ^{13}C NMR spectra which contained only the three peaks, separated by the usual isotope shifts, that would be expected for a carboxylic acid. (This result allowed oxygen exchange data to be conveniently acquired for the diacid.[5]) ^{18}O-Labeled succinic acid (natural abundance ^{13}C) was also prepared and it too was found to exhibit the usual spectrum with only three peaks.

Since it seemed likely that the complex spectrum of Figure 2A could be explained in terms of a coupling interaction between the two ^{13}C-enriched carboxyl carbons, a ^{13}C-^{13}C COSY experiment was performed on a sample of 52% ^{18}O-labeled [1,4-$^{13}C_2$]succinic acid. A contour plot from this experiment is shown in Figure 2C. It can be seen that off-diagonal peaks are indeed present, consistent with the coupling-induced splitting pattern interpretation given below (see Discussion).

In the course of our studies of oxygen exchange of succinic acid derivatives, we also observed an unusually large number of

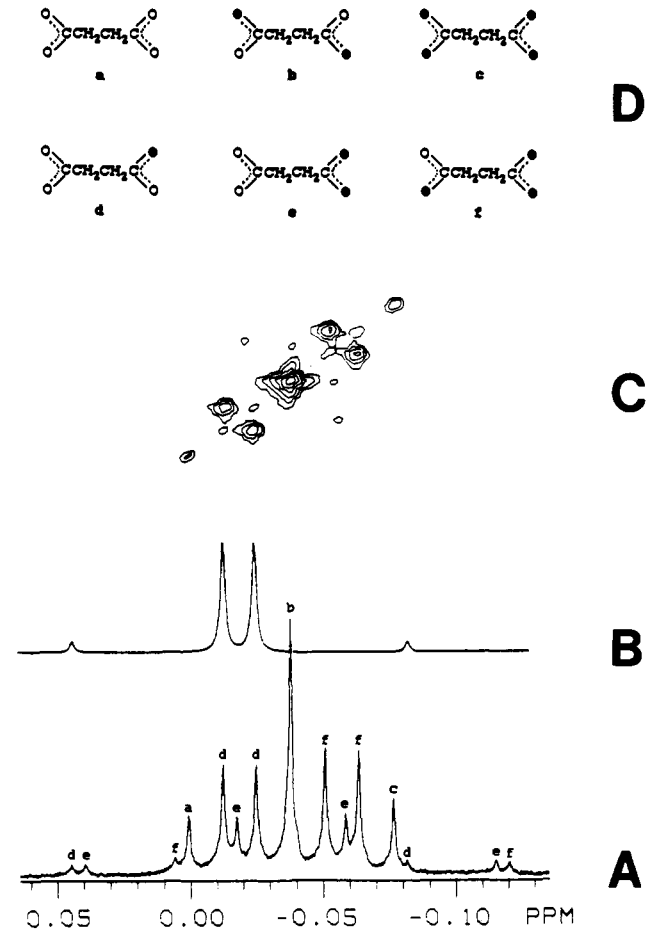

Figure 2. Coupling pattern interpretation. (A) Higher resolution spectrum of the sample referred to in Figure 1. Peaks labeled **a**, **b**, and **c** are assigned to the symmetrically ^{18}O-labeled succinate species shown in part D, and peaks labeled **d**, **e**, and **f** are assigned to the asymmetrically ^{18}O-labeled species. The simulated spectrum (B) was generated by assuming that two nuclei (such as the carboxyls of species **d**) separated by 0.0386 ppm (the isotope shift) were coupled with $J = 2.86$ Hz. The isotope shift is obtained from the chemical shift difference between peaks **a** and **b** (or **b** and **c**), and the coupling constant is obtained from the difference between the intense inner and weak outer peaks of any of the three AB systems (**d**, **e**, or **f**). Evidence in favor of the coupling pattern interpretation includes the observation of cross peaks in the ^{13}C-^{13}C COSY spectrum (C) for the intense inner peaks of the strongly coupled AB patterns.

signals in the ^{13}C NMR spectra of ^{18}O-labeled dimethylmaleic anhydride (Figure 3,A and B). As with the succinic acid spectra, the extra signals in the spectra for ^{18}O-labeled dimethylmaleic anhydride can be explained by considering the six possible ^{18}O isotopomers of these molecules (Figures 2D and 3C). However, in this case coupling interactions cannot be invoked since ^{13}C-enriched material was not used. Instead, the spectra in Figure 3 were interpreted in terms of isotope effects transmitted over two and even three bonds.

The various isotope shifts proposed to explain the spectra of [^{18}O]dimethylmaleic anhydride are summarized in Figure 4. Since the magnitudes of these isotope shifts are extremely small (as small as 0.00093 ppm = 0.93 ppb for an isotope shift over three bonds), additional procedures were utilized to verify that instrumental artifacts (nonhomogeneous magnetic fields) did not interfere with spectral interpretation. Thus, sharp reference peaks were included in the spectral windows during acquisition of the spectra to serve as "shim standards" to ensure that the fine splittings that were observed were not due to magnetic field inhomogeneities. The spectra of these signals are included in Figure 3, spectra A and B.

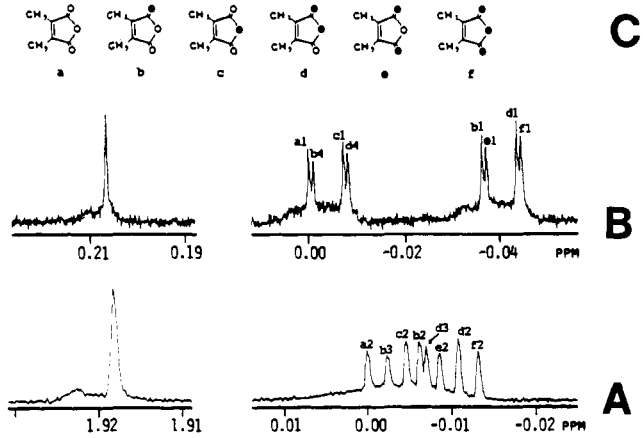

Figure 3. Multiple-bond ^{18}O-isotope shifts. 75.6-MHz ^{13}C NMR spectra of (A) the olefinic carbon region and (B) the carbonyl carbon region of 53% ^{18}O-labeled dimethylmaleic anhydride. Each peak is assigned to one of the eight lettered species in part C with a number corresponding to the particular carbon atom in that molecule which is proposed to give rise to that signal. Note that in both the carbonyl and olefinic regions the symmetrically labeled species (**a**, **c**, **e**, **f**) each give rise to one signal while the asymmetrically labeled species (**b** and **d**) each give rise to two signals. The signals for carbons 1 and 2 of the nonlabeled species **a** are referenced to 0 ppm; these peaks appear at 167.1 and 141.1 ppm, respectively, relative to dioxane-d_8 (66.5 ppm). The signals to the left in spectra A and B are from C-1 of citraconic anhydride and the quaternary carbon of benzyl alcohol, respectively. The sharp peaks were included in the spectral windows during acquisition of the spectra and served to verify that the fine splittings observed were not due to magnetic field inhomogeneities. In this sample, inhomogeneities that were present caused the broad tail to the left of each signal.

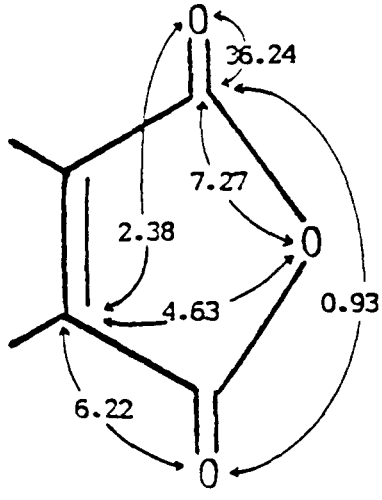

Figure 4. Dimethylmaleic anhydride isotope shift assignments. The magnitudes of the various isotope shifts assigned in Figure 3 for ^{18}O-labeled dimethylmaleic anhydride are given in ppb (1 ppb = 0.001 ppm).

Discussion

The pH dependence of the ^{13}C NMR spectra of [^{18}O,1,4-$^{13}C_2$]-succinic acid (Figure 1) is particularly intriguing. These spectral changes are clearly related to the ionization state of the carboxyl groups since spectra acquired above pH 7 and below pH 2 display no pH dependence. Initially, it was thought that hydrogen bonding between the carboxyl groups might account for these spectral changes, especially since hydrogen bonding has been shown to influence the magnitude of the ^{18}O-isotope shift in the ^{15}N NMR spectrum of pentane-2,3,4-trione 3-oxime.[8] However, several authors have argued against the presence of significant intramolecular hydrogen bonding in succinic acid.[9] Instead, the succinic acid spectra can be explained in terms of two recently reported phenomena working together in this system: (1) the differences in NMR pH-titration curves that result upon ^{18}O substitution of carboxylic acids, and (2) the strong coupling patterns that result when the degeneracy of magnetically equivalent nuclei is removed by ^{18}O labeling.

Substitution of ^{18}O into a carboxylic acid is accompanied by a small equilibrium isotope effect on its pK_a. As a result, slightly different titration curves are obtained when, for example, the ^{13}C NMR chemical shift of [^{16}O]- or [^{18}O]formic acid is measured as a function of solution pH.[10] The difference between these titration curves (i.e., the isotope shift) reaches a maximum near the pK_a of the acid (see Figure 5). Ellison and Robinson demonstrated how such measurements could be used to calculate the increased pK_a of [^{18}O]formic acid over that of [^{16}O]formic acid.[10] Similar variations in NMR isotope shifts have been observed in other systems involving ionizable groups.[11] In fact, using natural abundance ^{13}C, Perrin and Thoburn have measured the pH dependence of the ^{18}O-isotope shift in a series of dicarboxylic acids, including succinic acid.[12]

While the ^{18}O-induced perturbation of pK_a values influences the range spanned by the succinic acid spectral pattern, it is the presence of two sites of ^{13}C enrichment that leads to the unexpectedly large number of peaks. The carboxyl carbons of monomethyl [1,4-$^{13}C_2$]succinate exhibit a coupling constant of $^3J_{CC}$ = 2.3 Hz,[13a] and a coupling interaction of similar magnitude would be expected in [1,4-$^{13}C_2$]succinic acid. In the latter, however, the degenerate energy levels of the magnetically equivalent carboxyl groups prevent the coupling pattern from being observed.[14] The substitution of an ^{18}O atom into [1,4-$^{13}C_2$]succinic acid results in a small isotope shift for the attached carbon thereby removing the magnetic equivalence of the carboxyl groups (species **d**, Figure 2D). The ^{18}O labeling therefore allows a coupling pattern to be observed. Since the magnitude of the isotope shift in carboxyl groups is small (~1.3 Hz at 50 MHz)[3a] relative to the magnitude of the coupling constant ($^3J_{CC}$ = 2.3 Hz),[13a] a very strongly coupled AB pattern is expected (Figure 2B).[14]

The 15 peaks of Figure 2A therefore result from three overlapping AB patterns from the asymmetrically ^{18}O-labeled succinic acid species (**d**, **e**, and **f** in Figure 2D) plus three singlets from the symmetrically ^{18}O-labeled species (**a**, **b**, and **c** in Figure 2D). Similar observations of strong coupling have been made in ^{31}P NMR spectra of ^{18}O-labeled pyrophosphate[15] and tetraethyl pyrophosphate[16] and in ^{15}N NMR spectra of ^{18}O-labeled [^{15}N]-dimethylglyoxime.[8] The coupling pattern interpretation for our succinic acid spectra is also supported by the cross peaks observed (for the intense inner peaks of the AB systems) in the ^{13}C-^{13}C COSY experiment (Figure 2C) and by the less strongly coupled

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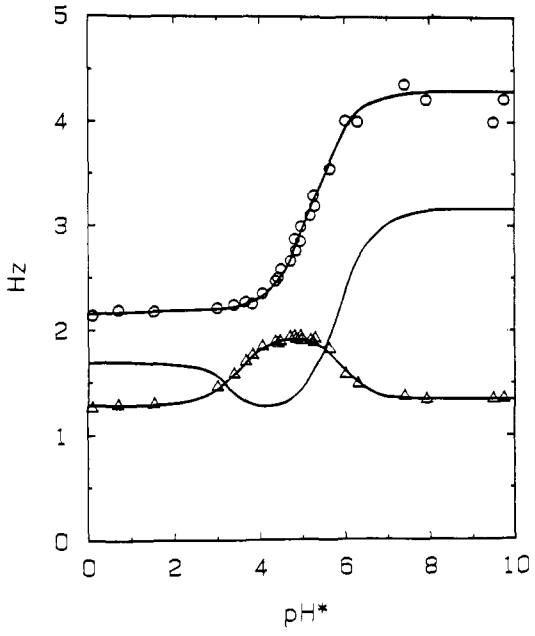

Figure 5. pH dependence of succinate isotope shift and coupling constant. 50.3-MHz ^{13}C NMR measurements of the magnitude of the (Δ) isotope shift and (○) coupling constant (described in Figure 2) were made for several D_2O solutions of ^{18}O-labeled [1,4-$^{13}C_2$]succinate. pH* values represent uncorrected pH meter readings. Also plotted is the ratio of the coupling constant to the isotope shift which defines the appearance of the AB systems.

AB systems that are observed in spectra acquired at higher field strengths (not shown).[5]

Knowledge of three-bond carbon–carbon coupling constants is important because of the relationship of $^3J_{CC}$ to the dihedral angle.[17] A dramatic change in succinic acid conformer populations upon ionization of the carboxyl groups has been demonstrated by Nunes *et al.* based upon an analysis of the ^{13}C satellites in ^1H NMR spectra of succinic acid.[18] The coupling constants of interest, $^3J_{HH}$, cannot be directly read from such spectra but must instead be calculated on the basis of several assumptions (indeed, the report by Nunes *et al.* corrected previous vicinal coupling assignments).[9a] In contrast, the three-bond carbon–carbon coupling constants, $^3J_{CC}$, can be read directly from the spectra reported in this study. A plot of these data (Figure 5) clearly shows the influence of solution acidity upon succinic acid conformer populations. Figure 5 also shows the pH dependence of the ratio of the coupling constant, $^3J_{CC}$, to the chemical shift difference between the coupled nuclei (the isotope shift). This ratio completely defines the relative positions and intensities of the peaks in the AB coupling patterns.[14] It is the variation in both this ratio and the isotope shift with pH that accounts for the appearance of the succinic acid spectra.

Menger and Lee demonstrated how the use of doubly ^{13}C-labeled compounds provides direct access to dihedral angle information for simple succinic acid derivatives.[13a] Such conformational information also provides insights into more complex systems such as DNA–ligand interactions, protein–ligand binding, and the analysis of lipid conformations.[13b-d] The additional use of ^{18}O labeling as reported here provides a method that complements Menger's approach and permits the study of even symmetrical species such as succinic acid. Furthermore, an extension of the pH-titration studies described here (Figure 5) to polar but non-hydrogen bonding solvents could provide useful information on the structure and properties of the strong hydrogen bonds formed by the monoanion of succinic acid that have recently been described.[12b,19]

Another example of how ^{13}C NMR can resolve all possible ^{18}O isotopomers in a complex solution is seen in the spectra of dimethylmaleic anhydride. As with succinic acid, a solution of 50% ^{18}O-labeled dimethylmaleic anhydride contains six distinct isotopomers as indicated in Figures 2D and 3C, respectively. These six species are obviously not resolved by the four peaks that are generally seen for an ^{18}O-labeled anhydride.[3b,20] (The four peaks arise from the following: (1) no ^{18}O's directly attached to the carbonyl carbon under observation, (2) one ^{18}O substituted at the bridge position, (3) one ^{18}O substituted at the carbonyl position, and (4) ^{18}O's substituted at both of the above sites.) However, under conditions of higher resolution, each of the four peaks is split by a very small isotope shift so that eight signals are seen (Figure 3B). Since ^{13}C-enriched material was not used, an explanation based upon coupling is not appropriate. Rather, it is proposed that these signals arise from the influence of an ^{18}O substitution at the carbonyl which is *not* under observation. This effect is presumably transmitted via the intervening bonds connecting the bridge oxygen, making it a 3-bond isotope shift.

Although ^{18}O-isotope shifts over two bonds have previously been observed, this appears to be the first observation in ^{13}C NMR of an ^{18}O-isotope shift over three bonds.[3b,5] ^{13}C NMR isotope shifts over three bonds have been observed as the result of ^2H substitution, and as might be expected, the magnitude of the isotope shift generally decreases significantly at sites further removed from the point of substitution.[21] Since the three-bond isotope shift seen here is so small (0.00093 ppm), it is valid to inquire whether the splitting arises from distinct nuclei or from the same nucleus at slightly different magnetic field strengths (i.e., poor shimming). To rule out the latter possibility, compounds containing sharp ^{13}C signals in the spectral window of interest were added to the sample (Figure 3, A and 3B). The lack of splitting in these "shim standard" peaks was taken as evidence of a sufficiently homogeneous field.

The above interpretation is also supported by spectra of the olefinic carbon signals of [^{18}O]dimethylmaleic anhydride (Figure 3A). Eight signals are again observed, but in this case all isotope shifts are necessarily two- or three-bond shifts since the olefinic carbons have no directly attached oxygens. It has been assumed that an isotope shift over two bonds is greater in magnitude than one over three bonds and that an isotope shift arising from a carbonyl oxygen ^{18}O substitution is of greater magnitude than an isotope shift arising from a bridge oxygen ^{18}O substitution.[3b,21a] Given these assumptions, the multiple-bond isotope shift assignments given in Figure 4 provide an internally consistent explanation for the [^{18}O]dimethylmaleic anhydride spectra.

Previous articles in this series have demonstrated the use of the ^{18}O-isotope shift in ^{13}C NMR spectroscopy to resolve species with varying degrees of isotopic enrichment. The examples presented here show that even isotopomers with identical ^{18}O enrichment (as determined by mass spectroscopy) may be distinguished if they differ in the locations of the ^{18}O labels within the molecule. In this way the ^{18}O-labeling pattern of even symmetrical compounds can be completely determined.

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