Two-dimensional N.M.R. Investigation of the Protonation Sequence in Spermidine

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Proton–carbon-13 two-dimensional correlation n.m.r. spectroscopy resolves the four protons adjacent to the nitrogen atoms of spermidine and permits determination of the protonation sequence.

Protonation sequences in polyprotic acids in solution have been studied by a variety of techniques, including calorimetry and potentiometry,[1] and i.r.,[2] electronic,[3] and n.m.r.[3,4,5] spectroscopy. When the successive macroscopic ionization constants differ by less than 2 log K units, overlapping populations of unprotonated, monoprotonated, and polyprotonated species make many of these techniques difficult to apply. In principle, the protonation sequence can be established by n.m.r. spectroscopy by determining the pH dependence of the relevant chemical shifts. However, this requires that each chemical shift be affected by the state of protonation of only one of several base sites. In practice, there are difficulties in the n.m.r. methods which have been used. Measurement of ^{15}N chemical shift changes in dilute solution requires enriched substrates, and long-range effects have been insufficiently studied.[6] ^{13}C Chemical shift para-

meters are particularly subject to long-range effects,[7] including the state of protonation of other more distant base sites. Most of the work in this field has used variation methods to refine the chemical shift parameters first approximated from model compounds.[4] The use of these approximation methods for analysis of the variation of ^{13}C chemical shifts with pH has given divergent protonation sequences for spermidine, *N*-(3-aminopropyl)-1,4-butanediamine (**1**). Kimberly and

a 3′ 2′ 1′ b 1 2 3 4 c
$NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NH_2$
(1)

Goldstein[8] report that for (**1**) at the point of macroscopic monoprotonation the primary N-c is 43% protonated, secondary N-b, 32%, and primary N-a, 32%, while Delfini and others[9] report (approximately) 50, 0, and 50% protonation respectively.

The chemical shifts of hydrogens adjacent to each base site are much less affected by the state of protonation of more remote sites, and thus approach 'unique resonances'.[4] For molecules whose ^1H n.m.r. spectra could be resolved, determination of the dependence of each of these chemical shifts on pH has been extensively used to elucidate the protonation sequence.[4] For the many cases in which the ^1H n.m.r. spectrum cannot be resolved, heteronuclear two-dimensional n.m.r. spectroscopy affords an elegant solution. The 200 MHz proton spectrum of spermidine is complex, making it difficult to assign the chemical shifts of the four (downfield) protons of interest on C-1, C-1′, C-3′, and C-4. This problem reflects both the close proximity of these four chemical shifts (less than 0.02 p.p.m. chemical shift difference between the protons on C-1′ and C-3′) and the highly coupled multiplet structure.

Two-dimensional n.m.r.[9–12] is a most appropriate technique for such spectroscopic problems for it permits overlapping resonances in a chemical shift dimension to be spread out in another dimension which is determined by the particular experiment chosen. An initial attempt to use a homonuclear two-dimensional *J*-coupled experiment,[13] which uses the J_{HH} coupling constants as the second dimension, failed to resolve fully the four downfield protons of spermidine. In the normal ^{13}C spectrum all four carbons of interest are fully resolved and have been unambigously assigned,[14] indicating that a proton–carbon-13-heteronuclear-correlated experiment might successfully resolve and assign the four protons. Using the pulse sequence described by Bax and Morris,[15] we were able to resolve fully all carbons and protons. Figure 1 shows a region of the 2-D spectrum representing the four carbons (and associated protons) which are adjacent to nitrogen atoms. The residual splittings in the proton dimension are due to ^1H–^1H homonuclear couplings which are not specifically suppressed in the pulse sequence. The power of this experimental technique is the ability to resolve proton resonances which are essentially identical in chemical shift. It has immediate applicability in following the course of sequential reactions where protons, otherwise identifiable only with difficulty, can be used as indicators.

These experiments were performed on a Varian XL-200 n.m.r. spectrometer using the standard hardware and two-dimensional software available. Sweep widths in the ^{13}C and ^1H dimensions were 2400 Hz and 800 Hz, respectively, with 1024 digital points in the carbon dimension. 256 Incremented ^{13}C spectra were taken for each run and zero-filling was used to obtain 512 digital points in the proton dimension. Exponential line broadening of 1.0—1.5 Hz was used in both dimensions.

All shifts (^1H and ^{13}C) were measured relative to dioxan as an internal standard and are reported relative to tetramethylsilane (TMS). Although two-dimensional displays such as Figure 1 were portrayed in the absolute-value mode, thus eliminating phase anomalies which give rise to negative peaks, individual spectral slices at single ^{13}C frequencies showing the ^1H resonances in the proton dimension were phased and recorded in the non-absolute-value mode to obtain narrower resonances and thus more accurate shift determinations.

Although full description of the protonation sequence requires a detailed analysis of pH–chemical shift plots, the approximate proton distributions for molecules that are not extensively protonated are readily derived from such plots. For example, at pH 11.2, where the relative amounts of unprotonated, monoprotonated, and diprotonated species, calculated from the known macroscopic ionization constants, are 0.70, 0.29 and 0.01, the fractions of the monoprotonated species protonated at primary N-c, secondary N-b, and primary N-a derived from pH–chemical shift plots are,

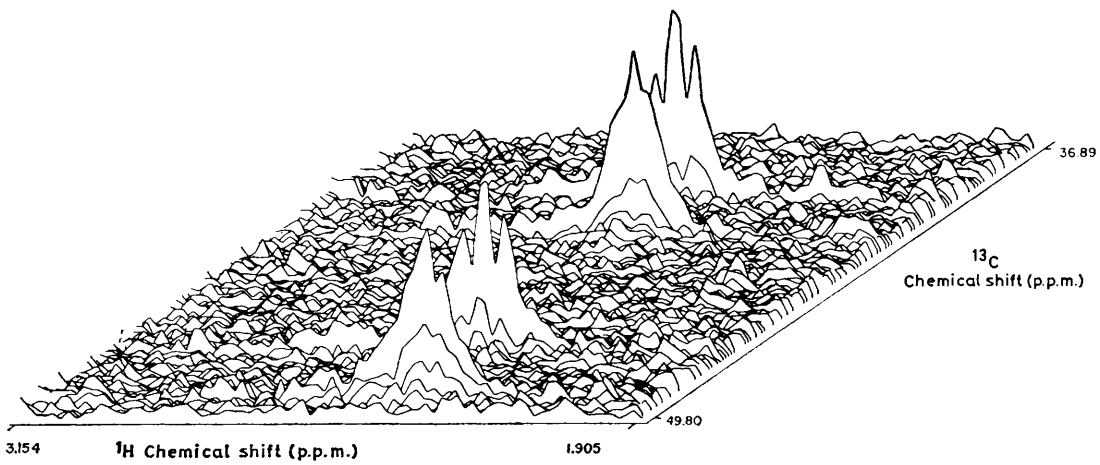

Figure 1. A portion of the heteronuclear C–H-correlated n.m.r. spectrum of spermidine showing the resonances due to the four downfield carbons (adjacent to nitrogen) and associated hydrogens. Data were collected as described in the text. Chemical shifts are relative to TMS.

respectively, 0.40, 0.35, and 0.22. The full protonation sequence is being elucidated in terms of microscopic constants and 2-D n.m.r. spectroscopy is being used to determine similar information for other bases.

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