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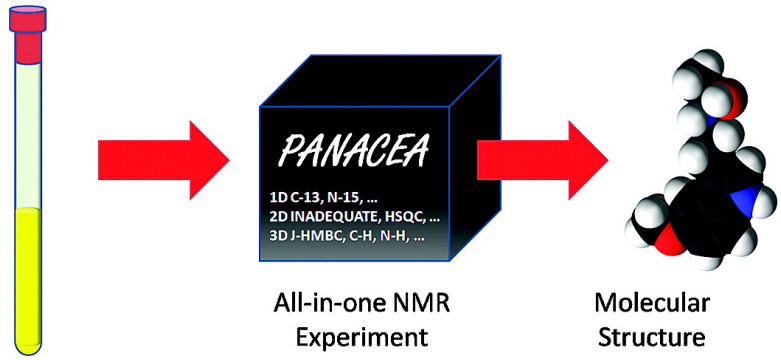

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Molecular Structure from a Single NMR Experiment

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Abstract: A procedure is described for determining the structure of a small molecule from a single NMR experiment. Several standard NMR sequences are combined so that the essential structural information is obtained in just one pass. Two-dimensional $^{13}C-^{13}C$ correlations ("INADEQUATE"), single- and multiple-bond $^{13}C-^{1}H$ correlations, and the conventional ^{13}C spectrum are recorded in parallel, making use of separate receiver channels for acquisition of ^{13}C and ^{1}H signals. The natural-abundance $^{13}C-^{13}C$ correlation measurements employ a high-sensitivity cryogenically cooled probe, optimized for ^{13}C detection. An extension of this "all-in-one" sequence with three parallel receivers permits the corresponding natural-abundance ^{15}N spectra to be included.

Introduction

Molecular structure determinations by NMR can be greatly facilitated if all the essential correlation information is derived from a single experiment. For small organic molecules, it can be argued that the two-dimensional $^{13}C-^{13}C$ and $^{13}C-^{1}H$ correlation spectra (together with the conventional ^{13}C spectrum) would normally be sufficient to determine the complete molecular structure. If other magnetically active (X) spins are present (such as ^{15}N), the corresponding X−H correlation spectra provide supplementary structural information. The single-bond $^{13}C-^{13}C$ correlation spectrum ("INADEQUATE") is the key that provides unambiguous information about the carbon skeleton of the molecule. In situations where the carbon chains are interrupted by a heteroatom, long-range $^{13}C-H$ or X−H correlations serve to bridge the fragments. The single-bond correlations identify the CH, CH_2, and CH_3 sites.

At present, these conventional investigations [INADEQUATE, HSQC (heteronuclear single-quantum correlation), HMBC (heteronuclear multiple-bond correlation)] would be carried out in a discontinuous sequence of measurements, often run under changing environmental conditions. We describe here a method that combines several standard pulse sequences into a single scheme, making use of a recent innovation that permits parallel acquisition of signals from two, three, or four different nuclear species. This PANSY[1,2] technique (parallel acquisition NMR spectroscopy) makes it feasible to record two-dimensional $^{13}C-^{13}C$ and $^{13}C-^{1}H$ correlation spectra and a standard one-dimensional ^{13}C spectrum, all at the same time. A great deal can be accomplished with just two receiver channels, because this still permits *indirect* measurement of the evolution of another heteronuclear spin. The experiment is easily extended to three or more receivers if necessary; we show an example that exploits parallel acquisition of ^{15}N signals.

This "all-in-one" experiment greatly simplifies the operating protocol, in line with other established procedures common in X-ray crystallography and mass spectrometry. It also ensures that all the measurements are performed under essentially identical conditions, thus mitigating the effects of any environment-induced changes or longer-term sample deterioration. By deriving all the structural information from a single pass, it offers the possibility that an entire sequence of different samples could be investigated without any operator intervention. An automatic NMR service could then take responsibility for investigating unknown samples and be confident of gathering all the relevant information without recourse to retrospective measurements.

Carbon−Carbon Correlation. In principle, the INADEQUATE experiment[3–6] offers the most reliable NMR information on the backbone structure of small organic molecules, since it establishes the connectivity of the carbon atoms that make up the basic framework. For proton-decoupled natural-abundance samples, each individual pair of directly bound ^{13}C spins exists in isolation, generating an NMR response that is unaffected by the other spin−spin interactions. Unfortunately the intrinsic sensitivity of this technique is best described as inadequate, being limited by the requirement that two low-abundance ^{13}C spins be present side by side in the same molecule. This means that only one molecule in about 8600 contributes to the observation of a particular interaction.

Although valiant attempts have been made to improve the sensitivity of this technique, they have had only limited success. Experiments that transfer polarization from protons to carbon[7,8] or from carbon to protons[9–12] falter or fail for quaternary

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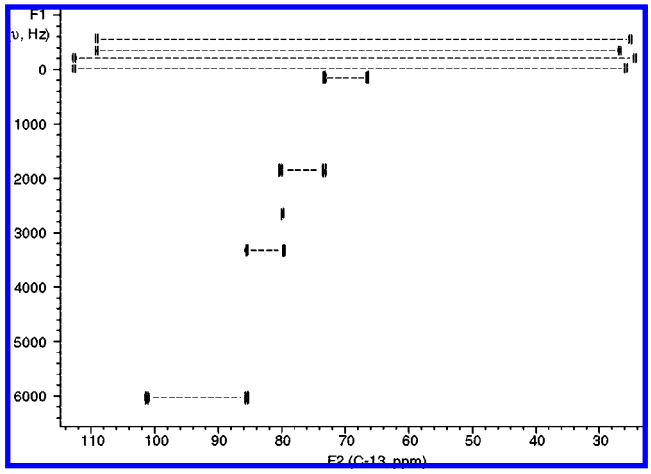

Figure 1. Demonstration of the sensitivity achievable for the INADEQUATE experiment using a cryogenically cooled probe optimized for ^{13}C detection. The sample was 10 mg of 2,3:5,6 di-*O*-isopropylidene-α-D-mannofuranose in $CDCl_3$, and the spectrum was acquired using the pulse sequence shown in Figure 2 in 9 h on a Varian 500 MHz (^1H) spectrometer. There is an AB spin system centered near 79 ppm. A symmetrization routine (ref 17) has been employed to enhance the signal-to-noise ratio (see text).

carbons, and these are just the sites most handicapped by slow spin−lattice relaxation. The addition of relaxation agents such as transition metal ions[13] may be unacceptable on chemical grounds and, unless the dosage is carefully controlled, may actually degrade signal strength by quenching the nuclear Overhauser effect.[14] A moderate enhancement of relaxation can be achieved by dissolving oxygen under pressure at a low temperature and by adding a substance (such as perfluorotertiarybutanol) that increases oxygen solubility.[15] Data-processing schemes that use a parametric fitting program,[16] or exploit the intrinsic symmetry[17] of the INADEQUATE traces, or concentrate the intensity of a J_{CC} doublet into a single line[17,18] offer further small improvements in signal-to-noise ratio. These rather modest palliatives have now been overshadowed by a recent advance in cryogenic probe technology, specifically designed for ^{13}C detection, which offers an additional, approximately 10-fold enhancement in sensitivity, restoring the practicality of the INADEQUATE technique. Figure 1 demonstrates that as little as 10 mg of a sample of 2,3:5,6-di-*O*-isopropylidine-α-D-mannofuranose in $CDCl_3$ can generate an INADEQUATE spectrum in only 9 h on a Varian 500 MHz spectrometer equipped with a cryogenically cooled probe optimized for ^{13}C detection.

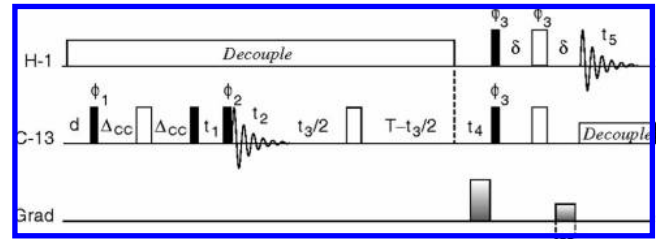

Figure 2. Pulse sequence designed for simultaneous observation of a decoupled one-dimensional ^{13}C spectrum, a two-dimensional $^{13}C-^{13}C$ INADEQUATE spectrum, two-dimensional multiplicity-edited $^{13}C-^1H$ HSQC spectra, and a three-dimensional $^{13}C-^1H$ J-HMBC spectrum in a spectrometer equipped for parallel acquisition of ^1H and ^{13}C signals. For details, see text.

$^{13}C-^1H$ Correlations (Single- and Multiple-Bond). Single-bond $^{13}C-^1H$ correlations[19–22] obtained by the HSQC sequence, offer a powerful structural tool, identifying the protonated carbon atoms in the molecule. These spectra are typically well-resolved, and the CH, CH_2, and CH_3 subspectra can be separated by a multiplicity-editing scheme.[23] Together with the ^{13}C chemical shift data, the multiplicity information provides indirect evidence for the presence of magnetically inactive heteroatoms in the molecule. Long-range $^{13}C-^1H$ couplings, derived from the HMBC pulse sequence, serve to link molecular fragments separated by a heteroatom. There have been many initiatives[24–47] to facilitate their detection, bearing in mind the low natural

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abundance of ^{13}C, and the practical complication that $^{1}H-^{1}H$ couplings and multiple-bond $^{13}C-^{1}H$ couplings are similar in magnitude.

Experimental Section

The key to the "all-in-one" measurement is to devise a radio-frequency pulse sequence that excites all the desired one-, two-, and three-dimensional spectra in a single pass, relying on the multiple-receiver facility. The innovation is to find a way to combine several standard pulse sequences in such a manner that any interaction is minimal and the individual detection sensitivities are not significantly compromised. The basic experiment (Figure 2) is conceived for a spectrometer equipped with two receivers (^{1}H and ^{13}C). It generates a one-dimensional ^{13}C spectrum, a two-dimensional $^{13}C-^{13}C$ INADEQUATE spectrum, three multiplicity-edited $^{13}C-^{1}H$ HSQC spectra, and a three-dimensional $^{13}C-^{1}H$ J-HMBC spectrum that can be projected to display the $^{13}C-^{1}H$ multiplets directly.

Clearly, the INADEQUATE experiment determines the overall experimental duration, owing to the low abundance of molecules containing two ^{13}C spins. However the greatly improved ^{13}C performance of the cryogenically cooled probe mitigates this problem, giving a sensitivity enhancement of roughly 10 times. In addition, a symmetrization routine[17] was applied to the INADEQUATE traces. It was based on the ^{13}C chemical shifts derived from the one-dimensional ^{13}C spectra recorded in parallel and made allowance for the small isotope shifts and the fact that the F_2 traces were not exactly symmetrical with respect to the double-quantum diagonal. Spectra shown in the Supporting Information demonstrate that this postacquisition processing affords a further 2-fold enhancement of signal-to-noise. The symmetrization routine used in this work is described in more detail in the Supporting Information. In particular, we show that local symmetrization becomes inefficient if the chemical shift difference between the two coupled sites is less than $2.2^{1}J_{CC}$.

The two-dimensional INADEQUATE stage employs an evolution interval t_1 and fixed delays of $\Delta_{CC} = 0.25/(^{1}J_{CC})$. Phase cycling ($\phi_1 = 4(x), 4(-x)$; $\phi_2 = x, y, -x, -y$; with the receivers set to $x, -y, -x, y, -x, y, x, -y$) is employed to suppress ^{13}C peaks that are not coupled to other ^{13}C sites; in this implementation these coherences are not rejected but are stored separately, and the residual magnetization is refocused by a second 180° pulse applied to ^{13}C. This is then utilized to record the $^{13}C-^{1}H$ correlations. Once the INADEQUATE signal has been acquired, the ^{13}C evolution is reversed in a constant-time experiment[48] (with $T = t_2 - t_4$ and variable parameter t_3). Nothing is wasted; the conventional ^{13}C spectrum is stored separately as a further aid to structure determination. Any residual magnetization from the INADEQUATE stage can now be safely neglected, because all the later stages utilize the far stronger signals from isolated ^{13}C spins.

The INADEQUATE stage is followed by free evolution of the ^{13}C chemical shifts and $^{13}C-^{1}H$ couplings with the proton decoupler switched off for an evolution interval t_4 in order to record the $^{13}C-^{1}H$ correlation spectra edited according to the ^{13}C multiplicities. The fact that we start with ^{13}C magnetization rather than an initial INEPT stage is not important since the sensitivity is determined by the INADEQUATE stage. The delay, T, is set to $T = t_2 - t_4$, where t_2 is the (constant) acquisition time in the directly detected dimension of the INADEQUATE experiment. The choice of t_2 is a compromise between the conflicting requirements of high resolution in F_2 and the signal losses due to spin−spin relaxation. The parameter t_4 is the J-evolution delay defining the spectral width in F_4, the indirect dimension of the multiplicity-edited $^{13}C-^{1}H$ HSQC experiments and the 3D $^{13}C-^{1}H$ J-HMBC experiment. The t_4 delay is initially set to $0.25/(^{1}J_{CH})$, and the spectral width in the F_4 dimension is set to approximately 4 times ($^{1}J_{CH}$) so that the evolution time (t_4) is incremented in steps of $0.25/(^{1}J_{CH})$. With $t_4 = 0.25/(^{1}J_{CH})$ the ^{13}C sites of all multiplicities have positive responses; with $t_4 = 0.5/(^{1}J_{CH})$ only the CH sites are detected; with $t_4 = 0.75/(^{1}J_{CH})$ the CH and CH_3 sites have positive responses while the CH_2 sites have negative responses. Thus, the multiplicity-editing is achieved by the first three increments. The decoupling is then switched off, and the experiment continues as a three-dimensional J-HMBC sequence with t_3 and t_4 as evolution parameters. The phase ϕ_3 is cycled $x, y, -x, -y$. The projection of this three-dimensional matrix onto the F_2F_3 plane displays the $^{13}C-^{1}H$ multiplets directly.

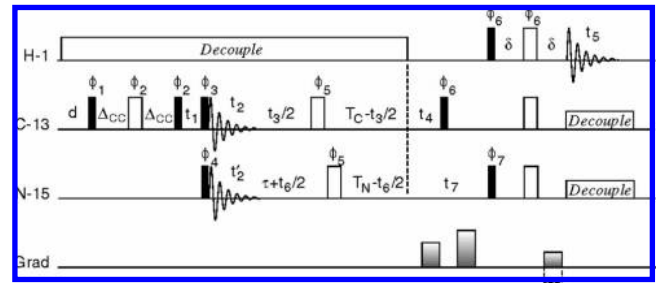

Figure 3. Pulse sequence corresponding to Figure 2, but with the additional capability to acquire the corresponding ^{15}N correlation spectra. An optional third receiver channel is used to acquire a directly detected one-dimensional ^{15}N spectrum. The constant-time parameters are T_C for the ^{13}C channel and T_N for the ^{15}N channel.

The ^{13}C coherence is then encoded by a gradient pulse and transferred to the protons for detection, exploiting the dual-receiver feature of the PANSY scheme. A gradient-decoding pulse during the second δ refocusing delay minimizes unwanted proton coherences. This delay is usually 1.0−1.5 ms in duration to allow for a reasonable gradient duration and recovery time. Phase-sensitive INADEQUATE spectra are obtained by incrementing the phases of the first three ^{13}C pulses in 45° steps, thus shifting the double-quantum coherences by 90°, giving two signal components in quadrature. An optional binomial filter[49,50] can be incorporated by incrementing δ in steps of $0.5/^{1}J_{CH}$ in successive scans to suppress the single-bond correlations in the three-dimensional J-HMBC spectrum.

A spectrometer equipped with a third receiver channel can also measure the corresponding ^{15}N information. Figure 3 shows the requisite pulse sequence. In the ^{15}N channel the new evolution parameter is t_7, and another field gradient pulse is applied after the ^{13}C coherence has been transferred to the protons. Because the extensive ^{15}N chemical shift range of small molecules (~400 ppm), it may be advantageous to use either composite or adiabatic inversion pulses[51] instead of hard 180° pulses. The same applies to the corresponding ^{13}C pulses. Some signal loss may occur due to molecular diffusion in the intense pulsed field gradient. In cases where the one-dimensional ^{15}N spectrum is not required, the remaining information can be obtained with a spectrometer having only two parallel receivers, as the ^{15}N evolutions can be observed indirectly.

The phase cycling is modified to allow separation of the ^{13}C and ^{15}N correlations: $\phi_1 = x, -x, x, -x, -x, x, -x, x$; $\phi_2 = x$; $\phi_3 = x, x, -x, -x$; $\phi_5 = x$; $\phi_6 = \phi_7 = y, y, -y, -y$ with ϕ_4 and the receivers set to $x, -x, -x, x, -x, x, x, -x$. The phases ϕ_1 and ϕ_2 are incremented by 45° on alternate increments to give phase-sensitive INADEQUATE spectra. The phases of both receivers and ϕ_3, ϕ_5, ϕ_6 are incremented by 90° every two increments. The T_C, t_2, and t_4 delays are set as described for the pulse sequence of Figure 2. In addition, the delay T_N is set to $T_N = t_2' - t_7$, where t_7 is the J-evolution delay of the $^{15}N-^{1}H$ J-HMBC experiment. To avoid excessive power levels that could potentially be damaging to the probe, the first ^{15}N pulse was displaced (not shown for simplicity

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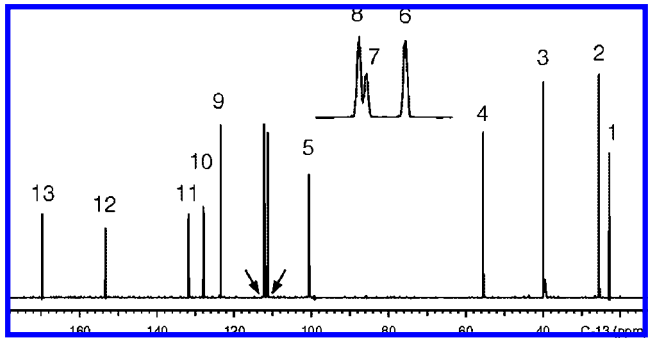

Figure 4. Decoupled one-dimensional ^{13}C spectrum of the test sample, consisting of 260 mg dissolved in 500 μL of DMSO-d_6 acquired with the pulse sequence of Figure 3. This identifies 13 chemically shifted sites. The region indicated by the arrows has been expanded to show two close resonances 7 and 8 (inset).

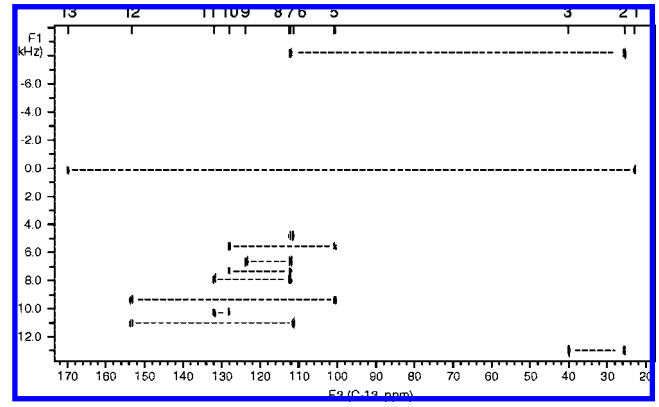

Figure 5. Two-dimensional INADEQUATE spectrum of the test sample acquired using the pulse sequence of Figure 3. This required a 12 h experiment on a Varian 600 MHz spectrometer equipped with two receivers and a standard room-temperature HCN triple-resonance probe. The trace at the bottom right has been aliased in the F_3 dimension. The numbering refers to the ^{13}C shifts derived from Figure 4. There is an AB system near 111 ppm (6 and 8).

reasons) and applied at the end of the t_1 delay when the latter becomes sufficiently long. The missing data points were recovered using backward linear prediction. The extra delay thus introduced was accounted for in the calculations of the subsequent delays. This also ensures that the ^{15}N and ^{13}C pulses that follow are not applied simultaneously. The parameter t_6 is the F_1 evolution delay of the multiplicity-edited $^{15}N-^{1}H$ HSQC experiments and the $^{15}N-^{1}H$ J-HMBC experiment. It is incremented independently of the t_3 delay, thus allowing independent adjustment of the ^{15}N spectral width. Similarly, t_7 and t_4 are set independently, thus allowing for differences in the $^{13}C-^{1}H$ and $^{15}N-^{1}H$ coupling constants. The τ delay is introduced to ensure that ^{15}N magnetization is fully refocused at the end of the t_7 evolution period of the first increment. Finally, the last ^{13}C 180° pulse is displaced (not shown for simplicity reasons) to avoid excessive power going into the probe and also to compensate for larger one-bond $^{13}C-^{1}H$ couplings as compared to the $^{15}N-^{1}H$ couplings.

Typical Experimental Parameters. The acquisition times in the directly detected dimensions were set to 82 ms. Longer acquisition times improve the resolution in these dimensions at the expense of reduced sensitivity of the proton-detected experiments. The repetition time was set between 1.0 and 1.5 s. The following delays were used: $\Delta_{CC} = 3.8$ ms, $\delta = 1.6$ ms, the initial t_4 delay was 1.6 ms, and the τ delay was 1 ms. All other delays were calculated based on these settings. The typical setting for the spectral widths was 25 kHz in the directly detected dimension, 32 kHz in the indirect ^{13}C dimension, 3.2 kHz in the indirect ^{15}N dimension, and 625 Hz in the J-dimension. Typical gradient levels were 10, 15, and $\pm$ 2.5 G/cm (Figure 3).

The sample temperature was 25 °C, and 16 dummy scans were used prior to acquisition. Proton decoupling was achieved using the WALTZ-16 sequence with radiofrequency level $\gamma B_2/2\pi = 5$ kHz. The ^{13}C decoupling was implemented with the WURST-40 adiabatic scheme using WURST-40 pulses of 1.3 ms duration, covering a 30 kHz bandwidth with decoupling level $\gamma B_2/2\pi = 2.8$ kHz. The ^{15}N decoupling employed constant-adiabaticity WURST-10 pulses of 2 ms duration, covering a 10 kHz bandwidth with $\gamma B_2/2\pi = 1.8$ kHz.

Typically, 16 increments were used in the indirect F_2 dimensions (t_4 and t_7), 128 increments were used in the F_1 dimensions (t_1, t_3, and t_6), and 2048 complex data points were acquired in the directly detected dimensions t_2, t_2', and t_5. For processing, the data were zero-filled to 16K (F_3), 2K (F_1), and 128 (F_2).

Building the Molecular Structure

The proposed method for structural determination is only applicable to relatively small organic molecules. We outline the protocol by reference to two small test molecules (mol mass: 232.28 and 324.44). The one-dimensional ^{13}C spectrum (see Figure 4) of the first sample establishes that this molecule

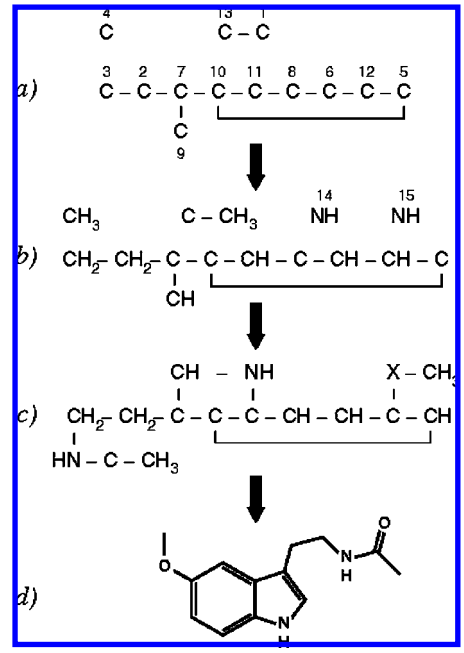

Figure 6. Building the molecular structure step by step. (a) The fragments identified from the INADEQUATE spectrum. (b) Attachment of protons from information in the multiplicity-edited HSQC spectra. (c) Linkage via the long-range $^{13}C-^{1}H$ and $^{15}N-^{1}H$ correlation information. (d) The final structure, melatonin.

contains 13 carbon atoms. The ^{13}C chemical shifts define the centers of symmetry of the INADEQUATE traces; allowance having been made for the secondary isotope shifts, thus facilitating sensitivity enhancement routines based on symmetry considerations.[17] The INADEQUATE spectrum of Figure 5 provides the carbon connectivity information, establishing the existence of one isolated carbon site and the two fragments shown in Figure 6a.

The two-dimensional $^{13}C-^{1}H$ HSQC spectra provide reliable information on the one-bond connectivities between carbon and hydrogen atoms. Figure 7 shows these spectra, edited according to multiplicity, thus identifying CH, CH_2, and CH_3 sites. Thus, the protons are assigned to the molecular fragments as shown in Figure 6b. In a similar manner the multiplicity-edited $^{15}N-^{1}H$ HSQC spectra (shown in the Supporting Information) indicate

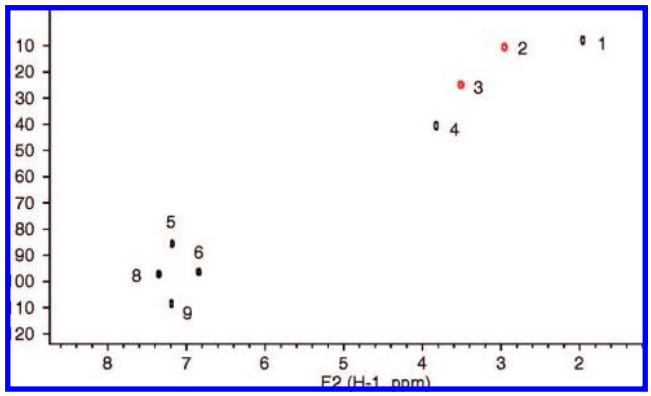

Figure 7. Multiplicity-edited two-dimensional $^{13}C-^{1}H$ HSQC spectrum of the test sample acquired with the pulse sequence of Figure 3 and $t_4 = 0.75/(^{1}J_{CH})$ shows positive CH and CH_3 peaks and negative CH_2 peaks (red).

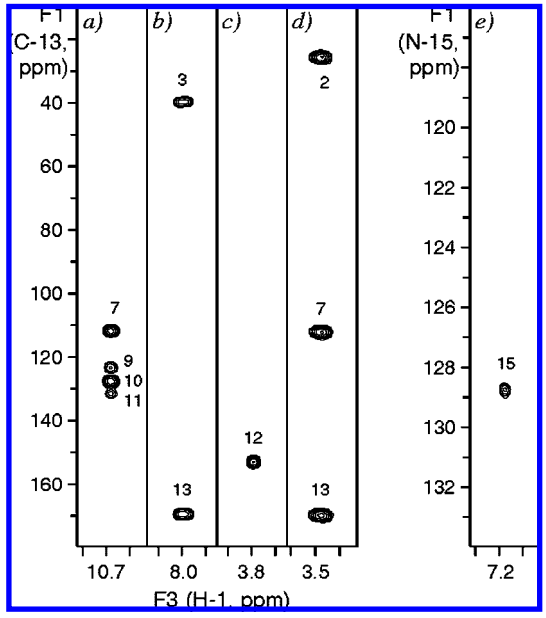

Figure 8. Long-range $^{13}C-^{1}H$ correlations in the test sample from indole (a), amide (b), methoxyl (c), and H-8 (d). (e) Long-range $^{15}N-^{1}H$ correlations. These strip plots were extracted from three-dimensional $^{13}C-^{1}H$ and $^{15}N-^{1}H$ J-HMBC spectra acquired with the pulse sequence of Figure 3. The numbering scheme refers to Figure 4.

the presence of two NH groups. On spectrometers equipped with a third receiver channel (see the Supporting Information) the one-dimensional ^{15}N spectrum identifies nitrogen atoms, provided that the sensitivity is sufficient.

These molecular fragments can now be linked together by means of the long-range $^{13}C-^{1}H$ (and $^{15}N-^{1}H$) correlation information derived from the three-dimensional J-HMBC spectra (Figure 8). Note that these spectra also provide complementary information for situations where the multiplicity-edited HSQC spectra show ambiguities as a result of significant differences in the single-bond scalar couplings. The HMBC spectra may have their own ambiguities, since distinction between two-bond and three-bond correlations is often problematic. In the present case only one-bond and three-bond $^{13}C-^{1}H$ correlations are involved in the H−C−X−C moieties, and hence, the molecular fragments can be connected unambiguously. On the other hand, two-bond and three-bond $^{15}N-^{1}H$ couplings in the H−N−C−C fragments, together with the INADEQUATE spectra, provide unambiguous information regarding the positions of nitrogen atoms within the molecule. Finally, long-range $^{15}N-^{1}H$ correlations in the H−C−N and H−C−C−N moieties supply further complementary information. On the basis of the long-range $^{13}C-^{1}H$ and $^{15}N-^{1}H$ correlations shown in Figure 8, it follows that our test molecule can be assembled as shown in Figure 6c. The two missing heteroatoms can be identified from an elemental analysis or on the basis of ^{13}C chemical shift evidence.

Consequently, our first test molecule is unambiguously determined to be melatonin, 5-methoxy-*N*-acetyltryptamine (Figure 6d), a naturally occurring hormone that regulates circadian rhythms. The structure of the second test molecule is unveiled in a similar manner in the Supporting Information.

We call the new structural determination scheme PANACEA (parallel acquisition NMR, an all-in-one combination of experimental applications). Although the proposed procedure is hardly a *universal* remedy—it is not intended for large molecules, and certainly not for proteins—it shows real promise for unlocking the structure of small organic molecules. It provides one-, two-, and three-dimensional spectra in a single measurement, displaying all the essential correlations. Not only does this speed up the acquisition of the structural information, but it also ensures that any slow changes (due, for example, to temperature variations or deterioration of the sample) have no differential effect on the various NMR results. PANACEA lends itself to unsupervised exploration of a set of different samples delivered by an automatic sample changer. All the relevant structural information is gathered without any need for retrospective measurements. A chemist would be able to say "run the NMR", in the same sense as "run the X-ray" or "run the mass spectrum."

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Supporting Information Available: Symmetrization scheme for enhancing sensitivity of INADEQUATE spectra, with an analysis of the AB case, test enhancement of signal-to-noise in the INADEQUATE spectrum of melatonin, one-dimensional ^{15}N spectrum and multiplicity-edited $^{15}N-^{1}H$ HSQC spectrum of melatonin, and PANACEA structure determination experiment applied to a second test molecule (quinine). This material is available free of charge via the Internet at http://pubs.acs.org.

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