Limitations in the Deduction of Carbon NMR Spectra from the f_1 Dimension of Standard 2D Heteronuclear Experiments When Applied to Natural Products

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The structure elucidation of a natural product requires a set of NMR spectra that includes both carbon observe experiments, such as 1D carbon and DEPT, and proton observe experiments, such as HSQC, HMBC, and COSY. Because NMR probes are optimized for either proton or carbon observe experiments, but not both, this often results in some experiments being acquired at a very suboptimal level of efficiency. An alternative is to deduce the carbon spectrum from the indirect, or f_1, dimension of the heteronuclear 2D experiments. This approach is sometimes being employed for the structure elucidation of newly isolated natural products in cases where the amount of material available precludes carbon observe experiments. However whether this approach is reliable in every case has not yet been established. This study applies the "indirect dimension" approach to a representative set of known natural products. The results are mixed. Analysis of E-HSQC spectra, in conjunction with COSY spectra, reliably defines the carbon spectra of the methyl, methylene, and methine carbons present. However, due to limits in resolution in the f_1 dimension of standard HMBC experiments, the presence and chemical shift positions of some quaternary carbons are fairly frequently obscured by those of other carbons. Thus it is often necessary to acquire a 1D carbon NMR spectrum to support the structure elucidation of a natural product.

High-resolution NMR is the essential analytical tool for the structure elucidation of natural products. Routinely a set of 1D and 2D proton and carbon NMR experiments, along with a mass spectrometry determination of molecular weight, are required in order to collect the data needed to determine the structure of a newly isolated natural product. The exact composition of the NMR experiment set needed will vary depending on the particular natural product in question; however a routine set of NMR experiments can be prescribed. In an excellent review on the subject of natural products NMR, Reynolds and Enriquez recommended a basic set of NMR experiments comprising 1D proton, 1D carbon, DEPT 135, DEPT 90, COSY, HSQC, HMBC or CIGAR, and NOESY or ROESY.[1] The basic procedure followed is to first use the carbon and DEPT spectra to define the carbon types present as methyl, methylene, methine, quaternary, or carbonyl and then to use the 2D experiments to connect the carbon units together and determine the configuration. However experiment sets of this type present a problem in terms of maximizing the efficiency of NMR data acquisitions. Several of the experiments in the set, 1D carbon and DEPT, are carbon observe experiments that are most efficiently acquired using a direct detection probe. While the other experiments, HSQC, HMBC, COSY, NOESY, etc., are proton observe experiments most efficiently acquired using an inverse probe. The cost in terms of efficiency related to these probe considerations is striking. In general it is estimated that inverse probes will be roughly twice as sensitive for proton observe experiments relative to the corresponding direct detection probe. In fact the Bruker probe specification sheets cite a 0.1% ethyl benzene sensitivity standard signal-to-noise ratio specification of $\geq$900 for their 500 MHz 5 mm z-gradient inverse BBI probe, but only $\geq$330 for their corresponding direct detection BBO probe.[2] The signal-to-noise ratio of a NMR peak increases linearly with the square of the number of scans averaged.[3] Acquiring a proton observe spectrum such as a HSQC to some adequate level of signal-to-noise using a direct detection probe will therefore require between 4 and 8 times the instrument time needed for the same experiment run on an inverse probe. Thus in order to acquire a complete set of NMR data for the structure elucidation of a natural product, either some of the experiments must be acquired at a very suboptimal level of efficiency or two separate sets of NMR experiments must be run, one carbon observe set acquired on a direct detection probe and one proton observe set acquired using an inverse probe.

Table 1. Set of Representative Natural Products Used

sample	isolated from	material	mol wt	formula
1[a]	*Illicium verum*	seed pod	148.20	$C_{10}H_{12}O$
2[b]	*Cinnamonum camphora*	tree bark	152.23	$C_{10}H_{16}O$
3[b]	*Illicium verum*	seed pod	174.15	$C_7H_{10}O_5$
4[c]	*Scutelloria baicalensis*	root	284.26	$C_{16}H_{12}O_5$
5[b]	*Camellia sinensis*	leaves	290.27	$C_{15}H_{14}O_6$
6[b]	*Cinchona officinalis*	tree bark	324.41	$C_{20}H_{24}N_2O_2$
7[d]	*Cannabis sativa*	leaves	332.48	$C_{21}H_{32}O_3$
8[b]	*Strychnos nux-vomica*	seeds	334.41	$C_{21}H_{22}N_2O_2$
9[b]	*Beta vulgaris L.*	root	504.43	$C_{18}H_{32}O_{16}$
10[e]	*Acanthostrongylophora sp.*	sponge	564.76	$C_{36}H_{44}N_4O_2$
11[f]	*Actaea podocarpa*	root	620.81	$C_{35}H_{56}O_9$

[a] Isolated by Rahul Pawar and Ikhlas Khan. [b] Purchased from Sigma-Aldrich. [c] Isolated by Jing Li and Ikhlas Khan. [d] Isolated by Mohamed Radwan and Samir Ross. [e] Isolated by Volodomyr Samoylenko and Muhammad Illias. [f] Isolated by Zulfiqar Ali and Ikhlas Khan.

In principle however it should be possible to obtain the information contained in the carbon observe experiments from the indirect dimension of proton observe 2D heteronuclear experiments. Multiplicity edited HSQC (E-HSQC) experiments should be able to detect the carbons with attached protons and classify them by type as methyls, methylenes, and methines, thus removing the need for DEPT experiments.[4] If quaternary and carbonyl carbons could consistently and reliably be observed in the HMBC spectra, then there would be no need for 1D carbon spectra. This approach of deducing the carbon spectrum from the indirect dimension of HSQC and HMBC experiments, without the acquisition of any carbon observe spectra, is in fact the standard approach recommended for complex carbohydrates.[5] This approach is also occasionally used for other classes of natural products in cases where the amount of compound available precludes the use of carbon observe experiments.[6–10] But how reliable and robust is this approach? Can the complete carbon spectra of some newly isolated natural product in fact be deduced from the indirect dimension of HSQC and HMBC experiments on a routine basis with confidence? This study applies

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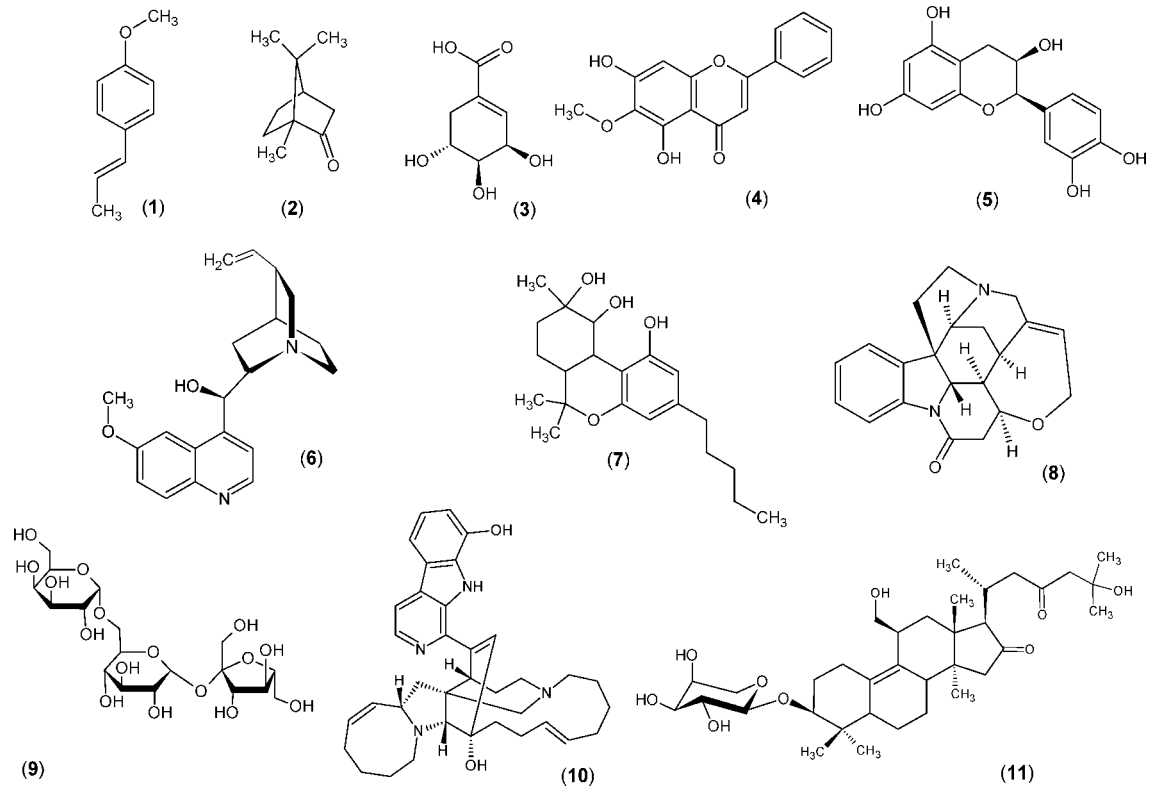

Figure 1. Representative set of natural products used in this study. See Table 1 for further details.

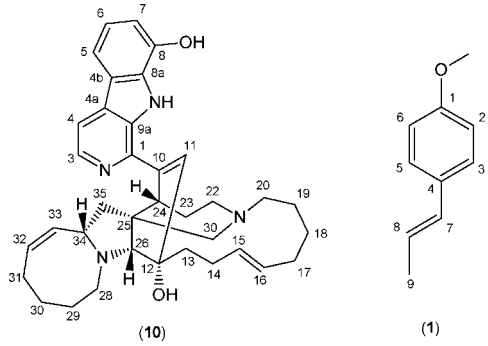

Figure 2. Carbon-numbering systems for 8-hydroxymanzamine (**10**)[11]and *trans*-anethol (**1**).

the "indirect dimension" approach to a representative set of known natural products. The results indicate that the reliability of the approach is more limited than might be supposed.

Results and Discussion

The representative set of natural products used in this study (**1**–**11**) is described in Table 1 and Figure 1. Five proton observe NMR experiments were performed for each of these compounds: a 1D proton, a COSY, an E-HSQC with multiplicity editing such that methyl and methine cross-peaks are positive and methylene cross-peaks are negative, an E-HSQC with only methine cross-peaks present, and a HMBC. The indirect carbon dimensions of the 2D spectra were then used to determine the number and type of carbons detectable for each compound. The case of 8-hydroxymanzamine (**10**) is a good illustration of the results obtained overall (Figure 2). The 13 methine carbons present in 8-hydroxymanzamine (**10**) are easily observed as positive cross-peaks in the first E-HSQC spectrum (Figure 3, top panel). There are no methyl carbons present in 8-hydroxymanzamine (**10**); however if any methyl carbons were present, they would be easily detected by comparing the positive cross-peaks of the two E-HSQC spectra. Analysis of the methylene

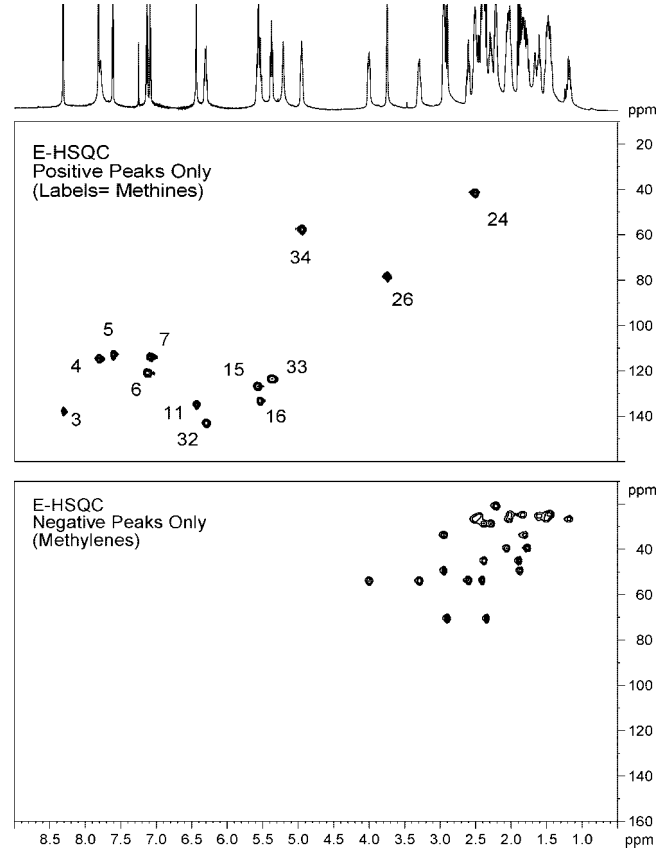

Figure 3. Edited HSQC of 8-hydroxymanzamine (**10**) with methines and methyls positive (top panel) and methylenes negative (bottom panel). Methine carbon assignments are from ref 11.

carbons is only slightly less obvious. Each methine or methyl carbon will result in only one cross-peak in the E-HSQC spectra. However

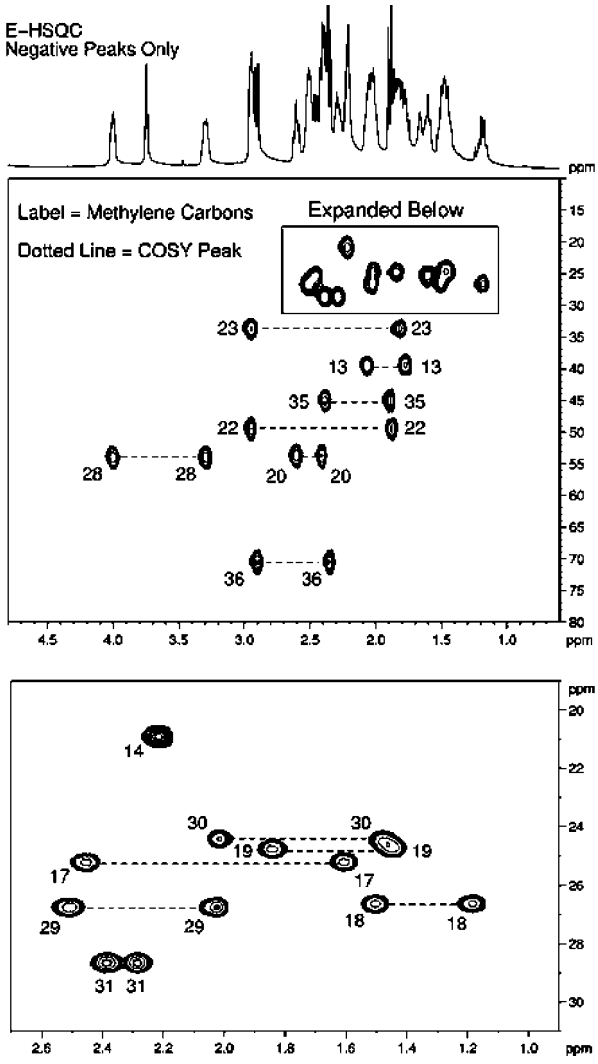

Figure 4. Edited HSQC of 8-hydroxymanzamine (**10**) from Figure 3 showing only negative peaks. Methylene carbon assignments are from ref 11.

the two geminal protons on each methylene carbon may be either degenerate or nondegenerate, and thus each methylene carbon may produce either one cross-peak or one pair of two carbon degenerate cross-peaks in the E-HSQC spectrum (Figure 4). In practice geminal COSY cross-peaks may be very useful in the analysis of the methylene cross-peak pairs. Thus in the case of 8-hydroxymanzamine (**10**) the methylene carbons at positions C-28 and C-20 are nearly degenerate, producing a line of four E-HSQC cross-peaks at 53.5 ppm; however a quick reference to the COSY spectrum allows assignment of the four E-HSQC peaks to two methylene carbons (Figure 4, top panel). A similar situation occurs with the methylene carbons at positions C-29 and C-18 (Figure 4, bottom panel). In this way the two E-HSQC spectra, analyzed in conjunction with the COSY spectrum, allow for the quick determination of all the methyl, methylene, and methine carbons present.

Analysis of the quaternary carbons using the indirect dimension of the HMBC spectrum was less successful. While the HSQC experiment gives either one or two cross-peaks per carbon, the HMBC spectrum will give, for each detected carbon, an indeterminate number of cross-peaks aligned along a single carbon ppm value, a structure in the spectrum that we might call a "skewer". Superimposition of 2D spectra on the computer screen readily allows for the detection of those HMBC skewers that originate on carbons other than those observed in the E-HSQC. These new HMBC skewers are those originating on quaternary and carbonyl carbons. However for 8-hydroxymanzamine (**10**) only seven of the nine quaternary carbons present can be readily observed using this approach (Figure 5). The problem is that HMBC skewers originating on the quaternary carbons at positions C-4b and C-9a overlap, respectively, with the skewers originating on methine carbons C-33 and C-16 (Figure 6). It should be noted that the problem here is not with detection limits or sensitivity, but with f_1 resolution. The skewers in question contain contributing peaks from carbons C-4b, C-33, C-9a, and C-16. However the C-4b skewer is not distinguishable as a separate skewer from the C-33 skewer, nor is the C-9a skewer distinguishable from the C-16 skewer. It should be noted here that C-4b and C-33 carbon lines, and likewise the C-9a and C-16 carbon lines, are easily resolved in the 1D carbon spectrum. Using the "indirect dimension" approach, without referring to a 1D carbon spectrum, or knowledge of the structure of 8-hydroxymanzamine (**10**), it would be concluded that only seven quaternary carbons are present, not nine.

Table 2 presents a comparison of carbon chemical shifts for 8-hydroxymanzamine (**10**) measured from a 75 MHz 1D carbon spectrum by Ichiba, Corgiat, and Scheuer,[11] with the chemical shifts measured from the E-HSQC and HMBC spectra in this study. The agreement of the two data sets is within 0.2 ppm for most carbons. Thus, although the indirect dimension approach gives a short count for the quaternary carbons present, the carbon chemical shifts measured for those carbons observed appear to be adequately precise and accurate.

The results obtained applying the "indirect dimension" approach to 8-hydroxymanzamine (**10**) are representative of the results obtained for the natural product sample set as a whole (Table 3). The E-HSQC spectra, analyzed in conjunction with COSY spectra, readily defined the presence and chemical shifts of all the methyls, methylene, and methine carbons present. However the HMBC skewers of the quaternary carbons present were fairly frequently obscured by those of other carbons (note bold entries in Table 3).This problem occurred not only in the larger molecules in the set, 8-hydroxymanzamine (**10**) and podocarpaside-1 (**11**), but in some of the smaller molecules such as *trans*-anethol (**1**) as well. The worst case was (–)-epicatechin (**5**), where only four out the seven quaternary carbons present could be deduced from the skewers of the HMBC. Again the problem with the detection of the quaternary carbons, in the representative sample set studied here, lies in the limited f_1 resolution of the HMBC spectra. If all possible natural product molecules were considered, there must certainly also be some cases where certain quaternary carbons are not detected by the HMBC spectrum at all because they are not adequately coupled to any proton in the molecule. That is to say that for certain quaternary carbons in a given molecule the largest J_{CH} present can be too small to produce an adequate antiphase magnetization during a delay of reasonable length. A good example of this would be found in the nucleotides. However judging from the results of this study, this is a much less common problem in natural products in general than that resulting from HMBC "skewer" overlap.

As noted by Reynolds and Enriquez, the use of linear prediction or zero filling of t_1 is absolutely necessary in order to achieve the complete f_1 resolution inherent in the experiment,[1] and all the 2D spectra used in this study were processed using zero filling in the t_1 domain (see Experimental Section below). However the f_1 dimension of the standard HMBC experiment is in general less well resolved than that of the HSQC experiment because the HMBC experiment, like the HMQC experiment, retains J_{HH} modulation in the indirect dimension.[1] This problem of a fairly limited f_1 resolution in the standard form of the HMBC experiment has previously been recognized in the literature, and high-resolution forms of the HMBC experiment have been developed.[12–15] By employing band selective carbon pulses to increase the f_1 digital resolution, and constant time t_1 domains to suppress J_{HH} modulation, these high-resolution HMBC

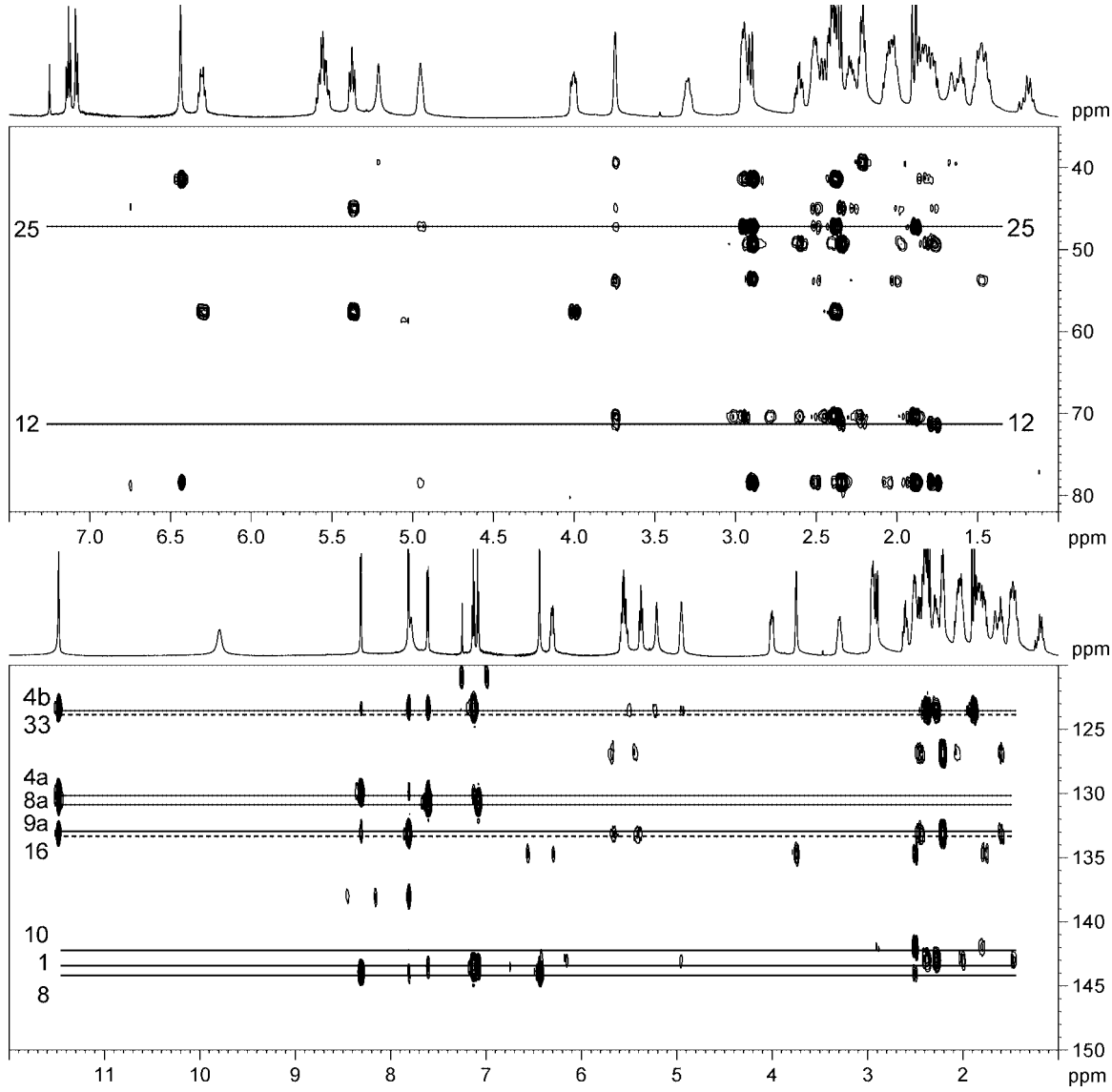

Figure 5. HMBC of 8-hydroxymanzamine (**10**) higher field region (top panel) and lower field region (bottem panel). Carbon assignments are from ref 11. Lines indicate quaternary carbon skewers. Dotted lines indicate selected methine carbon skewers.

experiments can achieve resolution comparable to that of a 1D carbon spectrum over selected portions of the f_1 domain.[14] While these experiments are very useful in resolving specific assignment problems, they are probably not, as part of a routine natural products NMR data collection program, viable substitutes for 1D carbon spectra. They will not, for instance, detect those relatively rare quaternary carbons that are not coupled to any protons in a given molecule. Some forms of the high-resolution experiments can be significantly less sensitive than standard forms of the HMBC experiment.[13,14] They may require multiple HMBC acquisitions to expand several different portions of the f_1 domain.[13,15] The use of band selective carbon pulses, or adiabatic carbon pulses, assumes either a knowledge of the 1D carbon spectrum or careful analysis of a standard low-resolution HMBC spectrum.[13–15] To date those published cases where the indirect dimension approach has been applied to the structure elucidation of newly isolated natural products have used only standard low-resolution forms of the HMBC experiment, rather than band selective high-resolution forms.[6–10]

How important is complete knowledge of all the quaternary carbons present to a correct structure determination of an unknown natural product? Consider the case of *trans*-anethol (**1**), where the HMBC skewer of quaternary carbon C-4 overlaps with that of carbon C-7. It might be argued that in a case such as this, where the molecule involved is small, that the presence and chemical shift of the missing quaternary carbon would be deduced from a careful analysis of the HSQC and HMBC spectra made in conjunction with a mass spectrometry determination of molecule weight. However the same certainly could not be said in the cases of longer molecules such as 8-hydroxymanzamine (**10**) or podocarpaside (**11**).

The key conclusion of this study is that the "indirect dimension" approach is not always a reliable method for determining the complete carbon spectrum of a newly isolated natural product. Acquisition of a 1D carbon spectrum will often be needed for the NMR-based structure elucidation of a new natural product. On the other hand it would be fair to say that the use of E-HSQC spectra removes the need for acquiring a set of DEPT spectra in most cases. Perhaps then the most efficient way to organize a high through-put natural products NMR data acquisition is to view it as a two-stage process. On a routine basis an initial proton observe NMR data set incorporating E-HSQC and standard low-resolution HMBC spectra would be acquired on all samples using inverse probes. For those samples where analysis of this initial NMR data set, made in conjunction with mass spectrometry data, indicates that there are unidentified quaternary carbons present, a 1D carbon spectrum

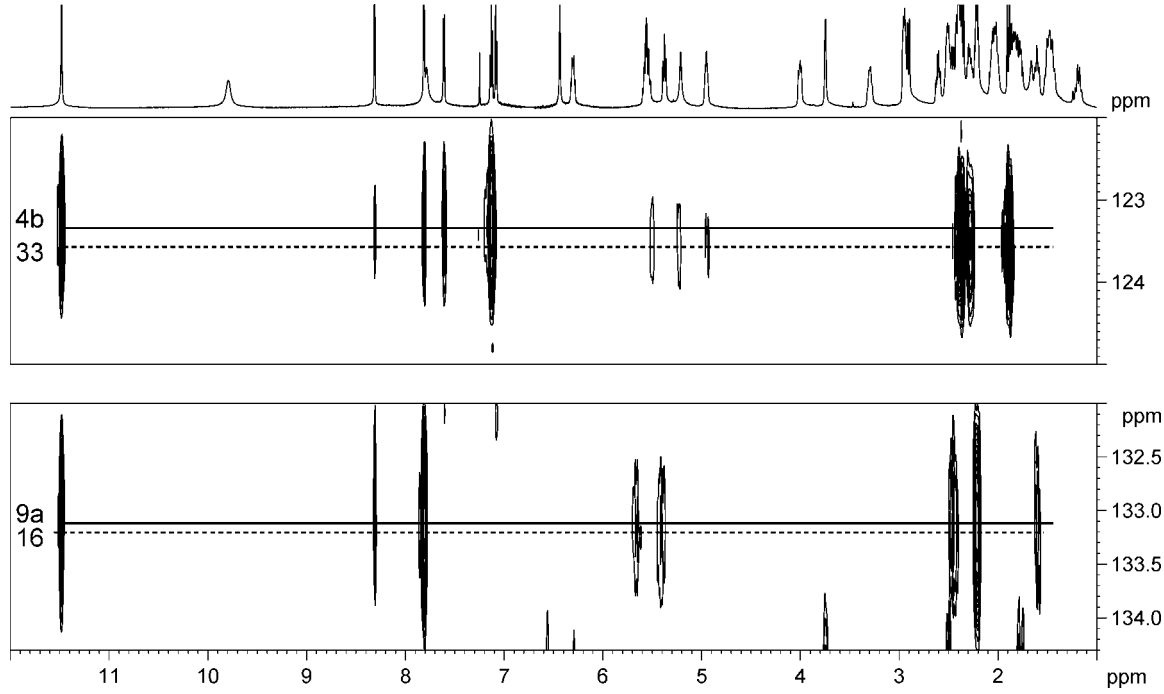

Figure 6. HMBC of 8-hydroxymanzamine (**10**). Selected carbon assignments are from ref 11. Lines indicate quaternary carbons. Dotted lines indicate methine carbons. Note that skewers from quaternary carbons C-4b and C-9a are overlapped by those of methine carbons C-33 and C-16, respectively.

Table 2. 8-Hydroxymanzamine (**10**) Carbon Chemical Shifts

position	type	literature[a]	observed[b]	position	type	literature[a]	observed[b]
3	CH	137.89	138.09	18	CH_2	26.52	26.59
4	CH	114.72	114.63	19	CH_2	24.54	24.76
4a	Q	129.76	129.96	20	CH_2	53.39	53.67
4b	Q	123.15	*c*	22	CH_2	49.12	49.34
5	CH	112.60	112.83	23	CH_2	33.39	33.60
6	CH	120.72	120.92	24	CH	41.25	41.60
7	CH	114.32	113.94	25	Q	47.06	47.10
8	Q	143.82	144.00	26	CH	78.26	78.50
8a	Q	130.55	130.77	28	CH_2	53.67	53.94
9a	Q	132.93	*c*	29	CH_2	26.42	26.72
10	Q	141.85	141.99	30	CH_2	24.15	24.40
11	CH	134.55	134.76	31	CH_2	28.41	28.59
12	Q	71.20	71.40	32	CH	142.78	142.98
13	CH_2	39.22	39.44	33	CH	123.34	123.59
14	CH_2	20.69	20.93	34	CH	57.44	57.70
15	CH	126.69	126.92	35	CH_2	44.79	45.01
16	CH	132.99	133.21	36	CH_2	70.19	70.53

[a] Ichiba, T.; Corgiat, J. M.; Scheuer, P. J. *J. Nat. Prod.* **1994**, *57*, 168–170. [b] As measured from the indirect dimension of E-HSQC and HMBC spectra. [c] Not cleanly resolved from other skewers in the HMBC spectra.

would then be obtained using a carbon-optimized instrument. Band selective high-resolution HMBC spectra would be employed only when needed to resolve specific assignment problems. In those cases where a 1D carbon spectrum is needed, but the sample size available is too limited to allow for a 1D carbon acquisition using a standard room-temperature direct detection probe, carbon-optimized cryoprobes or the new dynamic nuclear polarization (DNP NMR) techniques[16] could be employed. Adoption of this two-stage approach to natural products NMR data acquisition defines an efficient organization of a natural product NMR facility where most instruments are optimized for proton observe experiments with inverse probes, but one or two high-field instruments are reserved and dedicated to the acquisition of 1D carbon spectra using direct probes, carbon-optimized cryoprobes, or perhaps DNP NMR techniques.

Table 3. Carbons Resolved and Identified from Indirect Dimensions of the E-HSQC and HMBC Spectra

	methyls		methylenes		methines		quaternary		carbonyls	
sample	struct	obs	struct	obs	struct	obs	struct	obs	struct	obs
1	2	2	0	0	4	4	**2**	**1**	0	0
2	3	3	3	3	1	1	2	2	1	1
3	0	0	1	1	4	4	1	1	1	1
4	1	1	0	0	5	5	**7**	**6**	1	1
5	0	0	1	1	7	7	**7**	**4**	0	0
6	1	1	5	5	10	10	4	4	0	0
7	4	4	6	6	5	5	6	6	0	0
8	0	0	6	6	10	10	4	4	1	1
9	0	0	4	4	12	12	1	1	0	0
10	0	0	14	14	13	13	**9**	**7**	0	0
11	6(7)[a]	7	10	10	10	10	**6**	**5**	2	2

[a] Nearly degenerate methyls, 26 and 27, are resolved in the 1D proton spectrum but not in the HSQC.

Experimental Section

General Experimental Procedures. NMR spectra were acquired at 22 °C on a 600 MHz Varian INOVA instrument equipped with a 3 mm broad band direct detection z-gradient Nalorac probe. The standard pulse sequences and parameter sets available in the VNMRJ 1.1D software package were employed. Gradient forms of the 2D experiments were chosen. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). The spectra of compounds **1**, **4**, and **5** were acquired in d_6-DMSO with added TMS, the spectra of **2**, **6**, **7**, **8**, and **10** in $CDCl_3$ with added TMS, the spectra of **3** and **9** in D_2O with added TPS, and the spectra of **11** in d_4-pyridine. NMR spectra were processed on a personal computer using the Bruker Topspin 2.0 software package (Bruker-Biospin, Billerica, MA). The t_2 dimension of the E-HSQC and HMBC spectra were zero filled once and apodized with exponential windows using line-broadening factors matched to the Hz per point resolution. The t_1 dimension of the E-HSQC spectra were zero filled once and apodized with Gaussian windows using line-broadening constants matched to the Hz per point resolution. The t_1 dimension of the HMBC spectra were zero filled from 400 to 1024 and apodized using unshifted sine-squared windows. The COSY spectra were zero filled once and apodized with unshifted sine-squared windows in both dimensions.

Samples. (1*R*)-(+)-Camphor (**2**), shikimic acid (**3**), (–)-epicatechin (**5**), quinine (**6**), strychnine (**8**), and D-(+)raffinose (**9**) were purchased from Sigma-Aldrich (St. Louis, MO). The other compounds used in this study were research samples isolated by the faculty and research staff of the National Center for Natural Products Research (University, MS) as indicated in the footnotes to Table 1.

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