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Review

Advanced NMR Approaches for a Detailed Structure Analysis of Natural Products

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Some new nuclear magnetic resonance (NMR) approaches to elucidate chemical structures, which have not been determined by routine NMR methods, are presented. Selective detection of methine (CH), methylene (CH_2), or methyl (CH_3) signals in each subspectrum by editing NMR methods was utilized to reduce the complexity in crowded spectra. It also increased the peak separation and enhanced the sensitivity by limiting the measuring area of the 2D spectra. Several 2D methods to measure $^{2,3}J_{CH}$ values, which are useful for stereochemical assignment are then introduced. To determine the structure of a highly hydrogen-deficient molecule, efficient correlation methods for long-range ^{13}C–^{13}C coupling and ^{1}H-^{15}N HMBC are also described.

Key words: editing NMR; E-HSQC; E-HSQC-ROESY; DEPT C–C relay; natural product

Nuclear magnetic resonance (NMR) spectroscopy is one of the most important instrumental analysis methods for natural products. Routine one-dimensional (1D) and two-dimensional (2D) NMR methods could be used to determine the complex structure of a compound by both an improvement to the hardware and the development of multi-pulse sequences. However, there are still some cases of structural determination not being successful due to the limitations of conventional NMR methods. To solve these problems, some new NMR techniques involving modifications of the conventional methods have been developed. Some of these techniques and their application to the structural analysis of natural products are presented in this paper.

I. Selective Measurement of the Methine (CH), Methylene (CH_2), or Methyl (CH_3) Subspectrum by Editing NMR Methods

The standard procedure for a structural analysis of natural products involves the carbon multiplicity (methine (CH): *d* (doublet); methylene (CH_2): *t* (triplet); or methyl (CH_3): *q* (quartet)) of each signal from 1D

Table 1. NMR Methods, Key Words, and Related References in This Paper

Abbreviation	Meaning
1D	1 (one)-dimensional
2D	2 (two)-dimensional
2D-DEPT[22,23]	
2D-INEPT[21]	
C–C relay[70–74]	carbon-carbon relay
COLOC[59]	correlation *via* long-range coupling
COSY[36,37]	correlated spectroscopy
CT	constant time
CT-HMBC[34]	constant time-HMBC
D-HMBC[60]	decoupling-HMBC
DEPT[1]	distortionless enhancement by polarization transfer
DEPT-HMQC[7]	
DEPT C–C relay[72–74]	
DEPT-INADEQUATE[75,76]	
E	editing
E-HMQC[8]	
E-HMQC-TOCSY[30]	
E-HSQC[4,9]	
E-HSQC-NOESY[19]	
E-HSQC-ROESY[20]	
E-HSQC-TOCSY[18]	
F_1	1st frequency domain
F_2	2nd frequency domain
gradient-selected multiple quantum filter[10]	
heteronuclear NOE[83,84]	
HETLOC[42–44]	determination of heteronuclear long-range couplings (= ω_1 hetero half-filtered TOCSY)
HMBC[6,32,33]	heteronuclear multiple bond correlation
HMQC[5,6]	heteronuclear multiple quantum coherence
HMQC-NOESY[38]	
HMQC-ROESY[39]	
HMQC-TOCSY[4,28,29]	
HSQC[3,4]	heteronuclear single quantum coherence
HSQC-TOCSY[4,27]	
INADEQUATE[61–64]	incredible natural abundance double quantum transfer experiment
INEPT[2]	insensitive nuclei enhanced by polarization transfer
INEPT C–C relay[70,71]	
INEPT-INADEQUATE[77]	
J	*J*-coupling, scalar coupling
JBCA[40]	*J* based configuration analysis
J-HMBC[47,48]	*J*-resolved HMBC
J-IMPEACH MBC[46]	*J*-resolved improved performance accordion heterunuclear multiple-bond correlation spectroscopy

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Continued

J-res HMBC[45] J-resolved HMBC
n, n-ADEQUATE[67] adequate sensitivity double-quantum spectroscopy
NMR nuclear magnetic resonance
NOESY[13–16] nuclear Overhauser enhancement and exchange spectroscopy
phase-sensitive HMBC[56]
ROESY[13,17] rotating frame nuclear Overhauser effect spectroscopy (= CARMELSPIN cross-relaxation appropriate for minimolecules emulated by locked spins)
TOCSY[11–13] total correlation spectroscopy (= HOHAHA homonuclear Hartman-Hahn experiment)

^{13}C-NMR being determined by the DEPT[1] or INEPT[2] method. Appropriate setting of the measurement parameters and subsequent addition and/or subtraction with a suitable multiplying coefficient, if necessary, allows DEPT and INEPT experiments to provide each CH_2- and CH_3-selective subspectrum, and a subspectrum in which CH and CH_3 carbons have a positive phase whereas CH_2 carbons have a negative phase. These manipulation processes are referred to as "spectrum editing."

This idea has been extended to the 2D NMR methods to utilize the information of the carbon multiplicity to elucidate the structure of complex molecules and to reduce signal overlap in crowded spectra. 2D HSQC[3,4] and HMQC[5,6] spectra are widely used for detecting ^{1}H–^{13}C correlations directly connected. There are several related methods for the discrimination or selective detection of the CH, CH_2, and CH_3 signals by their phases (Fig. 1a) such as DEPT-HMQC,[7] E (editing)-HMQC,[8] E-HSQC,[4,9] and gradient-selected multiple quantum filter (MQF).[10] Figure 1a shows the E-HSQC spectrum of β-sitosterol (**1**), in which the CH and CH_3 signals have a positive phase (drawn by unfilled circles), whereas the CH_2 signals have a negative phase (drawn by filled circles). Each CH- (Fig. 1b), CH_2- (Fig. 1c), and CH_3-selective subspectrum can be provided by proper phase cycling and setting the measurement parameters. These methods for discriminating the carbon multiplicity by signal phase and getting subspectra are also called editing methods.

Combining E-HSQC with TOCSY (HOHAHA),[11–13] NOESY,[13–16] and ROESY[13,17] has provided the E-HSQC-TOCSY,[18] E-HSQC-NOESY,[19] and E-HSQC-ROESY[20] methods, which have similar advantages to those of E-HSQC. The introduction of editing methods to the traditional ^{13}C-observed 2D methods has also been developed as 2D-INEPT[21] and 2D-DEPT.[22,23]

The subspectrum of E-HSQC, in which the CH and CH_3 signals are positive and the CH_2 signals are negative (Fig. 1a), gives information about the one-bond C–H correlation and carbon multiplicity at the same time. However, overlapping of signals with opposite phases may cancel the signals. This problem can be avoided by separately measuring each CH (Fig. 1b) and CH_2 subspectrum (Fig. 1c). In Fig. 1a, there are close

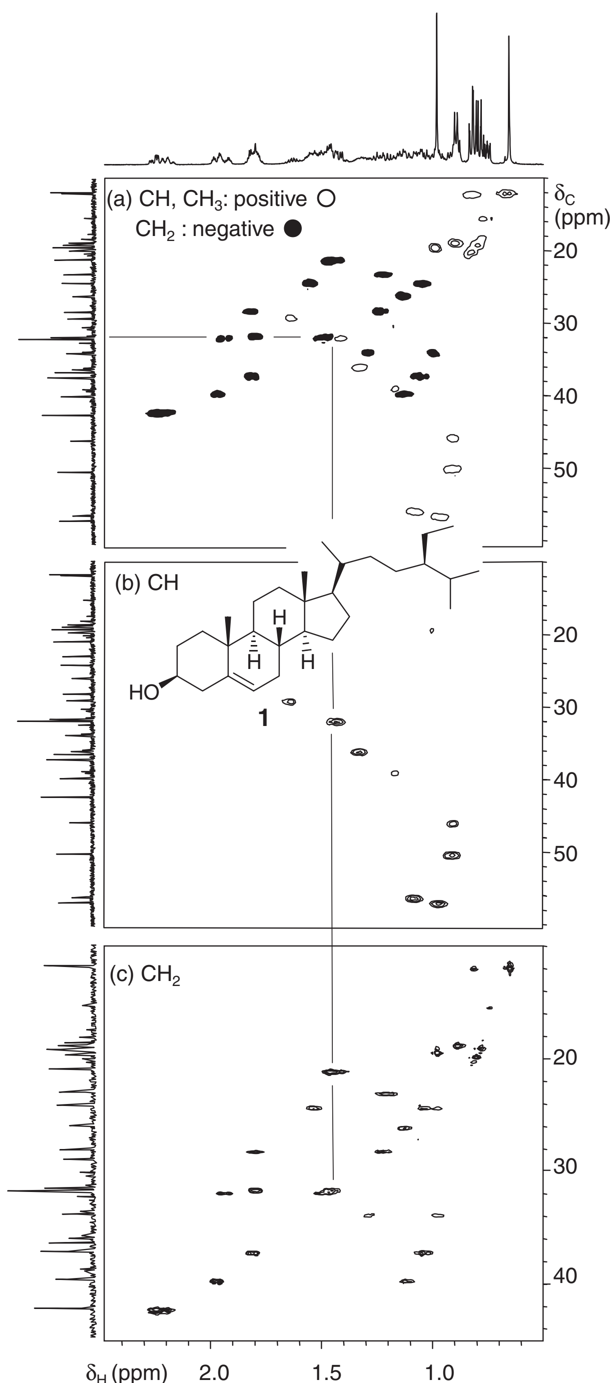

Fig. 1. E-HSQC Spectra of β-Sitosterol of **1** (3 mg/0.4 ml of chloroform-*d*) Recorded with a Bruker AMX-500 Spectrometer (^{1}H at 500 MHz, ^{13}C at 125 MHz).

(a) E-HSQC spectrum in which CH and CH_3 signals have a positive phase (drawn by unfilled circles) whereas CH_2 signals have a negative phase (drawn by filled circles). (b) CH subspectrum and (c) CH_2 subspectrum. The spectral width and data points were F_2(^{1}H) 4274 Hz for 1024 points and F_1(^{13}C) 19231 ((a) and (b)) and 4808 (c) Hz for 256 points, respectively. Forward linear prediction in the F_1 dimension was used to generate the final data matrix, F_2 1024 × F_1 512 point. Multiplication with squared cosine bell windows in both dimensions was performed before Fourier transformation. The number of transients was four, and the total measurement time was *ca.* 40 min for each.

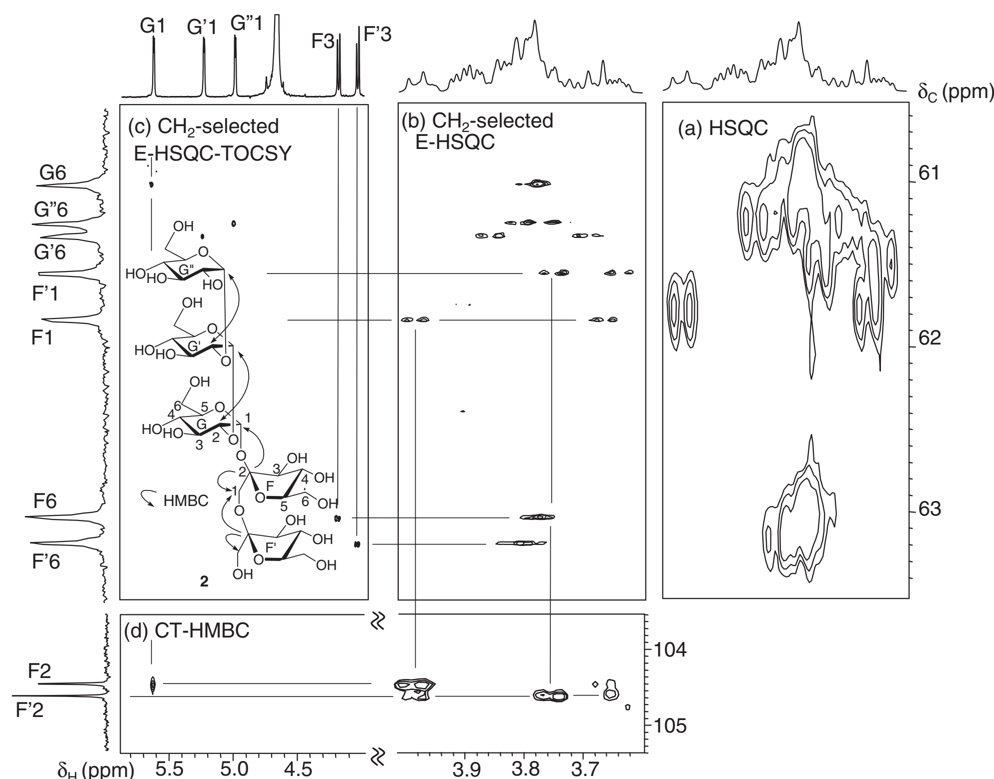

Fig. 2. HSQC, E-HSQC, E-HSQC-TOCSY, and CT-HMBC Spectra for Pentasaccharide **2** (10.8 mg/0.4 ml of D_2O).

(a) Conventional HSQC, (b) CH_2-selected E-HSQC, (c) CH_2-selected E-HSQC-TOCSY, and (d) CT-HMBC spectra. The spectral width and data points were $F_2(^1H)$ 1623 Hz for 1024 points and $F_1(^{13}C)$ 6757 ((a) and (d)) and 347 ((b) and (c)) Hz for 512 points, respectively. Forward linear prediction in the F_1 dimension was used to generate the final data matrix, F_2 1024 × F_1 1024 ((a) and (d)) and 512 ((b) and (c)) points. Multiplication windows were (a) squared cosine bell in both dimensions, (b) and (c) squared cosine bell in the F_2 and squared sine bell shifted by $\pi/6$ in the F_1 dimension, (d) Lorenz-Gaussian window (GB = 0.5, LB = −2) in F_2 and sine bell in the F_1 dimension. The number of transients and the total measurement time were (a) four and *ca.* 80 min, (b) 16 and *ca.* 5 hr, (c) 48 and *ca.* 14 hr, and (d) 96 and *ca.* 27 hr.

positive and negative peaks at around δ_C 32, δ_H 1.5 ppm. Figures 1b and c reveal these to be the CH and CH_2 signals. It seems that the intensity of the negative peak in Fig. 1a has been weakened by partial cancellation due to overlapping with the stronger positive peak.

Separation into the relevant subspectra is one of the strategies to reduce the complexity of crowded spectra. Another merit of measuring the subspectrum is the enhanced spectral quality. The resolution, one of the key factors of the spectral quality, is defined as spectral width in Hz per data point: the smaller the value, the higher the resolution. While an increase of F_1 (^{13}C axis) data points is restricted by the experimental time and data size, narrowing the F_1 spectral width is effective for improving the F_1 resolution. The F_1 spectral width for a conventional HSQC spectrum is usually set to cover the range of all protonated carbons. If there are signals outside the F_1 measuring range, they appear in the spectrum as unwanted folded signals. In respect of E-HSQC, the F_1 spectral width and data points can be optimized for each subspectrum. The chemical shifts of selected carbons for compound **1** are limited to a narrow region, enabling the F_1 spectral range to be set narrower, so that a high F_1 resolution results as shown in Fig. 1c. HSQC is preferable to HMQC for this high F_1 resolution subspectrum, since the contribution of proton–proton J coupling (J_{HH}) brings about broadening of the correlation peaks in the F_1 dimension[24] and, consequently, lowers the F_1 peak separation in the latter spectrum.

II. CH_2-Selected E-HSQC-TOCSY, a High F_1 Resolution Subspectrum. Application to the Oligosaccharides

The NMR spectra of oligosaccharides tend to be too complex to elucidate the structure. The ^{13}C chemical shifts of methylenes, especially, are concentrated in a narrow region, which makes the assignment difficult. This problem has been solved by measuring the high-resolution CH_2 subspectrum.[25] For example, in the HSQC spectra of pentasaccharides **2**[26] consisting of three units of glucose and two units of fructose, some correlation peaks of eight methylenes were overlapped (Fig. 2a), these were well separated in CH_2 subspectrum of E-HSQC (Fig. 2b), enabling the chemical shift of each methylene proton to be assigned. This unambiguous assignment is essential for a determination of the fructosyl linkage between the sugar units.

HSQC-TOCSY[4,27] and HMQC-TOCSY[4,28,29] are powerful methods for the structural elucidation of

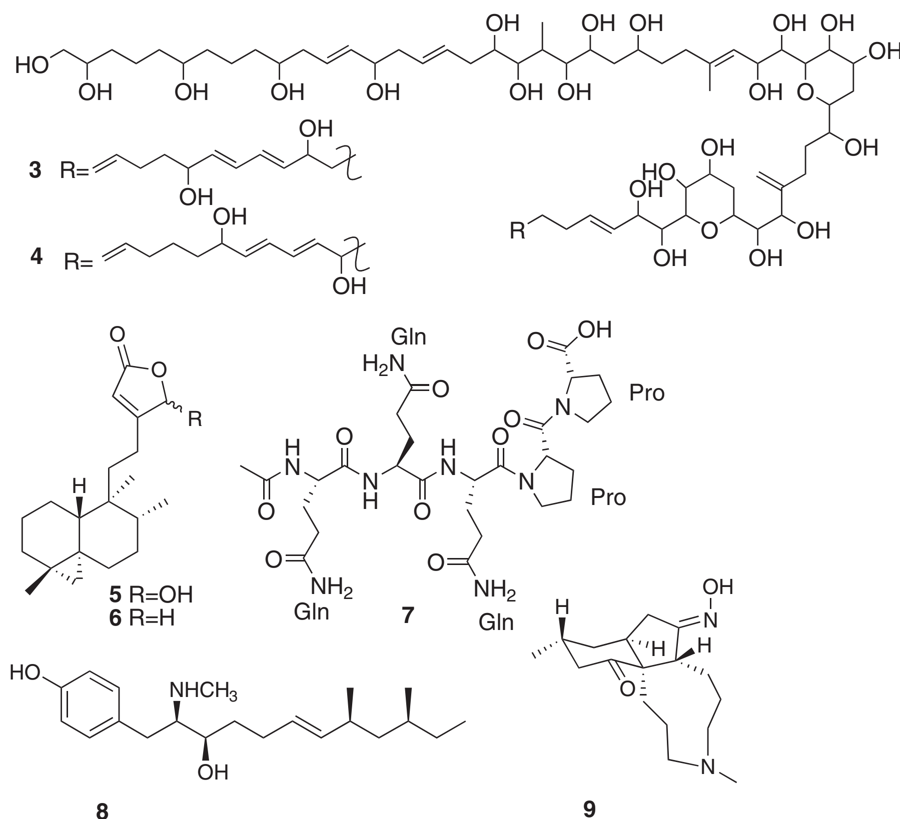

Fig. 3. Chemical Structures of **3**–**9**.

oligosaccharides. In these spectra, a carbon shows correlation signals to its directly attached protons (HSQC or HMQC signal) and to protons in the same spin system with them (HSQC-TOCSY or HMQC-TOCSY signals). These spectra of oligosaccharides can be used to distinguish most of the ^{13}C and ^{1}H signals that belong to the same sugar unit. The introduction of editing to these experiments has resulted in the E-HMQC-TOCSY[30] and E-HSQC-TOCSY methods. The resolution-enhanced CH_2-selected E-HSQC-TOCSY spectrum of **2** (Fig. 2c) could distinguish two methylene carbons close to each other. CH_2-selected E-HSQC and E-HSQC-TOCSY have been applied to the NMR analysis of several oligosaccharides.[18,25,26,31]

To detect the glycosidic linkage and assignment of the fructose units, the long-range ^{1}H–^{13}C correlated spectrum, HMBC,[6,32,33] is necessary. However, with conventional HMBC spectra, the contribution of J_{HH} coupling brings about broadening of the correlation peaks in the F_1 dimension like that in the HMQC spectra. This prevents an analysis of the HMBC spectra of oligosaccharides, in which splitting by J_{HH} is complex and ^{13}C signals are close to one another. In such a case, the CT-HMBC method proposed by Furihata[34] is useful. The pulse sequence contains a constant time (CT) for F_1 chemical shift evolution to suppress J_{HH} modulation in the F_1 dimension which results in a sharp line shape in the F_1 dimension. The correlation peaks in the CT-HMBC spectrum of **2** (Fig. 2d) are well resolved, clarifying the linkage of the sugar units.

The CH and CH_2 subspectrum of E-HSQC, and E-HSQC-TOCSY have also been exploited for the spectral analysis of other kinds of complex compounds: luteophanols B (**3**) and C (**4**) from a marine dinoflagellate[35] possessing many aliphatic methine and methylene carbons, and clerodane-type diterpenes, dytesinins A (**5**) and B (**6**),[19] in which methylene carbons overlap in a narrow region.

III. Application of the Editing Method to Low-Sensitivity HSQC-ROESY and HSQC-NOESY

When the ^{1}H-NMR signals of a compound overlap, it is difficult to analyze them by such homonuclear ^{1}H–^{1}H correlation methods as COSY,[36,37] TOCSY, NOESY, and ROESY. In this case, it is effective to use ^{13}C information by such heteronuclear 2D NMR methods as HMQC (or HSQC)-TOCSY and HMQC (or HSQC)-NOESY[38] (or ROESY[39]). While HMQC-TOCSY and HSQC-TOCSY are widely used, there is only limited application of HMQC (or HSQC)-NOESY (or ROESY) to natural products because of their low sensitivity. NOESY and ROESY experiments are much less sensitive than TOCSY. This problem can be

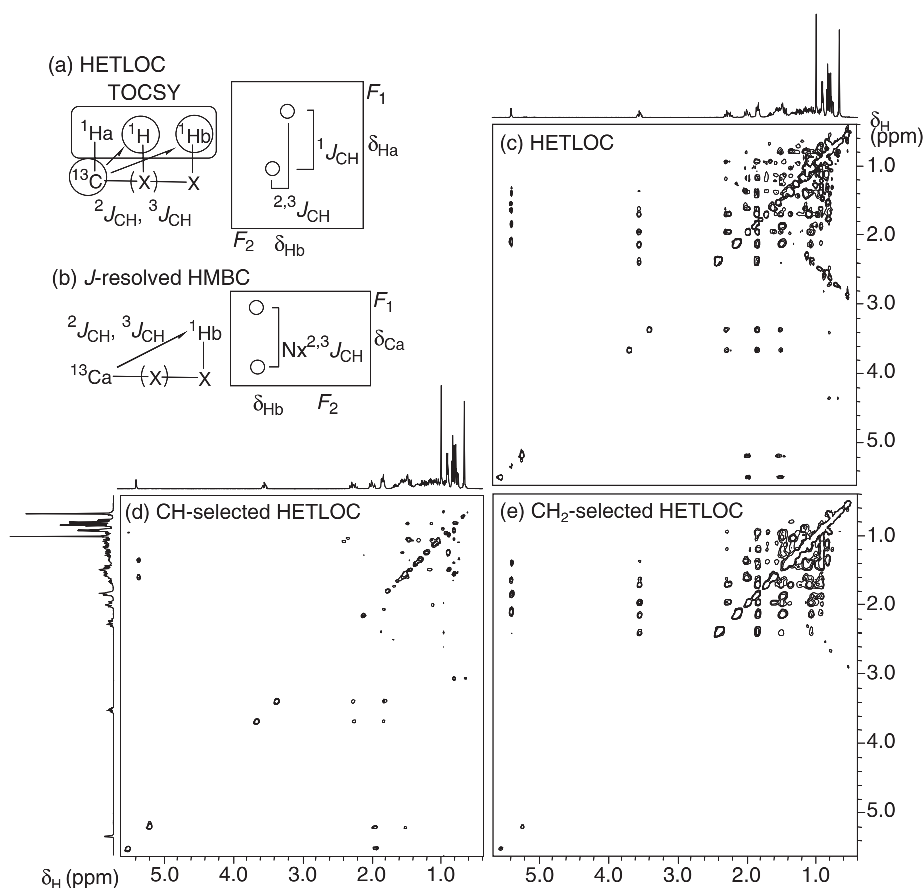

Fig. 4. HETLOC and J-Resolved HMBC Spectra of **1** (20 mg/0.4 ml of chloroform-d).

(a) and (b) The $^{2,3}J_{CH}$ values provided by HETLOC and J-resolved HMBC spectra. (c) HETLOC, (d) CH-selected HETLOC, and (e) CH_2-selected HETLOC spectra. The spectral widths in both dimensions were 4525 Hz. The data points were F_2 1024 × F_1 256 points. Zero-filling in the F_1 dimension was used to generate the final data matrix, F_2 1024 × F_1 512 point. Multiplication with squared cosine bell windows in both dimensions was performed before Fourier transformation. The number of transients, mixing time of TOCSY, and the total measurement time were respectively 64, 70 ms, and *ca.* 7 h each.

attenuated by using E-HSQC-ROESY and E-HSQC-NOESY. These F_1-limited methods can retain the same F_1 resolution with a smaller number of t_1 increments compared to the whole spectrum, since the effect from unwanted folded peaks can be ignored.

The structural analysis of a model peptide of the IgE-binding epitope in the wheat allergen, acetyl-Gln-Gln-Gln-Pro-Pro (**7**), has been achieved by using CH-selected E-HSQC-ROESY. Since **7** had only five CH carbons whose δ_C values were concentrated in the range of 10 ppm, the F_1 spectral width and data points could be reduced. As a result, the CH subspectrum with a sufficient signal-to-noise ratio revealed the configuration of the backbone amide bonds to be all-*trans*.[20]

CH-selected E-HSQC-NOESY has been measured for a determination of the relative stereochemistry of dytesinin A (**5**) from a marine tunicat. Since **5** contained only four methine carbons whose chemical shifts were relatively separated, a reduction of the F_1 data points to 16 and an increase in the number of transients provided a sufficient signal-to-noise ratio.[19]

IV. Application of Long-Range Coupling Constants for Determining Stereochemistry

Two- and three-bond coupling constants between protons or a proton and carbon show the dependence on their stereochemistry. Determination of the relative configuration by $^3J_{HH}$ and $^{2,3}J_{CH}$ has been developed as a J-based configuration analysis (JBCA).[40] Among the many 2D methods available for measuring $^{2,3}J_{CH}$,[41] the simplest and the most approproate experiments for JBCA are HETLOC[42–44] and J-resolved HMBC[45–48] which provide the magnitude of $^{2,3}J_{CH}$ values directly from the spectra. The HETLOC spectrum is based on TOCSY. It has both F_1 and F_2 ^{1}H-chemical shift dimensions. The diagonal and the correlation peaks are doublets split by $^1J_{CH}$ in the F_1 dimension. The long-range J_{CH} ($^{LR}J_{CH}$) values are measured from the signal displacement of each signal of the cross-peak doublet. This is a powerful method to measure the $^{LR}J_{CH}$ values between ^{1}H and ^{13}C in the same ^{1}H-spin network (Fig. 4a).

There are some variants of *J*-resolved HMBC: *J*-res HMBC,[45] *J*-IMPEACH MBC,[46] and *J*-HMBC.[47,48] These provide spectra like HMBC. The HMBC correlation peaks in these spectra are split in the F_1 dimension by multiplied $^{LR}J_{CH}$ with the *J*-scaling factor, *N* (Fig. 4b). The $^{LR}J_{CH}$ values between ^{1}H and ^{13}C *via* quaternary carbons or heteronuclei and the $^{LR}J_{CH}$ values on quaternary carbons are measurable by these methods. Scaling factor *N* is set according to the objective $^{LR}J_{CH}$ value as $N > 1/(^{LR}J_{CH} \times t_{1max})$. Setting a large *N* value results in sensitivity loss. In respect of HETLOC, the intervention of a small J_{HH} value weakens the peak intensity, and the $^{LR}J_{CH}$ values between ^{1}H and ^{13}C *via* quaternary carbons and $^{LR}J_{CH}$ values on quaternary carbons cannot be obtained. Those methods are therefore used complementarily. The benefits of editing are also utilized for HETLOC.[49–51] Measurement of the subspectrum according to the carbon multiplicity of HETLOC is helpful for reducing signal overlapping. Figures 4c, d, and e show conventional, CH-, and CH_2-selected HETLOC spectra of **1**, respectively. The CH and CH_2 protons are respectively selected in the F_1 dimension of each subspectrum d and e. 1D variants of HETLOC[52–54] have also been reported which provide two 1D spectra corresponding to the two F_1 slices (parallel to the F_2 axis) through each signal of the cross-peak doublet of HETLOC.

Early applications of JBCA[55] used HETLOC and phase-sensitive HMBC[56] to measure $^{2,3}J_{CH}$. In the latter spectrum, $^{2,3}J_{CH}$ values were determined by calculation of the intensity of the cross peaks. In later years, *J*-resolved HMBC methods were developed, and have been applied to the structural analysis of dytesinin A (**5**),[20] tyroscherin (**8**),[57] and lycoposerramine-B (**9**).[58]

V. Structural Determination of a Highly Hydrogen-Deficient Molecule: Application of Super-Long-Range Correlation between a Proton and Carbon *via* Four Bonds and Measuring the Long-Range ^{13}C–^{13}C Coupling Constants

The structural elucidation of highly hydrogen-deficient molecules might be a problem because the carbon network of such molecules cannot be completely followed by heteronuclear long-range C–H correlation spectroscopy, such as HMBC and COLOC,[59] which usually detect the long-range *J* coupling between a proton and carbon connected *via* three or less bonds. Several approaches have been reported to detect small $^nJ_{CH}$ (n > 3) or magnetization transfer *via* $^nJ_{CC}$ for the structural elucidation of such compounds. An example of the former is decoupling (D)-HMBC,[60] while the latter can be detected by conventional INADEQUATE[61,62] or its gradient-selected version[63,64] by optimizing for the observation of small $^nJ_{CC}$. Improvements to INADEQUATE and related methods have been reviewed by Buddrus.[65,66] The most serious shortcoming with these methods is the low sensitivity resulting from the low natural abundance of the isotopomer to be detected which has two ^{13}C nuclei in a molecule. The sensitivity can be enhanced by several strategies including ^{1}H detection such as 2D n, n-ADEQUATE,[67] using composite[68,69] pulses, magnetization transfer from ^{1}H, and elimination of the double-quantum filtration (C–C relay).[70–74] The methods combined with magnetization transfer from ^{1}H have resulted in two-step magnetization transfer *via* $^1J_{CH} \rightarrow {}^nJ_{CC}$ such as DEPT-INADEQUATE,[75,76] INEPT-INADEQUATE,[77] DEPT C–C relay,[72–74] and INEPT C–C relay.[70,71] The 1D methods[70–72,75–77] are the most advantageous from the viewpoint of the sensitivity. A signal overlapping problem arising from more than one correlation at the observed carbon can be resolved by measuring each subspectrum by DEPT-INADEQUATE, INEPT-INADEQUATE, DEPT C–C relay, and INEPT C–C relay.

In respect of khellin (**10**), only the connectivity represented by the bold line in Fig. 5 can be traced by HMBC, and hence, the direction of the γ-pyrone ring fused to the benzofuran ring cannot be determined. In the CH subspectrum from the DEPT long-range C–C relay method, two sets of doublets, $J = 11.8$ and 3.1 Hz, were clearly observed at C-4a, which would have been derived from two- and three-bond coupling with C-6 and C-3, respectively. Hence, the regiochemical connectivity between the γ-pyrone and benzofuran rings could be unambiguously determined.[74] The assignment of the acylated position of phenols is also a problem, since acyl protons located in a four-bond position away from the attached acyloxy carbons. In respect of methyl 3,4-diacetoxycinnamate (**11**), two acyl groups were assigned by the 2D DEPT long-range C–C relay method, in which key correlation peaks between the acyl protons and oxygenated benzene carbons indicated by arrows in the chemical structure were obtained.[78] The structural elucidation of 16-membered macrodiolide amphidinolide X (**12**) (*ca.* 5% ^{13}C enriched) isolated from a marine dinoflagellate showed that two ester linkages could not be determined from the HMBC spectrum because of the lack of correlation peaks between two ester carbonyl carbons and H-10 and H-17. However, the 2D DEPT long-range C–C relay spectrum gave correlation peaks for C-16/C-1 and C-10/C-6, resulting in the total structure of **12** shown in Fig. 5.[79]

^{1}H-^{15}N HMBC is useful for compounds containing nitrogen in the molecule. Due to the low natural abundance and low gyromagnetic ratio of ^{15}N, direct ^{15}N observation is very difficult at the natural abundance level. The advent of the inverse-detected method coupled with a pulsed-field-gradient has resulted in ^{1}H-^{15}N long-range heteronuclear correlation spectroscopy becoming practical at the level of natural abundance.[80,81]

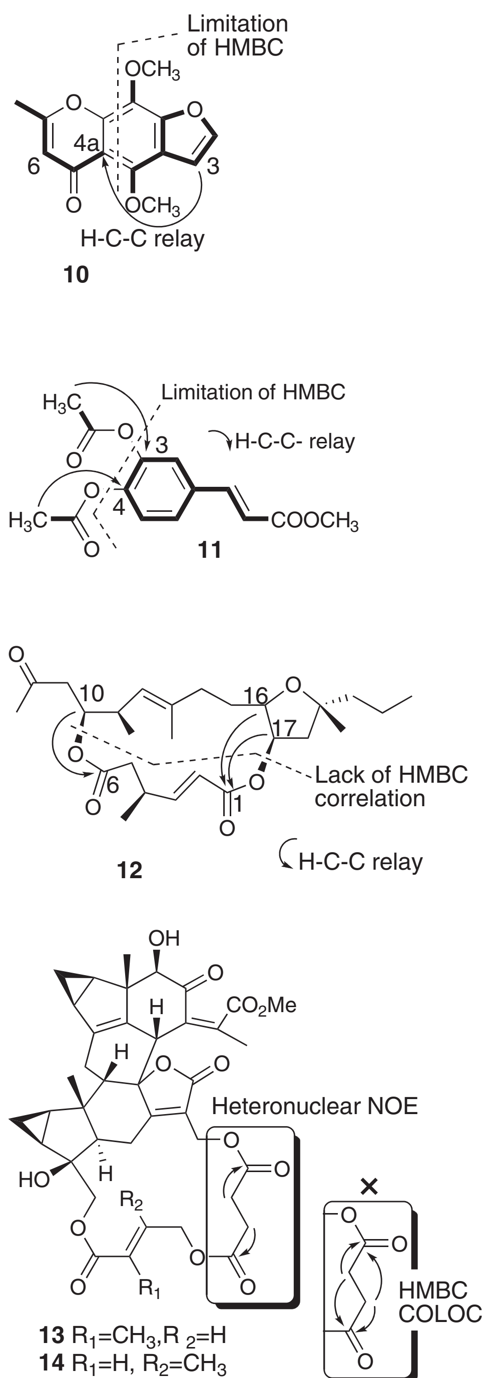

Fig. 5. Chemical Structures of **10–14**.

VI. Discrimination of $^{2}J_{CH}$ and $^{3}J_{CH}$ Correlations

Shizukaols B (**13**) and F (**14**), characteristic dimers from *Chloranthus japonicus*,[82] have a pendant macrocyclic ester substructure consisting of γ-hydroxytiglic or γ-hydroxysenecionic acid, and succinic or malic acid, respectively. Some problems may arise in the structural analysis of these compounds since an ester linkage separates the C–C connectivity in such a pendant ring. Two pairs of methylene protons in the succinate residue could not be unambiguously assigned by detecting $^{LR}J_{CH}$, since all the protons in two methylenes were correlated with both carbonyl carbons in the HMBC and COLOC spectra as shown in Fig. 5. Heteronuclear NOE[83,84] was found effective to selectively detect the two-bond ^{1}H-X-^{13}C (X = C or heteroatom) connectivity.[85] NOE from the two protons of each methylene was observed on the neighboring carbonyl carbon. These results revealed the assignment of the two methylenes in the succinate residue. If the objective two-bond correlation is that between a proton and protonated carbon, there are several methods to discriminate $^{2}J_{CH}$ and $^{3}J_{CH}$ by using J_{HH}.[86–88]

The sensitivity of NMR spectroscopy has been dramatically improved in recent years by the development of superconducting cryogenic probeheads[89] and high-field magnets. The other factor for enhancing sensitivity is to reduce the sample volume by concentrating the solution. A variety of micro- and submicroprobes of less than 5 mm in diameter[90] are commercially available. Combined use of such probes with Shigemi NMR micro cells[91,92] achieves a further reduction in the sample volume.

NMR spectroscopy has become a daily tool. Routine experiments can provide high-quality spectra more easily due to the improved hardware and software now available. Structural elucidation of many compounds therefore demands less time than before. However, there are still some structural problems which require unique approaches to overcome the limitations of the conventional NMR methods. NMR provides various information by tailored pulse sequences which comprise pulses and delay times. Since the combination of pulses and delays is infinite, NMR would satisfy the present and future needs for new methods.

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