Published in final edited form as:

Magn Reson Chem. 2015 December ; 53(12): i. doi:10.1002/mrc.4384.

Structure elucidation of uniformly ^{13}C labeled small molecule natural products

Mikhail Reibarkh^{a,*}, Thomas P. Wyche^b, Josep Saurí^a, Tim S. Bugni^b, Gary E. Martin^a, and R. Thomas Williamson^a

^a Process and Analytical Chemistry, NMR Structure Elucidation Group, Merck Research Laboratories, Rahway, NJ, United States

^b School of Pharmacy, University of Wisconsin—Madison, Madison, WI, United States

Abstract

Utilization of ^2H , ^{13}C , and ^{15}N isotopically labeled proteins and peptides is now routine in biomolecular NMR investigations. The wide-spread availability of inexpensive, uniformly ^{13}C enriched glucose now makes it possible to isolate uniformly ^{13}C labeled natural products from microbial fermentation. We now wish to describe an approach for the rapid structural characterization of uniformly ^{13}C labeled natural products that avoids the pitfalls of relying on parameters typically employed in biomolecular NMR studies.

Keywords

U- ^{13}C labeled; CT-HSQC; ^{13}C – ^{13}C COSY and COSYLR; natural products; structure elucidation; enterocin

Introduction

Natural products from microbial and fungal fermentations are a rich source of bioactive molecules that can provide an effective entry point into the development of therapeutic agents for a wide variety of human and animal health issues.^[1] Although the field of natural products isolation and structure elucidation is, in many respects, mature, it is still a generally time-consuming process that sometimes may lead to structural ambiguity. In the past decade, natural product-based drug discovery has fallen into disfavor with most major pharmaceutical research companies.^[2] In this report, we present a streamlined approach to rapid structure characterization of natural products utilizing uniform (U) ^{13}C labeling. This strategy has become feasible because of the increased availability and affordability of labeled substrates such as U- ^{13}C labeled glucose. Using labeled substrates in fermentation media produces metabolites highly enriched with ^{13}C . With the ^{13}C label in place, the

* Correspondence to: Mikhail Reibarkh, Merck Research Laboratories, Process and Analytical Chemistry, NMR Structure Elucidation Group, Mail stop: RY800-D133P, 126 E. Lincoln Ave, RY800-D231, Rahway, NJ 07065, United States. mikhail_reibarkh@merck.com.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

backbone of virtually any natural product can be quickly established by standard and long-range optimized ^1H -decoupled ^{13}C - ^{13}C COSY experiments.^[3–5] The ^1H NMR chemical shifts can be subsequently assigned through the use of an F_1 -decoupled HSQC experiment. The constant time CT-HSQC described by Bax and coworkers^[6] provides a method to achieve F_1 decoupling in the HSQC experiments of uniformly ^{13}C -labeled peptides and proteins. Herein, we expand the theoretical framework of the CT-HSQC approach to make it universally applicable to practically any U- ^{13}C labeled organic small molecule. The resulting structure elucidation strategy is demonstrated both theoretically and experimentally using the natural product enterocin,^[7] **1**, as an example. Practical limitations of the experimental approach will also be described in this report.

^1H - ^{13}C HSQC: a cornerstone of structure elucidation

Natural abundance ^1H - ^{13}C HSQC experiments^[8,9] have represented a cornerstone of small molecule structure elucidation protocols for more than 20 years. The experiment offers high sensitivity, straight-forward and robust setup, while providing a $^1\text{H}/^{13}\text{C}$ fingerprint of the molecule. Multiplicity-editing adds additional information content relating to the number of protons covalently attached to each carbon atom. Recently developed pure shift analogs of the HSQC experiment have served to further increase resolution and sensitivity.^[10–12] In addition to ^1H and ^{13}C chemical shifts, HSQC spectra provide a wealth of structurally relevant information. In fact, there are successful examples of using just ^1H and multiplicityedited ^1H - ^{13}C HSQC data for automated structure verification purposes.^[13]

Uniform ^{13}C labeling of small molecules offers opportunities to access quaternary ^{13}C chemical shifts, as well as ^1H - ^{13}C and ^{13}C - ^{13}C coupling constant information, the latter of which are not easily accessible at ^{13}C natural abundance.^[14] At the same time, U- ^{13}C labeling introduces additional J_{CC} and J_{CH} pathways during t_1 evolution in the HSQC experiment, which are negligible at natural abundance. Evolution of J_{CC} and J_{CH} couplings affects sensitivity, lineshape, and the phase of HSQC cross-peaks and complicates data interpretation, and therefore is undesirable and to be avoided. The constant time (CT) approach was first introduced in 1992 to avoid J_{CH} evolution and to minimize J_{CC} evolution in ^1H - ^{13}C HSQC of uniformly ^{13}C -labeled proteins and peptides.^[6] The success of CT-HSQC experiment is heavily reliant on the structural uniformity of amino acid residues, which equates to uniformity of the corresponding J_{CC} coupling constants.

Applying the same approach to a much broader range of ^{13}C - ^{13}C coupling constants and chemical shifts is non-trivial. Recently, a modified version of ^1H - ^{13}C HSQC for U- ^{13}C labeled small molecules was published.^[15] However, the modified experiment still focuses on the same narrow range of J_{CC} couplings as the original CT publication and hence has limited general applicability. We believe that the issue can benefit from a thorough

theoretical analysis of potential modulations introduced by J_{CC} couplings, similar to the analysis performed for the ADEQUATE experiments.^[16]

CT-HSQC and its limitations

The constant time (CT) pulse sequence element and an example of an HSQC pulse sequence are shown in Fig. 1. Constant time pulse sequence elements simplify, but do not remove, J_{CC} evolution. Evolution caused by J_{CC} introduces a modulation to the magnetization that is a cosine function of the J_{CC} coupling constant and CT value:

$$M(J) = \cos\left(\pi \times {}^AJ_{CC} \times CT\right) \times \cos\left(\pi \times {}^BJ_{CC} \times CT\right) \times \cos\left(\pi \times {}^CJ_{CC} \times CT\right). \quad (1)$$

Here, the terms ${}^AJ_{CC}$, ${}^BJ_{CC}$, and ${}^CJ_{CC}$ denote one-bond ^{13}C – ^{13}C homonuclear coupling constants between the carbon of interest and up to three neighboring carbons, A, B, and C. Long-range ${}^nJ_{CC}$ couplings can also contribute to the modulation significantly in some cases because some ${}^nJ_{CC}$ couplings can be rather large, ranging up to >16 Hz.^[17]

In the case of amino acids and, consequently, peptides and proteins, the mathematical description of the CT-HSQC experiment can be significantly simplified because of structural uniformity, which translates into uniformity of the corresponding J_{CC} coupling constants. All aliphatic ${}^1J_{CC}$ values will be within a 32–40 Hz range, and long-range ${}^nJ_{CC}$ couplings are small enough in amino acids to consider them negligible. One-bond and long-range couplings to the carbonyls of the peptide backbone are selectively suppressed, which is possible because of narrow and predictable range of their ^{13}C chemical shifts.^[6] The simplified formula uses average aliphatic ${}^1J_{CC}$ value and a number of covalently bound carbon neighbors, N:

$$M(J) \approx \cos^N\left(\pi \times J_{av} \times CT\right). \quad (2)$$

Choosing CT to match $1/J_{av}$ (26.6 ms) allows multiplicity-editing based on the number of covalent carbon neighbors. CT matched to $2/J_{av}$ (53.2 ms) leads to correlation responses with the same phase.^[6] Long-range ${}^nJ_{CC}$ are again considered negligible with a corresponding cosine term close to 1.

One can easily see that while this approach is powerful, provided that the assumption regarding J_{CC} is valid, the method quickly fails, becoming unusable in the more general case of an organic small molecule. Small molecule ${}^1J_{CC}$ values typically are in 25–70 Hz range,^[18,19] with known outliers as low as 6–15 Hz for cyclopropyl and 15–20 Hz for cyclobutyl carbons and as high as 85–95 Hz for protonated acetylenic carbons.^[19,20] Similarly, long-range ${}^nJ_{CC}$ constants are also more highly variable and can range to as large as 16 Hz,^[17] thus contributing significantly to the modulation of magnetization. As an example, at an optimization of 53.2 ms for the constant time period as in the original publication,^[6] a long-range coupling of 9 Hz will essentially null the modulation function, thereby eliminating an HSQC correlation.

Analysis of the limits of CT approach

In the general case of an organic molecule there are four possible situations: 0, 1, 2, and 3 carbon neighbors. We will provide a mathematical treatise for every case. The effect of $^nJ_{CC}$ couplings will be considered later.

N= 0: No J_{CC} couplings.

$$M_0(J) \equiv 1. \quad (3)$$

N= 1: One J_{CC} coupling.

$$M_1(J) = \cos(\pi \times J_{CC} \times CT). \quad (4)$$

N= 2: Two J_{CC} couplings.

$$M_2(J) = \cos(\pi \times {}^AJ_{CC} \times CT) \times \cos(\pi \times {}^BJ_{CC} \times CT). \quad (5a)$$

Applying a trigonometric transformation $\cos(x) \times \cos(y) = 0.5 \times [\cos(x+y) + \cos(x-y)]$ transforms (5a) from the multiplicative to the additive function:

$$M_2(J) = 0.5 \times \left[\cos\left(\pi \times CT \times ({}^AJ_{CC} + {}^BJ_{CC})\right) + \cos\left(\pi \times CT \times ({}^AJ_{CC} - {}^BJ_{CC})\right) \right]. \quad (5b)$$

The resulting arguments are now $({}^AJ_{CC} + {}^BJ_{CC})$ and $({}^AJ_{CC} - {}^BJ_{CC})$ instead of ${}^AJ_{CC}$ and ${}^BJ_{CC}$.

It is now easy to see that within the range of one-bond carbon–carbon couplings typically encountered with small organic molecules the function $M_2(J)$ can adopt any value between -1 and $+1$, including null. Moreover, even small changes in the ${}^1J_{CC}$ value may dramatically impact the result and thereby response intensity. For example, consider a CT value of 53.2 ms used in the original publication^[6] (matched to the average aliphatic ${}^1J_{CC}$ coupling for proteins and peptides):

$$\text{If } {}^AJ_{CC} = 50 \text{ Hz and } {}^BJ_{CC} = 38\text{Hz} \rightarrow M_2(J) = -0.5, \quad (6)$$

$$\text{If } {}^AJ_{CC} = 47 \text{ Hz and } {}^BJ_{CC} = 38\text{Hz} \rightarrow M_2(J) = -0.0, \quad (7)$$

$$\text{If } {}^AJ_{CC} = 44 \text{ Hz and } {}^BJ_{CC} = 38\text{Hz} \rightarrow M_2(J) = +0.5. \quad (8)$$

Considering that 38, 44, 47, and 50 Hz are all within the typical range of $^1J_{CC}$ coupling constants for organic molecules, one can easily see how an otherwise minor 3 Hz difference can cause dramatic variations in both sign and relative intensity of cross-peaks in a CT-HSQC spectrum. This hypothetical example clearly illustrates challenges of using CT-HSQC for a uniformly ^{13}C -labeled organic molecule such as a natural product.

N= 4: Three J_{CC} couplings.

$$M_3(J) = \cos\left(\pi \times {}^AJ_{CC} \times \text{CT}\right) \times \cos\left(\pi \times {}^BJ_{CC} \times \text{CT}\right) \times \cos\left(\pi \times {}^CJ_{CC} \times \text{CT}\right). \quad (9a)$$

Equation (9a) can be simplified considering that the only case when a protonated carbon can have three other covalently bound carbons if it is an sp^3 carbon. In this case, direct carbon-carbon $^1J_{CC}$ couplings will be confined to a relatively narrow range, and original CT assumption can still be used here:

$$M_3(J) = \cos^3\left(\pi \times J_{CC} \times \text{CT}\right). \quad (9b)$$

Graphical representation of $M_1(J)$ and $M_3(J)$ are shown in Fig. 2, and $M_2(J)$ behavior is shown in Fig. 3. For all cases CT is set to 53.2 ms as in the original publication.^[6] All functions have intensity and sign variations that are dependent on $^1J_{CC}$ coupling constants.

Zero crossings are especially undesirable because it means that certain coupling constants will result in null magnetization, effectively *erasing* the corresponding HSQC cross-peak.

Optimization of CT for a general case

It is clear from the analysis of Eqns (3–9) that there is no ‘ideal’ setting of CT that will adequately cover the typical range of one-bond carbon-carbon couplings in organic molecules without modulation of the function crossing zero (often more than once) within this range. Figure 4 provides a visual aid showing the $M_2(J)$ function at varied CT values.

As a first approach to identifying a ‘universal’ CT setting, one could try to find a compromise CT value that would be sufficient for most but not all possible one-bond couplings, similar to the approach utilized for the inverted $^1J_{CC}$ 1, n-ADEQUATE experiment.^[21] Using similar methodology, we calculated that the CT value of 20 ms would represent such compromise. With this optimization, $M_2(J)$ will cross zero at 25 Hz and 75 Hz (see Fig. 5), which may be considered acceptable because 25 and 75 Hz are on the edges of the typical range of one-bond couplings encountered for most natural products.^[18–20] However, further analysis of $M_3(J)$ behavior shows that choosing a 20-ms optimization for CT is a rather poor choice in terms of a universal optimization. With 20-ms CT optimization, $^1J_{CC}$ coupling constants in the range from 19 to 31 Hz (as well as from 69 to 81 Hz) will result in response intensity below 5% of maximum possible value, which for practical purposes can be considered null (see Fig. 5). Having $^1J_{CC}$ coupling constants ranges of 19–31 Hz and 69–81 Hz as null regions is very undesirable because they overlap

with the typical range of one-bond ^{13}C – ^{13}C couplings in organic small molecules and natural products. Large long-range ^{13}C – ^{13}C couplings would also attenuate down the signal intensity, although less drastically. With the optimization of CT for 20 ms, the intensity loss because of long-range carbon–carbon couplings, $^nJ_{\text{CC}}$, of 16, 10, and 7 Hz is calculated to be 40%, 20%, and 10%, respectively. Of course an investigator could still use a 20-ms optimization for the CT parameter if no $^1J_{\text{CC}}$ couplings are smaller than 32 Hz or larger than 68 Hz are expected in the molecule, but it is definitely not universal for organic small molecules and natural products.

A closer look at the formula (5b) makes it clear that in order to decouple J_{CC} evolution in a truly uniform fashion, one has to satisfy all possible ranges of $(^AJ_{\text{CC}} + ^BJ_{\text{CC}})$ and $(^AJ_{\text{CC}} - ^BJ_{\text{CC}})$. The only way to accomplish this goal is by creating a very slow cosine wave, which is achieved with a 5 ms optimization of the CT interval (see Fig. 6). This setting represents truly universal optimization: in a coupling range of 0–96 Hz it yields both constant phase and reasonable signal intensity (zero crossing is at 100Hz). Additionally, this optimization is very insensitive to smaller $^nJ_{\text{CC}}$ couplings: even the largest $^nJ_{\text{CC}}$ of 16 Hz would cause an intensity loss of less than 3%.

Optimization of the CT interval for a universal value of 5 ms has one major disadvantage: a short constant time interval limits t_1 , which in turn limits the resolution of the indirectly determined frequency domain F_1 to 200 Hz. Linear prediction during processing can somewhat improve F_1 digital resolution. However, this disadvantage does not affect the structure elucidation protocol for ^{13}C -labeled molecules, because high resolution ^{13}C data are available via 1D ^{13}C and 2D ^{13}C – ^{13}C COSY spectra. These points will be further considered below in the results and discussion section.

Results and discussion

^1H decoupled ^{13}C – ^{13}C COSY

The first step in the carbon backbone determination of a uniformly ^{13}C labeled natural product is to acquire a ^1H decoupled ^{13}C – ^{13}C COSY spectrum.^[4] These data quickly provide information on the carbon–carbon connectivity of all neighboring carbon atoms. Because of the high incorporation of ^{13}C label, these experiments can be acquired in a short experiment time as shown below using a 7.8-mg sample of enterocin (**1**). One-bond carbon–carbon correlations afford partial structures, which can be successively linked together through heteroatoms utilizing long-range ($^nJ_{\text{CC}}$, $n = 2, 3$) ^{13}C – ^{13}C correlations via a ^{13}C – ^{13}C COSYLR^[5] experiment. In the case of ^{13}C – ^{13}C COSY, the choice of the t_1 evolution time governs the size of the ^{13}C – ^{13}C coupling constant leading to the observation of a cross-peak. As the t_1 interval becomes longer, progressively smaller ^{13}C – ^{13}C coupling constants will afford correlations. For a given spectral width, the t_1 evolution time can be increased by either increasing the number of F_1 increments or by using COSYLR with an explicitly specified t_1 evolution delay. Both of these approaches provide comparable data, but COSYLR proved to be more practical because it allows one to set any t_1 evolution interval while keeping the number of F_1 increments small and governed only by needed resolution in F_1 indirectly acquired dimension.

Figure 7 shows the ^1H decoupled ^{13}C – ^{13}C COSY spectra optimized for one-bond and for long-range ^{13}C – ^{13}C couplings. The spectra were acquired in 35 min and in 4 h and 45 min, respectively using a Bruker 600-MHz AVANCE III triple resonance spectrometer equipped with 1.7-mm MicroCryoProbe.TM

Universally optimized ^1H – ^{13}C CT-HSQC

Structure elucidation paradigms for U- ^{13}C labeled natural products generally utilize a ^1H -decoupled ^{13}C – ^{13}C COSY as the primary structural tool, while CT-HSQC data are employed in a supplementary role to establish ^1H – ^{13}C direct correlations. Figure 8 shows CT-HSQC spectra acquired with CT intervals of 5 and 26.6 ms acquired on a 7.8 mg sample of U- ^{13}C labeled enterocin. The acquisition times were 16 and 85 min, respectively, using a Bruker 600-MHz spectrometer equipped with a 1.7-mm MicroCryoProbe.TM

One can easily appreciate the robustness of the universally optimized CT-HSQC spectrum acquired with CT optimized to 5 ms: no correlations are missing, phase characteristics are good, and there are only small variations in relative peak intensities. Other CT settings are noticeably less reliable. For example, a CT-HSQC spectrum acquired with 7.5 ms optimization has some peaks missing and other peaks exhibit severe phase distortions (see Figure S1 in the Supplementary Information). The CT optimization of 26.6 ms (default value for proteins and peptides^[6]) results in a spectrum with multiple missing correlations and with inverted phase for other responses. Experimental results in these three spectra confirm the theoretical considerations developed above. All missing or inverted responses are in quantitative agreement with nulls and inversions of the response intensity governed by the Eqn (1) (see Figures S1–S4 in the Supplementary Information).

Structure elucidation of enterocin

A ‘rough’ carbon skeleton of the enterocin molecule was assembled using ^1H decoupled ^{13}C – ^{13}C COSY optimized for large couplings (see Figs. 7 and 9). This skeleton consisted of several substructures that are formed whenever a contiguous carbon–carbon chain is interrupted by a heteroatom (Fig. 9). The resultant substructures were connected together using long-range ^1H decoupled ^{13}C – ^{13}C COSYLR data and ^1H – ^{13}C connectivities from the optimized CT-HSQC spectrum (see Figs. 8 and 9). The total acquisition time for all necessary experiments (short-range ^1H ^{13}C – ^{13}C COSY, long-range ^1H ^{13}C – ^{13}C COSYLR, CT-HSQC, 1D ^1H , and 1D ^{13}C) was 5 h 35 m for a 7.8 mg sample of U- ^{13}C labeled enterocin, with ^{13}C – ^{13}C COSYLR requiring 4 h 45 m, which represents 85% of total acquisition time.

Enterocin fermentation and isolation

The ascidian, *Didemnum psammathode*, was collected in October, 2010, in the Florida Keys (24° 37.487', 81° 27.443'). For cultivation, a sample of ascidian (1 cm³) was rinsed with sterile seawater, macerated using a sterile pestle in a micro-centrifuge tube, and dilutions were made in sterile seawater, with vortexing between steps to separate bacteria from heavier tissues. Dilutions were separately plated on three media: ISP2, R2A, and M4. Each medium was supplemented with 50 µg/ml cycloheximide and 25 µg/ml nalidixic acid. Plates

were incubated at 28 °C for at least 28 days. Strain WMMB285 was identified as a *Streptomyces* sp. based on the 16S rDNA sequence (GenBank JX960758.1).

A 10 mL seed culture (25 × 150 mm tubes) in medium ASW-A (20-g soluble starch, 10 g glucose, 5 g peptone, 5 g yeast extract, 5 g CaCO₃ per liter of artificial seawater) was inoculated with strain WMMB285 and shaken (200 RPM, 28 °C) for seven days. The seed culture was used to inoculate 100 mL of ¹³C-enriched ASW-A media (10 g U-¹³C-glucose/L) containing Diaion HP20 (4% by weight). After 7 days, filtered HP20 and cells were washed with water and extracted with acetone. The acetone extract was subjected to liquid-liquid partitioning using 30% aqueous methanol and chloroform (1:1). The chloroform partition was fractionated by size exclusion chromatography applying Sephadex® LH-20 as stationary phase and a solvent mixture of chloroform:methanol 50:50 as mobile phase. Five fractions were obtained, and the fraction containing enterocin was purified by HPLC.

A Shimadzu LC system was used, equipped with a LC-20AT pump, SPD-M20A diode array detector, a SIL-20AC HT auto sampler, and a FRC-10A fraction collector. The LC time program consisted on a gradient of methanol 15%/water 85% to methanol 60%/water 40% in 8 min, then increased from methanol 60%/water 40% to methanol 100% in 1 min, and held for 3 min. The column used was a Phenomenex Onyx monolithic column, 100 × 4.6 mm. The flow rate used was 3 mL/min. Deoxyenterocin eluted at 2.45 min and was collected in fractions 3 to 7, while enterocin, eluted at 3.70 min and was collected in fractions 9 to 11.

Conclusions

We have demonstrated a rapid and efficient structure elucidation protocol for U-¹³C labeled natural products. The protocol we propose consists of ¹H decoupled ¹³C-¹³C COSY optimized for short and long-range couplings and CT-HSQC optimized for use with natural products and organic small molecules. We developed a theoretical framework and have demonstrated the experimental verification of the techniques for expanding the scope of CT-HSQC experiment from narrow amino acid applications^[6] to a protocol that is universally applicable for labeled natural products and organic small molecules. The only trade-off of the universally optimized CT-HSQC is lower *F*₁ resolution, which, should not be a major impediment to the use of the protocol for structure elucidation because of the availability of high resolution ¹³C information from 1D ¹³C and 2D ¹³C-¹³C COSY data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by the NIH, grant GM092009 (T.S.B.) and grant GM107557 (T.S.B.)

References

- [1]. a) Reynolds, WF.; Mazzola, EP. Progress in the Chemistry of Organic Natural Products. Kinghorn, AD.; Faul, H.; Kobayashi, J., editors. Vol. 100. Springer International Publishing; Switzerland: 2015. p. 223-309. b) Breton RC, Reynolds WF. Nat. Prod. Rep. 2013; 30:501–524. [PubMed: 23291908]
- [2]. a) Petkewich R. C&EN. 2007; 85(20):56. b) Jarvis L. C&EN. 2012; 90(8):30.
- [3]. Aue WP, Bartholdi E, Ernst RR. J. Chem. Phys. 1976; 64:2229–2246.
- [4]. Martin, GE.; Zekter, AS. VCH Publishers, Inc.; New York: 1988. p. 59
- [5]. Bax A, Freeman R. J. Magn. Reson. 1981; 44:542–561.
- [6]. Vuister G, Bax A. J. Magn. Reson. 1992; 98:428–435.
- [7]. Miyairi N, Sakai HI, Konomi T, Imanaka H. J. Antibiot. 1976; 29:227–235. [PubMed: 770404]
- [8]. Claridge, TDW. High-Resolution NMR Techniques in Organic Chemistry. Pergamon Press; New York: 1999. p. 229-245.
- [9]. Castañar, L.; Parella, T. Ann. Rep. NMR Spectrosc. Webb, GA., editor. Vol. 84. Elsevier; Amsterdam: 2015. p. 163-232.
- [10]. Adams RW. eMagRes. 2014; 3:1–15.
- [11]. Zangger K. Prog. Nucl. Magn. Reson. Spectrosc. 2015;86–87. 1–20. [PubMed: 26282197]
- [12]. Castañar L, Parella T. Magn. Reson. Chem. 2015; 53:399–426. [PubMed: 25899911]
- [13]. a) Golotvin SS, Vodopianov E, Lefebvre BA, Williams AJ, Spitzer TD. Magn. Reson. Chem. 2006; 44:524. [PubMed: 16489552] b) Golotvin SS, Vodopianov E, Pol R, Lefebvre BA, Williams AJ, Rutkowske RD, Spitzer TD. Magn. Reson. Chem. 2007; 45:803–813. [PubMed: 17694570] c) Elyashberg, ME.; Williams, AJ.; Martin, GE. Prog. NMR Spectrosc. Feeney, J.; Sutcliffe, L., editors. Vol. 53. Pergammon, Oxford; 2008. p. 1-104.
- [14]. a) Williamson RT, Buevich AV, Martin GE. Org. Lett. 2012; 14:5098–5101. [PubMed: 22994381] b) Sauri J, Parella T, Williamson RT, Martin GE. Magn. Reson. Chem. 2015 in press: DOI: 10.1002/mrc4322.
- [15]. Foroozandeh M, Giraudeau P, Jeannerat D. Magn. Reson. Chem. 2013; 51:808–814. [PubMed: 24123384]
- [16]. Reibarkh M, Williamson RT, Martin GE, Bermel W. J. Magn. Reson. 2013; 236:126–133. [PubMed: 24035003]
- [17]. Krivdin, LB.; Della, EW. Prog. NMR Spectrosc. Feeney, J.; Sutcliffe, M., editors. Vol. 23. Pergammon, Oxford; 1991. p. 301-610.
- [18]. Wray V. Prog. Nucl. Magn. Reson. Spectrosc. 1979; 13:177–236.
- [19]. Marshall, JL. Carbon-Carbon and Carbon-Proton NMR Couplings: Applications to Organic Stereochemical and Conformational Analysis. VCH; Deerfield Beach, FL: 1983. p. 65-199.
- [20]. Kamie ska-Trela, K. Ann. Rep. NMR Spectrosc. Webb, GA., editor. Vol. 30. Academic Press; New York: 1995. p. 131-230.
- [21]. Martin GE, Reibarkh M, Buevich AV, Blinov KA, Williamson RT. eMagRes. 2014; 3:215–234.

Figure 1. Pulse sequence elements of the CT-HSQC experiment and the principle of CT evolution.

Figure 2.
Graphical representation of response intensity $M_1(J)$ (green) and $M_3(J)$ (red) at CT of 53.2 ms.

Figure 3. Graphical representation of response intensity $M_2(J)$ at CT of 53.2 ms. Ten $M_2(J)$ functions are shown with J_{CC} incremented from 30 Hz to 75 Hz with 5 Hz step and with variable J represented by $^BJ_{CC}$.

Figure 4. Graphical representation of response intensity $M_2(J)$ at different CT values. For each CT value, ten $M_2(J)$ functions are shown with $^A J_{CC}$ incremented from 30 Hz to 75 Hz with 5 Hz step and with variable J represented by $^B J_{CC}$.

Figure 5.

Graphical representation of $M_1(J)$ (grey), $M_2(J)$, and $M_3(J)$ (black) response intensities at 20 ms CT value. For visualization purposes, ten $M_2(J)$ functions are shown with $^A J_{CC}$ incremented from 30 Hz to 75 Hz in 5 Hz step and with the variable J represented by $^B J_{CC}$.

Figure 6.

Graphical representation of $M_1(J)$ (grey) and $M_2(J)$ response intensities at 5 ms optimization of the constant time interval. For visualization purposes, ten $M_2(J)$ functions are shown with $^AJ_{CC}$ incremented from 30 Hz to 75 Hz with 5 Hz step and with variable J represented by $^BJ_{CC}$.

Figure 7.

^1H decoupled ^{13}C - ^{13}C COSY spectra of the enterocin. A.) ^1H decoupled ^{13}C - ^{13}C COSY spectrum optimized for one-bond correlations. The experiment was acquired with 2048 points in the direct F_2 dimension and 256 points in the indirect F_1 dimension for a maximal t_1 interval of 8.5 ms. Pulses affording a 45° flip angle were used. Each FID was acquired with four scans giving a total acquisition time of 35 min. Every possible one-bond ^{13}C - ^{13}C correlation has been observed (see Fig. 9) with an average signal-to-noise ratio of 82:1. B.) ^1H decoupled ^{13}C - ^{13}C COSYLR spectrum optimized for 2- and 3-bond correlations. The experiment was acquired with 2048 points in the direct F_2 dimension and 256 points in the indirect F_1 dimension, the t_1 delay was set 50 ms. Again, pulses affording a 45° flip angle were used. Each FID was acquired with 32 scans giving a total acquisition time of 4 h 45 m.

Figure 8.

CT-HSQC spectra of U-¹³C labeled enterocin shown with normalized vertical scales. Positive contours are black and negative contours are red. Enterocin structure with highlighted groups, corresponding to the highlighted HSQC correlations, is shown. A.) CT-HSQC spectrum with CT interval of 5 ms. The data were acquired with 2048 points in the direct F_2 dimension and 128 points in the indirect F_1 dimension. Four transients were accumulated per t_1 increment giving a total acquisition time 16 m. B.) CT-HSQC spectrum acquired with the CT interval optimized for 26.6 ms. The data were acquired with 2048 points in the direct F_2 dimension and 682 points in the indirect F_1 dimension. Four transients were accumulated per t_1 increment giving a total acquisition time of 85 m. Two missing correlations and two correlations with the inverted phase clearly illustrate the importance of employing the optimized CT interval.

Figure 9.

Structure of enterocin derived directly from the ^{13}C - ^{13}C COSY data. Bonds shown in red represent one-bond carbon-carbon correlations observed in ^1H decoupled ^{13}C - ^{13}C COSY experiment. Arrows represent multiple-bond correlations observed in ^1H decoupled ^{13}C - ^{13}C COSYLR experiment acquired with a 50 ms delay. Strong $^nJ_{CC}$ correlations are shown using solid arrows, and weak correlations are denoted by dashed arrows.