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Ultra-high Resolution Band-Selective HSQC for Nanomole-Scale Identification of Chlorine-Substituted ^{13}C in Natural Products Drug Discovery

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Abstract

Ultra-high resolution band-selective HSQC (bsHSQC) has been employed for detection of ^{35}Cl - ^{37}Cl isotope shifted ^{13}C NMR signals for assignment of regioisomerism in bromo-chloro natural products. Optimum pulse sequence and instrumental parameters for maximization of detection of the isotope shifts were explored. The chlorine isotope shifts (δ) were detected within crosspeaks and were shown to vary with hybridization of ^{13}C , substitution of ^{13}C , presence of β -chloro substituents and their relative configuration. Deconvolution of Cl-substituted CH bsHSQC crosspeaks may provide other useful information, including a potentially MS-independent method for quantitating $^{37}\text{Cl}/^{35}\text{Cl}$ isotopic fractionation during the biosynthesis of halogenated natural products.

Graphical Abstract

Ultra-high resolution band-selective HSQC was refined to detect ^{35}Cl - ^{37}Cl isotope shifts in ^1H - ^{13}C crosspeaks of chlorinated CH. The scope and limitations, including optimum experimental parameters for detection of isotope shifts induced in ^{13}C (δ), were explored by measurements of five chlorinated compounds, including polychlorinated natural products from the red alga, *Plocamium cartilagineum* from California.

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Supporting Information. High-resolution ^{13}C NMR and bsHSQC spectra of compounds 3-7. Additional supporting information may be found in the online version of this article at the publisher's web site.

Keywords

NMR; ^1H ; ^{13}C ; ^{35}Cl ; ^{37}Cl ; natural product; HSQC; terpene; Rhodophyta; isotope shift

Introduction

Many organisms within marine Phyla, especially red algae (Rhodophyta), are replete with polyhalogenated compounds,^[1] many with profound biological activity.^[2] For example, the potent cytotoxic compound, halomon from *Portiera hornemannii*,^{3,4} was entered into NIH sponsored pre-clinical trials as an anti-cancer agent.^[5] More recently, halomon and five analogs were shown to be modest to potent inhibitors of the DNMT-1 isoform of DNA methyl transferase (halomon; IC_{50} 1.25 μM).

The structures of polyhalogenated terpenes from red alga may include up to eight Cl and Br atoms, and some compounds are known to contain all three commonly occurring halogens. Outside of Rhodophyta, the occurrence of bromochloro-compounds from marine sponges is rare. For example, the cytotoxic mollenyne A (**1**, HCT-116 cells, IC_{50} = 1.3 $\mu\text{g} \cdot \text{mL}^{-1}$), from the sponge *Spirastrella mollis*, is the first in a family of bromochloro-long chain guanidine-substituted lipids from sponges.^[6] Recently, we had need for an independent NMR method to assign the positions of Cl and Br substituents in new analogs of **1**, including mollenyne B (**2**),^[7] the structure of which appeared to have interchanged the positions of Br and Cl with respect to the former. In simple bromochloroalkanes, discrimination between Cl-substituted and Br-substituted ^{13}C signals is usually trivial; the former are observed at lower field (δ ~8–9 ppm) due to the larger α -deshielding effect of the more electronegative Cl atom. In more highly substituted compounds, like **1**, additional β -deshielding effects may confound simple assignments made by inspection and estimations of empirical ^{13}C chemical shift increments, alone. For example, in halohydrin **1**, the Cl- and Br-substituted methine ^1H and ^{13}C NMR signals have almost identical δ values.^[6] The structures of a surprising number of bromochloroterpenes from red algae, including violacene⁸ and obtusallenes V-VII,^[9] were originally misassigned, partly due to mistaken interpretation of Cl- versus Br-substituted ^{13}C NMR signals. Finally, contemporary methods for NMR assignments by DFT calculations, based on gauge-independent atomic orbitals (GIAO),¹⁰ which have achieved reliable levels of accuracy in predicting ^1H and ^{13}C chemical shifts in many organic structures, are of little help with Br- and Cl-substituted ^{13}C because they poorly estimate the spin-orbit effects of heavy atoms.¹¹

Analysis of stereostructure of natural products cannot proceed without unambiguous assignment of regiochemistry. We sought to expand a potential solution to this problem that relies on the isotope shift (δ) observed for ^{13}C NMR signals substituted by ^{35}Cl - ^{37}Cl at natural abundance (3:1). While this isotope effect has been long known,¹² it was first exploited for natural product assignments by Suzuki^[13] and Braddock.^[14] Under high digital resolution acquisition conditions, a Cl-substituted ^{13}C NMR signal sometimes separates into a split peak due to the $^{35}\text{Cl}/^{37}\text{Cl}$ induced isotopic shift (ν ~0–0.9 Hz). The Cl-isotope shift can be used for identification and assignment of Cl-substituted ^{13}C ; a result conditional upon being able to acquire ^{13}C NMR spectra with adequately high signal-to-

noise ratio (SNR) and with sufficient digital resolution (ppm/pt). The latter is easy to achieve, but meeting the former criterion is almost impossible when the total sample size is limited to only a few nanomole, as was the case for **2**.

In order to overcome this limitation, we refined and deployed a band-selective HSQC experiment. Band selective HSQC sequences have been proposed before, but these earlier implementations required addition of extra pulses and field gradients to the standard sequence to select the resonances of interest.^{[15],[16]} Our implementation simply replaces a broad-band 180° ¹³C refocusing pulse in Boyer's multiplicity edited HSQC sequence,¹⁷ employing Bodenhausen's Q3 Gaussian cascade shaped pulse,^[18] with a selective pulse. The resulting band-selective HSQC experiment (bsHSQC, see Figure 2 for the pulse sequence) allowed dramatically improved resolution in F1, close to absolute magnet resolution (ν ~0.2 Hz), for direct visualization of isotopically split H-C-Cl crosspeaks.⁷ This ultra-high resolution bsHSQC, combined with measurements using an NMR spectrometer equipped with a microcryoprobe (600 MHz, 1.7 mm probe), resolved the bromochloro regioisomerism in **2** using entire available sample (43 μ g, 68 nanomole) within conventional acquisition times. Here, we explore the scope and limitations of bsHSQC for resolving isotopically split H-C-Cl crosspeaks, the instrumental and pulse parameters for optimal split crosspeak detection (δ), and a discussion of possible application to determination of isotopic fractionation of ³⁵Cl/³⁷Cl during the biosynthesis of chlorinated natural products.

Results and Discussion

For the purposes of surveying ³⁵Cl/³⁷Cl isotope effects on ¹³C, we chose to conduct side-by-side comparisons of both high-resolution ¹³C NMR (hr¹³C NMR) and indirect-detected bsHSQC experiments on selected compounds: regioisomeric synthetic bromochloroalkanes ($\pm$)-**3-4** (Figure 1) and abundant bromopolychlorinated natural products **5-7**, first isolated from the Californian red alga, *Plocamium cartilagineum* by Mynderse and Faulkner.^[19] For the sake of convenient experiment times, the measurements described here were carried out on 'strong samples' (~5–10 mg/5 mm tube); but, as we have shown in our prior work,⁷ bsHSQC is easily adapted to "nanomole-scale samples" within conventional time frames. Investigation of compounds **3-4** allows an estimation of the effect of vicinal quaternary halogen substitution, while compounds **5-7** possess an ideal variety of halogen structural qualities within a single molecule: Cl-CH couplets in different electronic and steric environments, mono- and dichloro-substituted carbons, vicinal dichloro-substitution, and the differences of sp² and sp³ hybridized Cl-CH carbons. Additionally, stereochemical differences on isotope shifts – diastereomeric and geometric isomer pairs (*E*- and *Z*-terminal vinyl chlorides) represented by the pairs **5-6** and **6-7**, respectively – were examined.

The bsHSQC experiment achieves high SNR and improved resolution in F1 by limiting the ¹³C spectral window to a narrow band of the ¹³C signals of interest. During the reverse INEPT a selective ¹³C 180° pulse refocuses only the ¹³C signals of interest. This prevents ¹³C signals outside of the desired spectral width from being folded back in. We also found it advantageous to reduce the ¹H spectral width to a region around the resonances of interest. Digital filtering in the F2 dimension eliminated strong solvent signals and enabled the full dynamic range of the receiver to be applied to digitization of the signals of interest.

Typically, ^{13}C resolution of 0.17–0.34 Hz was obtained (see the Experimental section for full details of spectral widths, offsets, number of points collected and processing details).

An expansion of the H-3–C-3 crosspeak for **3** from an optimized bsHSQC (see below, and Supporting Information for full-scale 2D NMR plots) is shown in Figure 3, clearly showing near base-line separation of the minor ^{37}Cl -CH component from the major ^{35}Cl -CH crosspeak. Here, and in every other case discussed in this report, the minor ^{37}Cl -CH isotopic component is shielded with respect to the dominant ^{35}Cl -related signal due to the slightly greater spin-orbit effect and shorter C-Cl bond length for the heavier isotope. A comparison of slices taken through the bsHSQC contour map (Figure 3b) and hr ^{13}C NMR C-3 signal (Figure 3c, note the poorer SNR) reveals similar structures and ^{35}Cl - ^{37}Cl isotope splitting. Measurement of separations, taken at the apices of the split peaks, of the C-3–H-3 bsHSQC slice and C-3 1D ^{13}C NMR signal, gives comparable values for the isotope shift ($\delta = 0.0074$ and 0.0083 ppm, respectively), although, if required, more accurate estimates of δ could be obtained by deconvolution and best-curve fit as we have shown previously.⁷ The ^{35}Cl - ^{37}Cl isotope shift is clearly visible from the bsHSQC spectrum, alone, and confirmed by direct-detected high-resolution ^{13}C NMR. We would proffer that hr-bsHSQC offers comparable resolution to direct- ^{13}C NMR for detection of ^{35}Cl - ^{37}Cl isotope shifts, but clearly with the advantage of reduced cost of experimental time.

A brief investigation was carried out of factors influencing the SNR in the ^1H -bandwidth selection steps of bsHSQC by monitoring the C-3–H-3 crosspeak slice of compound **3** through F1. As expected, the SNR of the slice shows a strong dependence upon the tip-angle of the ^1H excitation pulse (Figure 4). By comparison, HSQC recorded with conventional parameters (F1 resolution = 94 Hz/pt, non-selective excitation) and deviations of the excitation pulse, P1 of ± 0.6 μs had minor, tolerable effects on the SNR and lineshape. A similar effect upon SNR was observed upon variation of the first ^{13}C pulse in the reversed INEPT sequence embodied within the bsHSQC pulse sequence. Figure 5 shows maximal response – almost equal to non-selective excitation – by using a “G3.256” shaped ^1H excitation pulse corresponding to 2500 μs .

Lack of frequency filtering and foldback in the evolved dimension increases noise which places an upper limit on the narrowest sweepwidth in F1 (SW) that can be used. The bsHSQC spectra of a ‘strong’ sample of **3** (~4 mg/ml, 600 MHz) were measured with different values of SW that were progressively halved from 9.2 ppm to 1.15 ppm, while compensating by a reciprocal increase in the number of scans (NS = 1 to 8), achieving constant digital resolution (DR = 5.4 Hz/pt) and constraining total experimental times to within the range 6.6 – 3.4 min (Figure 6). No effective losses of SNR were observed over these ranges of values (Figure 6a–d). Even reduced experimental times (NS = 2 or 1, Figure 6e,f) at nominal values of SW (1.15 or 6.0 ppm) that halved the SNR (Figure 6e,f), still gave an acceptable peak signal.

Measurements of the isotope splitting of Cl-CH crosspeaks from optimized bsHSQC spectra of compounds **5–7** (CDCl_3) are shown in the composite Figure 7. Each of these compounds contains three chlorinated CH groups – one of them, the sp^2 -hybridized terminus, C-1. The bsHSQC spectra reveal split ^{13}C -H crosspeaks only for the signals due to sp^3 hybridized C-5

and C-10. Notably, the C-10–H-10 crosspeak in compounds **6** and **7** revealed three components due to the three possible isotopologues – $^{35}\text{Cl}_2\text{-CH}$, $^{35}\text{Cl},^{37}\text{Cl-CH}$ and $^{37}\text{Cl}_2\text{-CH}$ – in order of diminishing intensity, and decreasing δ_{C} . The third component, C-10–H-10 $^{37}\text{Cl}_2\text{-CH}$, was not observed in **5**, possibly due to overlap under the second component. Because **5** and **6** are diastereomers (epimeric at C-5), the weaker isotope shift for the latter component suggests a possible dihedral angle dependence of the transmitted spin-orbit interaction in this 1,2-vicinal dichloride that may be predictive of *threo* versus *erythro* diastereoisomerism.

The signal due to the vinyl chloride (sp^2 C-1–H-1 signal, Supporting Information) showed no splitting in **5-7**; only weak broadening, in both the bsHSQC spectra and the 1D ^1H ^{13}C NMR spectrum, suggesting here, transmission of spin-orbit effects on the ^{13}C nucleus in vinyl chlorides is relatively inefficient. Also noteworthy is the lack of splitting for Br-substituted $^{13}\text{C-H}$ couplets in **3-7** (not shown), demonstrating a clear discrimination of Br-CH from Cl-CH signals.²⁰

The ease of measurement of ^{35}Cl - ^{37}Cl ^{13}C -isotope splitting has immediate utility in equivocal structural assignments of polyhalogenated organic compounds, but also opens an opportunity to investigate an underexplored phenomenon; chlorine isotope fractionation during biosynthesis of chlorinated natural products. It is known that enzyme-mediated biosynthesis induces fractionation of isotopes of lighter elements – e.g. ^{12}C versus ^{13}C , ^{14}N versus ^{15}N – through, cumulative small differential rate constants at each elemental reaction step. ^{13}C and ^{15}N isotope fractionation has been used to characterize crop-types and geographic origins of plants. Seawater has essentially an invariant $^{35}\text{Cl}/^{37}\text{Cl}$ ratio, r , that is independent of geographic location,²¹ but it might be expected that halogenation reactions requiring Cl^- (or possibly, formal “ Cl^+ ”, or “ $\text{Cl}^\bullet$ ”: two a electrophilic reactants generated by oxidation of Cl^- by different classes of ‘chlorinase’ enzymes²²) in the biosynthesis of halogenated natural products may also induce fractionation of ^{35}Cl and ^{37}Cl . In principle, ratio r , (Eqn 1), where n are the respective amounts of Cl isotopes, can be accurately obtained by careful deconvolution and integration of isotope-split bsHSQC cross peaks.⁷

$$r = \text{mole fraction}(^{37}\text{Cl}) = \frac{n(^{37}\text{Cl})}{n(^{37}\text{Cl}) + n(^{35}\text{Cl})} \quad \text{Eqn 1}$$

The true power of bsHSQC for this application would be the ability to interrogate r values at *individual* Cl-CH couplets, unlike the conventional method for measuring r – isotope-ratio mass spectrometry (IRMS) – that suffers from the requirement of mg-sized samples of organic compounds needed for sample processing for accurate MS measurements, and Cl atom averaging of r for polychlorinated compounds. Consequently, IRMS can only provide averaged r values in polychlorinated natural products. Finally, it remains to be tested whether measurements of $^{37}\text{Cl}/^{35}\text{Cl}$ mole fractions by bsHSQC could be made with sufficient precision to detect native biosynthetic Cl-isotopic fractionation and define a lower limit for measurable ‰. This is a subject worthy of independent study.²³

In conclusion, we have demonstrated the scope and limitation of bsHSQC for indirect detection of chlorine isotope-shift ^{13}C chemical shifts (δ) in a series of models – both synthetic compounds and natural products. The method yields comparable results to directly detected 1D ^{13}C NMR, but at greatly reduced experimental times, making it suitable for ‘nanomole-scale’ natural products structure elucidation when paired with microcryoprobe NMR. We note some early trends: lower δ of signals for Cl-substituted sp^2 ^{13}C compared with sp^3 ^{13}C , and possible diastereotopic differences for *erthyro* versus *threo* vicinal dichlorides. The possible application of bsHSQC to detection of $^{37}\text{Cl}/^{35}\text{Cl}$ isotope fractionation in biosynthesis of natural products may be feasible, provided measurements of split-peak ^{13}C signal integrals are sufficiently precise.

Experimental Section

General Experimental Procedures. Measurements of high-resolution ^{13}C NMR and bsHSQC

spectra are described below. High-resolution ESITOF analyses were carried out on an Agilent 1200 HPLC coupled to an Agilent 6350 TOFMS. Low-resolution MS measurements were made on a Thermoelectron Surveyor UHPLC coupled to an MSD single-quadrupole detector. Purification and analyses were performed on an Agilent 1100 HPLC or a Jasco PU-286 Plus HPLC instruments, coupled to UV-vis and ELSD detectors using reversed phase (C_{18}) or normal phase (SiO_2) columns (10×250 mm).

High-Resolution ^{13}C NMR spectra

The high-resolution 1D ^{13}C NMR spectra were recorded on a Varian NMR spectrometer equipped with a 5 mm Xsens $^{13}\text{C}\{^1\text{H}\}$ cryoprobe at 303 K. Solvent signals were used as internal standard (CDCl_3 : $\delta_{\text{H}} = 7.26$ ppm, $\delta_{\text{C}} = 77.0$ ppm). The pulse conditions were as follows: spectrometer frequency (SF) = 125.70 MHz, spectral width (SWH) = 2520.2 Hz, pulse 90° width (P1) = 16.75 μs , Number of complex points = 10000 or 32000, Processed using linear prediction, SI = 16384 or 65536, line broadening (LB) = 0.00 Hz, Gaussian broadening (GB) = 0.13 Hz, shifted sine bell (SSB) = $\pi/2$, acquisition time (AQ) = 4.34 s or 12.7 s, relaxation delay (D1) = 5.00 s, and number of scans (NS) = 100 to 1000. The sample concentration was 2–6 mg/mL.

High Resolution bsHSQC spectra

Spectra were recorded on a Bruker Avance III 600 NMR spectrometer equipped with a 1.7 mm $^1\text{H}\{^{13}\text{C}/^{15}\text{N}\}$ microcryoprobe at 298 K or on a Bruker Avance III 600 NMR spectrometer equipped with a 5 mm $^1\text{H}\{^{13}\text{C}/^{15}\text{N}\}$ cryoprobe at 298 K. Residual solvent signals were used as internal reference (CDCl_3 : $\delta_{\text{H}} = 7.26$, $\delta_{\text{C}} = 77.0$). A modification of the multiplicity edited 2D ^1H - ^{13}C HSQC sequence described by Boyer and coworkers,¹⁷ available as a standard Bruker pulse sequence (hsqcedetgpsisp2.3), was employed. The pulse conditions were as follows: pulse sequence = hsqcedetgpsisp2.3, SWH ^1H = 2397.7 Hz, SWH ^{13}C = 693.4 Hz, composite pulse decoupling = bi_p5m4sp_4sp.2, shaped pulse 180° for refocusing = G3.256, 180° shaped pulse for refocusing (P24) = 2500 μs , $^1J_{\text{CH}}$ optimized for 145.0 Hz, TD ^1H = 4096, TD ^{13}C = 2048 or 4096, acquisition time (AQ) = 0.43 s, D1 = 1.00 s, and dummy scans (DS) = 16. The spectra were processed using linear prediction on

^{13}C dimension: LB = 0.00 Hz, NCOEF = 8, SI = 4096 or 8192, SSB ^1H and ^{13}C = $\pi/2$. For the 1.7 mm probe, P1 = 9.30 μs , ^{13}C pulse 90° width (P3) = 11.70 μs , SF ^1H = 599.45 MHz, SF ^{13}C = 150.74 MHz, the sample concentration was 30–90 mg/mL, and NS = 1; For the 5 mm probe, P1 = 8.00 μs , P3 = 10.40 μs , SF ^1H = 600.11 MHz, SF ^{13}C = 150.90 MHz, the sample concentration was 10–20 mg/mL, and NS = 4. *Caution.* Check power and duration of your ^{13}C decoupling to avoid probe damage.

(±)-2-Bromo-3-chloro-2-methylbutane (3) and (±)-3-Bromo-2-chloro-2-methylbutane (4)

Compounds **3–4** were prepared by bromochlorination of the 2-methyl-2-butene (*N*-bromosuccinimide, HCl gas, CH_2Cl_2)^[24] and purified by silica gel chromatography (elution with hexanes) to give an inseparable mixture of **3:4** (ratio: 0.22:1). The ^1H NMR and ^{13}C NMR data matched the literature values.^[25]

Bromopentachloroterpenes 5–7.

Pure samples of **5–7** were isolated from extracts of red alga *Plocamium cartilagineum*, collected at Bird Rock, San Diego (January 2011), using a modification of the procedure described by Mynderse and Faulkner.^[19] ^1H , ^{13}C NMR and MS data of the purified compounds matched the literature values.^[19]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mollenynes A (1)⁶ and B (2),⁷ synthetic bromochloroalkanes **3** and **4**, and polyhalogenated terpenes (**5-7**) from *Plocamium cartilagineum*.¹⁹

Figure 2.

Pulse sequence of the bsHSQC. Filled narrow bars represent 90° pulses. Open rectangular bars represent 180° ^1H pulses. Open shaped bars represent shaped ^{13}C 180° pulses. The Bruker shape files used were as follows: Chirp, Crp60,0.5,20.1; Xfilt, Crp60_xfilt.2; G3, G3.256. Delta was set to $1/(4 \times {}^1J_{\text{CH}}) = 1724 \mu\text{s}$ (${}^1J_{\text{CH}} = 145 \text{ Hz}$). "trim" represents a 1ms trim pulse, "EA" identifies the gradient whose sign was alternated to implement echo-antiecho detection, and "CPD" represents the composite pulse decoupling sequence (Waltz64). Gradient strengths were set as recommended in the Bruker pulse sequence hsqcedetgpsisp2.3.

Figure 3.

(a) High-resolution bsHSQC of the C-3–H-3 crosspeak of **3**. (b) 1D column slice (through δ_{H} 4.224, q, $J = 6.7$ Hz) of (a). (c) High-resolution ^{13}C NMR of C-3.

Figure 4.

1D column slices of the bsHSQC C-3-H-3 crosspeak in **3** (F1 digital resolution = 0.09 Hz/pt (a)–(c) or 10.8 Hz/pt (d)–(f) showing the effect of imperfect ^1H pulse width (P1) on the SNR and line shape. (a) and (d); 7.9 μs (calibrated, 90 $^\circ$); (b) and (e); 8.2 μs (c) and (f) 8.5 μs .

Figure 5.

1D column slices of C-3–H-3 crosspeak of **3** with F1 resolution 5.4 Hz/pt showing the effect of the selective band width to the SNR in bsHSQC. (a) Non-selective excitation pulse. (b)–(e) Selective excitations. The first 180° ^{13}C pulse in the reverse INEPT employed a “G3.256” shape of 1250, 2500, 3000, 5000 μs duration respectively.

Figure 6.

1D column slices of the C-3-H-3 crosspeak of **3** (2500 μ s “G3.256” pulse; F1 digital resolution = 5.4 Hz/pt) showing the effect of SW in F1 and total experimental time on the SNR of bsHSQC. (a) SW (F1) = 9.2 ppm, NS = 1, time = 6.6 min. (b) SW (F1) = 4.6 ppm, NS = 2, time = 6.6 min. (c) SW (F1) = 2.3 ppm, NS = 4, time = 6.6 min. (d) SW (F1) = 1.15 ppm, NS = 8, time = 6.6 min. (e) SW (F1) = 6.0 ppm, NS = 1, time = 4.4 min. (f) SW (F1) = 2.3 ppm, NS = 2, time = 3.4 min.

Figure 7.

High-resolution bsHSQC of C-CH crosspeaks of compounds **5–7** (CDCl₃). (a) C-5–H-5 crosspeak of **5**. (b) C-10–H-10 crosspeak of **5**. (c) C-5–H-5 crosspeak of **6**. (d) C-10–H-10 crosspeak of **6**. (e) C-5–H-5 crosspeak of **7**. (f) C-10–H-10 crosspeak of **7**.