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NMR based quantitation of cycloartane triterpenes in black cohosh extracts

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Abstract

The cycloartane triterpene content in the roots/rhizomes (RR) and aerial parts (PX) of *Actaea racemosa* (AR), *A. podocarpa* (AP), and *A. cordifolia* (AC) have been investigated by quantitative ^{1}H NMR (qHNMR). Thereby, it was demonstrated that qHNMR represents a powerful methodology for the analysis of crude plant extracts as it does not rely on the rarely available identical reference triterpenes. Specifically, the presence of the characteristic C-19 cyclopropane (*exo*/*endo*) hydrogen signals made it possible to quantify the less common/not ubiquitously present group of cycloartane triterpenes, directly in extracts. As an example, ARPX and ARRR were shown to contain, 3.8 – 20.8% ± 8.2% and 7.2 – 19.3% ± 4.0% of cycloartane triterpenes, respectively. The cycloartane concentration in ACPX and ACRR was 7.5 – 8.7% ± 0.8% and 13.9 – 28.5% ± 7.3%, respectively, based on the weight of the extract. AP was shown to contain notably lower amounts of the cycloartane triterpenes as compared to AR and AC in the roots/rhizomes. The content for APPX and APRR was only 2.1 – 3.3% ± 0.7% and 1.1 – 4.0% ± 1.5%, respectively. In addition, an example is presented for the identification of specific cycloartanes as marker compounds for AR within crude extracts based on the same qHNMR spectra and 2D NMR methods.

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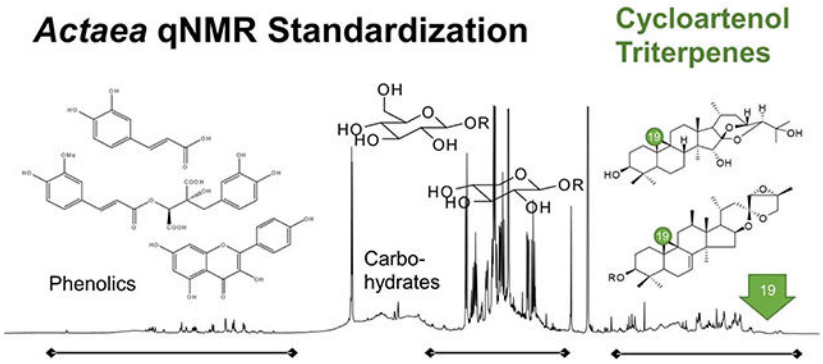
Conflict of interest
The authors declare no conflict of interest.

Dedication
The authors dedicate this article to the late Dr. Norman Farnsworth and his wife, Priscilla Farnsworth, for their support of botanical research, on the occasion of what would have been Norman's 90th birthday (March 23, 2020).

Appendix A. Supplementary Data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.xyz.
Furthermore, the original NMR data (FIDs) are made freely available at https://dx.doi.org/10.7910/DVN/TL11QU (Harvard Dataverse).

Graphical Abstract



Keywords

Quantitative NMR; qHNMR; *Actaea racemosa* L.; *Actaea podocarpa* DC.; *A. cordifolia* DC.; Cycloartane triterpene

1. Introduction

Black cohosh (*Actaea racemosa* L., AR) is a popular dietary supplement, widely used for (peri-)menopausal women's health in the U.S. and Europe. Because plant sourcing continues to rely on natural harvest, adulteration of the plant material by related plant species is of concern. *A. podocarpa* DC. (AP), *A. cordifolia* DC. (AC) are related species, for example, that share the same habitat.

With triterpenes being the most abundant as well as the most well-studied class of constituents in *Actaea* plant materials, common commercial preparations of black cohosh involve powdered dry extracts, which are standardized for their content of triterpene glycosides by high-performance liquid chromatography-evaporative light-scattering detection (HPLC-ELSD); this is typically done with reference to one of the most abundant congeners, (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (s*yn.* 23-*epi*-26-deoxyactein, or sometimes 27-deoxyactein) (**1**) [1] [2]. For example, BNO 1055, a dry extract prepared with 58% ethanol, which is contained in Klimadynon® and Menofem® [3], purportedly contains 2.5% triterpene glycosides using this analytical method. In a clinical study for the management of vasomotor symptoms [4], tested AR extract was standardized to 7.27 mg of triterpenes per daily dose (two capsules) using a similar HPLC method. More precisely, 64 mg of AR 75% EtOH extract was analyzed by HPLC-ELSD and shown to contain 5.6% (3.64 mg) of major cycloartane spiroketal triterpene glycosides, such as 1.42 mg of **1**, 0.36 mg of (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 26-deoxyactein) (**2**), 1.38 mg of (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*S*)-26-hydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 26*S*-actein) (**3**), and 0.47 mg (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*R*)-26-hydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 26*R*-actein) (**4**). AR extracts

that were used in a pharmacokinetics study contained 7.0% of the major triterpenoids. More precisely, the extract contained 4.4% of **1**, 2.2% of **3**, and 0.4% of **4** [5][6]. As seen in the above examples, AR extracts contain 2.5 – 7.0% of the major triterpenes. However, standardization is made to a single or several triterpenes only and typically does not consider other triterpene compounds also present. In contrast, assessment of the total triterpene content in AR extract is not feasible when using classical LC-based standardization methods.

Reports describing the use of qHNMR for analysis of natural product have increased considerably in recent years [7][8]. Together with the growing number of chemical applications, more validation studies on the use of quantitative NMR (qNMR) and quantitative conditions including acquisition and processing parameters have been proposed [9][10]. There are three principal qHNMR methods that are commonly used with regard to their approach to quantitative reference calibration, *viz*; 1) internal calibration (IC), 2) external calibration (EC), and 3) a hybrid of both (ECIC) [11]. Each method has its own advantages. The major advantage of EC and ECIC methods includes not having to contaminate a precious sample by introduction of a calibrant. The objective of the present study is to investigate the application of qHNMR to the analysis of AR and related species / plant part extracts for the elucidation of the total content of the cycloartane triterpenes.

2. Experimental

2.1 Plant Material

Three North American *Actaea* species were used for this study: *A. racemosa* L. (AR), *A. podocarpa* DC. (AP) and *A. cordifolia* DC. (AC). Samples were separated into the aerial parts and roots/rhizomes and plant parts coded PX and RR, respectively. A total of 23 samples (ARPX 6, ARRR 6, APPX 3, APRR 3, ACPX 2, ACRR 3) were investigated. The collected samples were originally authenticated as *C. racemosa*, *C. americana*, and *C. rubifolia*, respectively. However, the revised naming system was used here because the genus *Cimicifuga* has since been reclassified to genus *Actaea* and many of the species names have changed [12]. These three American species were in part selected due to the high probability of finding them intermixed during the wild collection and because of their shared habitat and morphological similarities.

As shown in Table 1, samples denoted BC624, BC404, BC582, BC626, and BC581 were cultivated and collected from the Dorothy Bradley Atkins Medicinal Plant Garden at UIC. Sample BC629 was cultivated in Chillicothe, Ohio, and the specimen was authenticated and deposited in the Herbarium of the Field Museum of Natural History, Chicago, Illinois. Other American species were acquired through wild collections in Virginia, North Carolina, and Tennessee, and the voucher specimens are housed at the Searle Herbarium, Field Museum of Natural History and the Ramsey-Freer Herbarium, Lynchburg College (Lynchburg, Virginia).

2.2 Sample Preparation

The extract samples were prepared by taking a 10 mL aliquot of 70% aqueous MeOH, which was added to 1 g of dried, ground plant material and the mixture sonicated for 30 minutes. The extracts were left overnight at room temperature and then filtered. The residue plant material was similarly extracted and the two filtrates combined. The combined extracts were dried in forced air, to yield the crude extract for metabolomic analysis. A volume of 650 μL of DMSO-d_6 (Cambridge Isotopes, lot#8L-052, 99.9% D) was added to between 15 and 27 mg of dried extract in an Eppendorf vial, followed by sonication for 5 min. After sonication, the vial was centrifuged and 600 μL of supernatant were transferred to a 5 mm NMR tube, leaving any insoluble residue in the vial. Additionally, BC036 was also prepared in pyridine-d_5 in the same fashion for the purpose of identifying reference compounds. Following sample preparation, the residual extract in the Eppendorf vial was air dried (over a longer period of time, to evaporate DMSO) and then weighed to calculate the sample concentration in the NMR tube. Exact qHNMR sample concentrations were calculated based on using 600.0 μL of the 650 μL solutions.

2.3 qNMR Analysis

One dimensional ^{1}H and homonuclear 2D ^{1}H NMR experiments (gCOSY) were recorded on a Bruker Avance 600 NMR spectrometer equipped with a TXI cryogenic probe with the sample temperature maintained at 25.0 °C (298.0 K). Acquisition parameters were as follows: acquisition time (aq) = 4 s, relaxation delay (d1) = 60 s, pulse width (PI) = 3 us (30° flip angle), time domain data point digitization (TD) = 144k, number of scans (NS) = 64, and dummy scans (DS) = 4.

The NMR data were processed in TopSpin (versions 3.0 and 3.2; Bruker, Karlsruhe, Germany), NUTS (Professional version 20070706; Acorn NMR Inc., San Francisco, CA), and Mnova (version 10.0.2; Mestrelab Research S.L., Santiago de Compostela, Spain). All ^{1}H spectra were individually and identically processed with the following parameters: GM (LB −2.0 Hz, GB 0.1), zero filling to SI=512k and with application of automatic phasing, using Bruker Topspin prior to quantitation. The integral value was obtained following baseline correction with Mnova. Appropriate full auto baseline correction method was chosen within Whittaker smoothing, polynomial, ablative and cubic splines.

Quantitative qHNMR was employed to obtain the quantity of specific types of compounds in the crude extract by using the residual DMSO-d_5 resonance as an internal calibrant. To make the integrated intensity of a ^{1}H NMR signal accurately proportional to the number of observed nuclei, acquisition parameters were chosen appropriately. Accordingly, “quantitative experimental conditions” [7], including appropriate parameter selection such as relaxation delay and digitization, were carefully chosen for *Actaea* extract analysis. The calibration curve was generated as part of our work in the UIC/NIH Botanical Center [10], using dimethylsulfone ($DMSO_2$) as an external calibrant (EC). The residual DMSO-d_5 signal was then used as an internal calibrant (IC) for both the $DMSO_2$ EC samples as well as the analyzed extracts. To verify the calibration curve, a mixed sample of $DMSO_2$ and caffeine was used.

$$y = 0.0127x$$
$$R^2 = 0.9975$$

Where y is the mM concentration, and x is the ratio of the ^{1}H integral values of the analytes relative to that of the residual DMSO-d_5.

2.4 Reference compound identification

Two of the major *Actaea* triterpenes (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 23-*epi*-26-deoxyactein) (**1**) and (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*R*&*S*)-26-hydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (*syn.* 26*R*/*S*-actein) (**3** and **4**) were obtained from USP and their spectra in DMSO-d_6 were obtained on the same instrument (600 MHz) under the same conditions. Identification was performed by visual comparison of spectra of the crude extract and the reference [13][14].

3. Results and Discussion

3.1 General observations

The ^{1}H NMR spectra of *Actaea* extracts exhibit the presence of numerous constituents, such as cycloartane triterpenes, carbohydrates, phenolic compounds. These three major constituent classes are spectroscopically characterized in the following three regions of a typical ^{1}H NMR spectrum of *Actaea* extract (BC036) in Figure 1: (**A**) triterpene aglycone region between 0.0 to 2.2 ppm; (**B**) carbohydrate region between 3.0 to 4.0 ppm, (**C**) phenolic region between 6.0 to 8.0 ppm. Other samples exhibited similar profiles. In addition to these three regions, a broad hump in the spectral region between 4.0 and 5.0 ppm was observed in all spectra. This is likely to be associated with overlap of numerous dynamically exchanging hydroxy groups of the highly oxygenated *Actaea* compounds. Even though the extracts were carefully dried, the H_2O/HDO signal was observed as a broadened peak (exchange) overlapped with the carbohydrate signals appearing between 3.3 to 3.6 ppm to different degrees across the samples. The residual DMSO-d_5 peak was observed as a 1:2:3:2:1 quintet and the chemical shifts in the spectra were referenced to the center peak at δ = 2.500 ppm. Regardless of the species/plant parts, the most crowded region is B, followed by the A region. The C region tends to be the least crowded.

3.2 Quantitative Analysis of Cycloartane Triterpenes

The obvious major difficulty with which qHNMR has to contend in the analysis of natural products is the problem of overlapping resonances. In the case of purity assessment, which is one of the most important applications for qHNMR, the target NMR spectrum is relatively clean, and it is not difficult to differentiate signals of the target compounds and those of impurities. On the other hand, the application of qHNMR for the assessment of crude extracts/fractions is more challenging because of their chemically complex nature. However, conditions exist where direct quantitation of whole extracts is possible and include the presence of target signals that are cleanly separated from other signals. For example, Li et al. [15] reported the quantitation of camptothecin and trigonelline in a 1D ^{1}H NMR spectrum.

Quantitation in this instance was made possible because the characteristic peaks appeared within a 9.5– 5.5 ppm window, where the spectrum is not crowded. Chauthe et al. [16] demonstrated that the standardization and quantitation of medicinal plant constituents. For example, anthocyanins (delphinidin-3,5-diglucoside, petunidin-3,5-diglucoside and malvidin-3,5-diglucoside) from *Eugenia jambolana* fruit extract and imperatorin from *Aegle marmelos* fruit extract were selected as marker constituents. Olefinic or aromatic hydrogen signals of the above-mentioned compounds were used for quantitation. Other food applications have been examined and reviewed [17].

A. racemosa is known to contain a characteristic class of compounds, specifically the cycloartane triterpenes. The quantity of total cycloartane triterpenes can be calculated from the integral value of one of the cyclopropane hydrogen signals using qHNMR. Accumulation of the H-19 *exo* signals of various cycloartane triterpenes is observed in the spectral region of the samples between 0.20 to 0.45 ppm (Figure2). COSY cross peaks of all of signals between 0.20 to 0.45 ppm are coupled to signals between 0.46 to 0.62 ppm, which confirms that resonances between 0.20 and 0.45 ppm arise only from H-19 *exo*, and resonances between 0.46 to 0.62 ppm are only from H-19 endo hydrogens. Since no other correlations were observed in the COSY spectrum for signals < 0.62 ppm (note: H-7b resonances can appear at very high fields in pyridine-d_5, but not in DMSO-d_6) at the current signal-noise ratio, it was reasonable to assume that the total integral of this region represents only the triterpene C-19 hydrogens. The quantity of cycloartane triterpenes was calculated based on the integral value between 0.20 – 0.45 ppm as one hydrogen of a representative cycloartane, for example (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (**1**) ($C_{37}H_{56}O_{10}$ 660.83 g/mol), which has been used as a marker compound for AR in HPLC-ELSD method. By using the residual DMSO-d_5 signal as an internal calibrant (IC), the quantity of cycloartane triterpene aglycones was calculated as **1**. There are two advantages in this method over integrating a broader ppm range of the spectrum: First, as this spectral region (0.20 – 0.45 ppm) contains hydrogen resonances that are very specific to the cycloartanes and are isolated (out in the open), the chance of integrating unrelated signals is low. Secondly, because the ^{1}H resonances are isolated and this region is therefore surrounded by baseline, this makes the baseline correction operation easier and reduces baseline correction errors. Baseline correction was performed using the MNova NMR software using several methods including Whittaker smoother, polynomial, ablative, and cubic splines. Within these four methods, Whittaker smoothing and cubic splines reduced the baseline to zero when focusing on the spectral region that is relevant to the cycloartane C-19 hydrogens. For the DMSO-d_5 signal, there was no major difference observed in the four methods. The Whittaker-method was chosen for this study.

In addition to ARRR, other representative North American *Actaea* species/plant parts were quantitated for their cycloartane content in the 70% methanolic extracts (Table 2), including ARPX, APRR, APPX, ACRR, and ACPX.

As a result, ARPX and ARRR were found to contain 3.8 – 20.8% ± 8.2% and 7.2 – 19.3% ± 4.0%, respectively, of cycloartane triterpenes. Two of the ARPX samples were found to contain over 20% of cycloartane triterpenes. Further experiments might be necessary to

substantiate the particularly high values. In contrast, the remainder of the samples were found to contain between 3.8% and 5.9% cycloartane triterpenes. The two samples with the highest content do not have a significant common point, such as collection season or collection site. Sample collections were made in different locations and at different time periods throughout the growing season to specifically analyze for the degree of variation within the botanical. The cycloartane content in ACPX and ACRR was found to be 7.5 – 8.7% ± 0.8% and 13.9 – 28.5% ± 7.3%, respectively. In terms of cycloartane content, the difference between AR and AC was not able to be determined; however, AP showed a consistently lower number.

3.3 *Quantitative Results for* A. podocarpa

Within three North American species, AP exhibited different features. AP exhibited a lower content of cycloartane triterpenes as compared with AR and AC in the roots/rhizomes. The amount for APPX and APRR was 2.1 – 3.3% ± 0.7% and 1.1 – 4.0% ± 1.5%, respectively. The presence of cycloartane triterpenes in AP has previously been reported [18]. However, 13 representative AR cycloartane triterpenes, such as **1**, **3**, or **4** were not detected by HPLC–PDA/MS/ELS in a fingerprinting study that examined *Actaea* species as adulterants of black cohosh samples [19]. Other research [20] compared the phytochemistry of *A. racemosa*, *A. pachypoda*, *A. podocarpa*, and *A. rubra*. Seven representative AR triterpenes were not found in AP under the conditions of the present study. On the other hand, AP specific triterpenes that are not cycloartanes and lack the characteristic high-field H-19 resonances of the cycloartanes, such as podocarpaside J, podocarpaside H, podocarpaside I, and cimiside A were identified along with 9,10-*seco*-9,19-cyclolanostanes such as podocarpasides A-G. Several 9,10-*seco*-9,19-cyclolanostanes have also been reported from AP. The origin of this unique triterpene skeleton was suggested to be biosynthetically derived from a cycloartane triterpene precursor [21][22]. Thus, the present results suggested that AP contains a lower amount of AR specific cycloartane triterpenes and greater amount of the *seco*-cycloartanes.

AP is also referred to as *Cimicifuga americana* or yellow cohosh. It shares the same habitat as AR in the eastern United States. Moreover, the dry, dark rhizomes alone are difficult to distinguish outside of the laboratory. The differences found in triterpene content demonstrates that qHNMR can be used for avoiding misidentification of the two species, and hence accidental adulteration of “Black Cohosh”.

3.4 Reference Compound Identification

In addition to the quantitation of a particular compound class as a whole, individual reference compounds specific to *A. racemosa* could be identified in the hydrogen NMR spectra of the crude extracts. For this identification, the signals of seven skeletal methyl groups in *Actaea* triterpenes were used. These methyl groups serve as “surveillance units” for the neighboring segments of the molecules. The characteristic patterns (chemical shifts) for methyl group signals in *Actaea* are very sensitive to their chemical environment so that dereplication without reliance on reference data is possible only using classification binary trees [23].

In this study, two of the reference compounds, (12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (**1**) and (12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*R*/*S*)-26-hydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside (**3/4**), were identified in the ^{1}H NMR spectrum of the extracts by comparison with the data of reference material. Even though the NMR data of these compounds in DMSO-d_6 was not available from the literature, the signal assignments were performed using COSY, HMBC and HSQC correlations as well as the reported data for these compounds in pyridine-d_5 as a guide [13] [14]. The NMR data of **1** and **3/4** obtained in DMSO-d_6 are summarized in Table 3. When the ^{1}H NMR spectra of the two reference compounds were compared to a resolution enhanced spectrum of the extract (BC036), all of the seven methyl group signals could be identified and matched as shown in Figure 3.

The presence and positions of particular methyl signals is a strong indication of the existence of specific compounds as shown by Qiu et al.[23]. In addition to the ^{1}H spectral evidence, the COSY spectra were examined for compounds **1** and **3/4**. The spectra of the reference compounds were superimposed on that of BC036. As a result, all of the key cross peaks of compounds **1** and **3/4** were observed in the BC036 COSY spectrum. Specifically, correlations H-16/H-15a, H-16/H-15b, and H-16/H-17 were clearly observed in both cases. These are characteristic for actein types of side chains. Furthermore, the following COSY correlations were observed: H-12/H-11a COSY and the chemical of C-12 confirmed an OAc moiety at C-12; The H-3/H2a COSY correlation and the chemical shift of the anomeric C-1' confirmed the sugar position. The H-19 *exo/endo* COSY correlations confirmed the cycloartane moiety. Altogether, the presence of **1** and **3/4** in the BC036 extract was revealed as each correlation cross peak provides information about part of the structure. COSY cross peaks that did not match with the reference materials were cross peaks between hydroxyl groups and sugar hydrogens that were shown in a solid square in Figure 4. While hydroxyl groups can engage in hydrogen bonding with other components within the mixture and thus may exhibit slightly different behavior than in pure reference compounds, it is quite reasonable to assume that such cross peaks may not match up. Hence, from the ^{1}H, COSY and HMBC evidence, **1** and **3 & 4** were identified in the ARRR extract (BC036).

3.5 Solvent Effects

The extract, BC036, which showed a typical ARRR ^{1}H NMR pattern in DMSO-d_6, was dissolved in pyridine-d_5 and the 1D ^{1}H and 2D COSY NMR spectra were acquired at 600 MHz (Figure 4). The pyridine-d_5 extract sample was prepared in the same manner as the DMSO-d_6 sample. As previously noted, the ^{1}H spectrum of the DMSO-d_6 sample exhibited the characteristic A, B and C regions which covers the three major classes of structure types: triterpenes, carbohydrates, and phenolics. However, in the pyridine-d_5 spectrum of the same sample has triterpene and carbohydrate signals but only a trace of signals associated with the phenolic compounds. Moreover, the residual solvent peaks in pyridine-d_5 and their respective ^{13}C satellites appear in the same region as the phenolic peaks and partially overlap with them. A DMSO-d_6 solvent signals appears at 2.500 ppm in a less crowded spectral region. Hence the DMSO-d_6 is shown to be a more efficient solvent for the *Actaea* metabolomics studies of species for three reasons: (1) it dissolves the three known major

classes of *Actaea* compounds; (2) the residual solvent peak does not overlap significantly with peaks of interest in the spectrum; and (3) DMSO-d_6 is a near-universal solvent and possibly dissolves additional compounds.

Although the diagnostic signals of the triterpene skeleton appear in the same range (0.5 ppm – 2.5 ppm) of both DMSO-d_6 and pyridine-d_5 spectra, the carbohydrate signals are clearly affected by the solvent. The carbohydrate signals in pyridine-d_5 are shifted about 1.2 ppm downfield compared to their position in DMSO-d_6. The ^{1}H resonances of the carbohydrate fall in the range of 3.0 – 4.0 ppm in DMSO-d_6 vs 4.2 – 5.2 ppm in pyridine-d_5.

3.6 Summary

The ^{1}H NMR spectra of crude extracts from a total of 23 *Actaea* plant samples were obtained and analyzed for their species -and plant part- specific metabolomic characters by qHNMR. This study demonstrates that qHNMR is a powerful methodology for the analysis of crude plant extracts. Specifically, in *Actaea* species, the presence of the characteristic C-19 cyclopropane signals made it possible to quantify the cycloartane triterpene class as a whole and without the necessity for the identical reference standards. ARPX and ARRR contained 3.8 – 20.8% ± 8.2% and 7.2 – 19.3% ± 4.0%, respectively, of cycloartane triterpenes. The cycloartane content in ACPX and ACRR was 7.5 – 8.7% ± 0.8% and 13.9 – 28.5% ± 7.3%, respectively. AP contains notably lower amounts of cycloartane triterpenes as compared to AR and AC in the roots/rhizomes. The amount for APPX and APRR were 2.1 – 3.3% ± 0.7% and 1.1 – 4.0% ± 1.5%, respectively. In addition to the quantitative ability of ^{1}H NMR, the same spectrum was used for extracting information about the identification of marker compounds. The marker compounds **1** and **3/4** were identified in AR crude extracts by utilizing the seven methyl peaks in the 1D ^{1}H spectrum and 2D COSY.

The use of the residual hydrogen signal from the NMR solvent DMSO-d_6 was found to be suitable for the quantitation of cycloartane triterpenes of *Actaea* extract. While this report was being prepared as part of the dissemination of the 2012 PhD dissertation results of A. Imai [24], Çiçek et al. published a qHNMR study in which the total triterpene content of *A. racemosa* was assessed using methanol-d_4 solution and 1,2,4,5-tetrachloro-3-nitrobenzene as IC [25]. The choice of solvent is a critical parameter in qHNMR as it determines how existing qualitative, structural NMR data can be applied to quantitation. As building of a knowledge base of reliable chemical shift information is laborious, the ability to use historic NMR data is invaluable. Thus, from the perspective of available structural assignments, pyridine-d_5 is more suitable than DMSO-d_6 for future development of targeted quantitation of individual *Actaea* triterpenes in crude extracts, because the vast majority of published structural data are based on NMR measurements performed in pyridine-d_5. In particular, this knowledge base allows for the mining of Me group chemical shift data for structural barcode patters, as has been shown previously [26]. Future development of targeted qHNMR-based, metabolomic standardization methods will require the translation of the ^{1}H NMR profiles of known triterpenes from pyridine-d_5 to DMSO-d_6, or their ab initio establishment. Alternatively, external calibration (EC) [27] combined with use of existing pyridine-d_5 reference data could be a viable path.

Finally, potentially limiting factors associated with this study should also be mentioned. The plant samples had been collected some time before analysis and stored under ambient conditions. According to our observations working with Actaea botanicals, however, all indications are that the triterpenes are stable, especially in intact plant material. Furthermore, the available number of distinct samples for each species have limited the statistical significance of the species and plant part quantitative triterpene content values. However, the study demonstrated that quantitation of crude black cohosh materials can be achieved by qHNMR without authentic reference materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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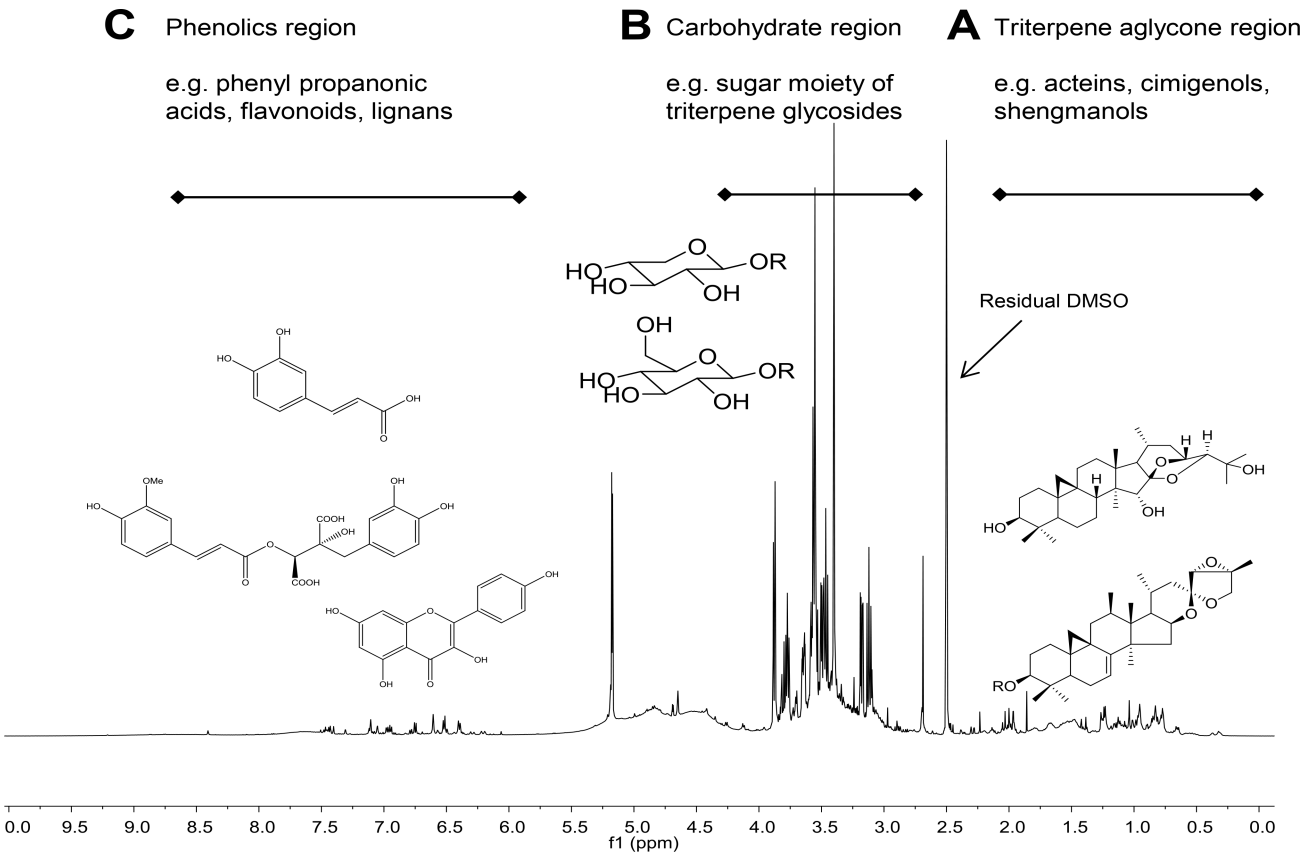

Fig. 1.

A typical 600 MHz ^{1}H spectrum of a 70% aqueous MeOH extract of ARRR; 12.60 mg of extract (BC036) in 600 μL of DMSO-d_6. Chemical shifts were calibrated to 2.500 ppm for the residual DMSO-d_5 signal.

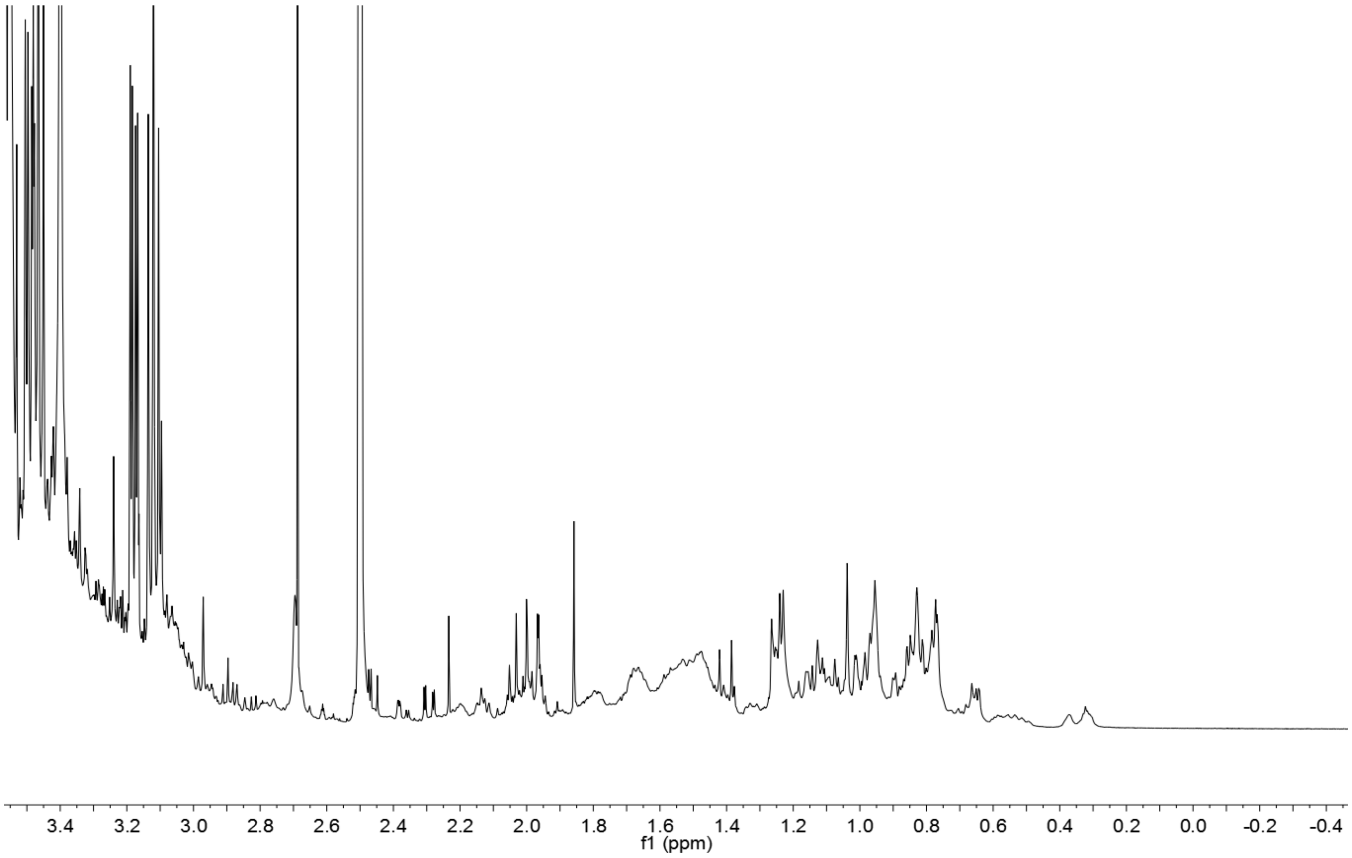

Fig. 2.

The structure of a typical cycloartane triterpene and its H-19 *exo* signal on ^{1}H spectrum of a 70% aqueous MeOH extract of ARRR. Signals between 0.20 – 0.45 ppm arise from the H-19 *exo* hydrogens as confirmed by 2D COSY.

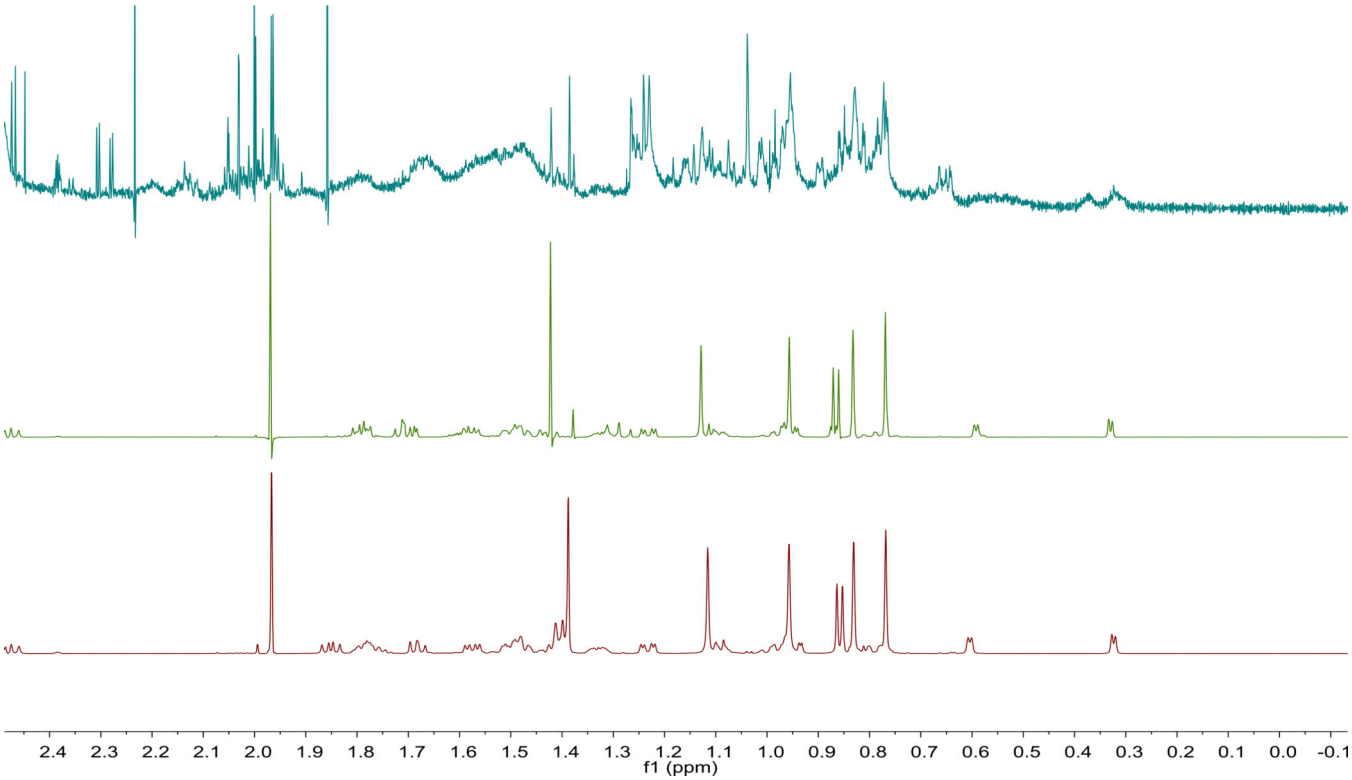

Fig. 3.

Identification of 1 and 2 by ^{1}H NMR (600 MHz) as Two of the Major constituents of ARRR Extract (BC036). Processing was performed as follows: BC036, GM, LB −3.0 Hz, GB 0.1; compounds **1** and **2**, GM, LB −1.0 Hz, GB 0.1.

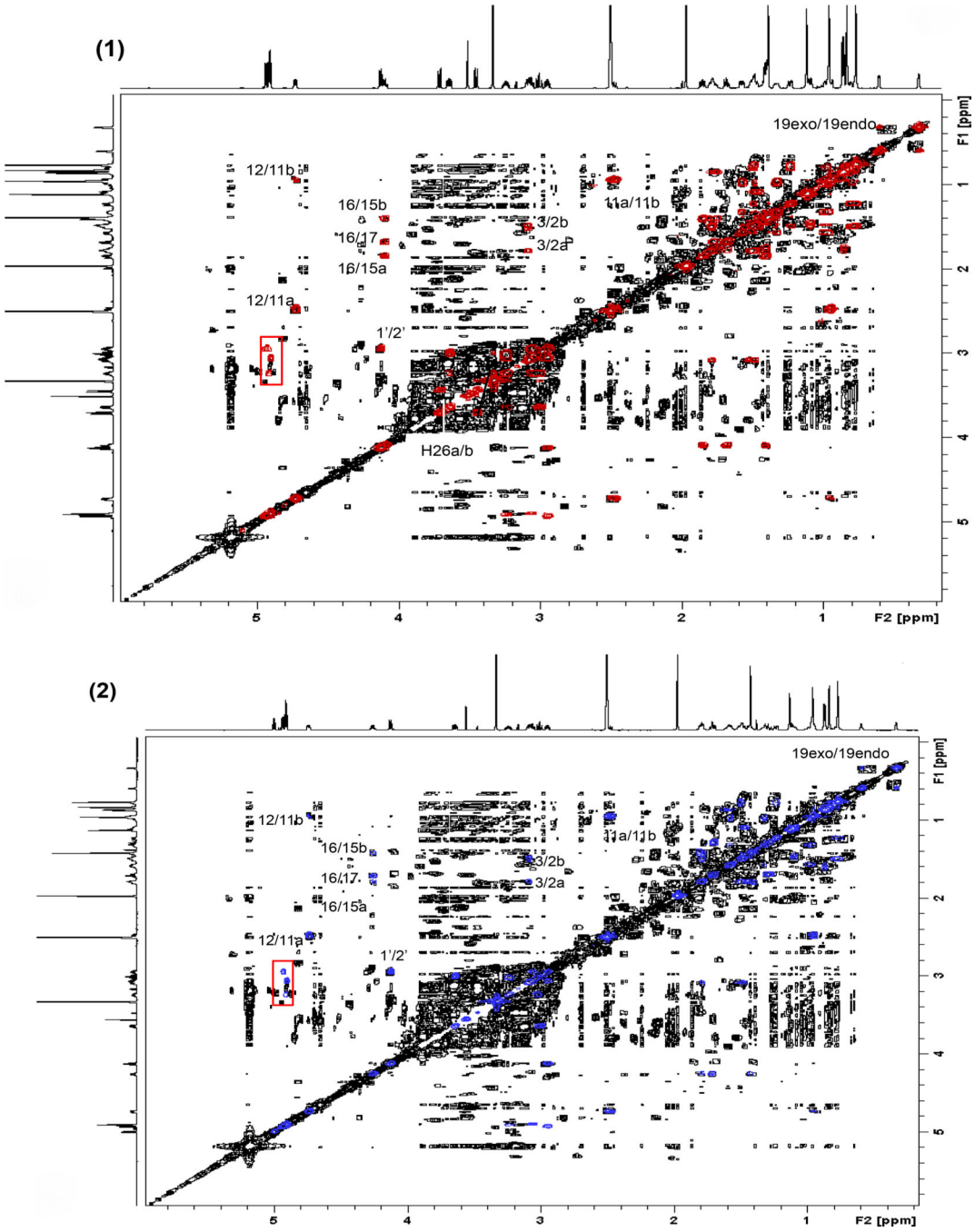

Fig. 4.

Identification of **1** and **2** in ARRR extracts (BC036). The 600 MHz COSY spectra of compounds **1** and **2** are each superimposed with the ARRR COSY spectrum, respectively. External projections are the ^{1}H spectra of each reference compound. Cross peaks in the solid rectangle are OH-CH couplings.

Table 1.

List of plant material used for this study.

Genus	Species	Plant Parts [a]	BC number [b]	Collection date	Collection Site
Actaea	*racemosa*	PX	BC026	6/29/1999	Sevier County, TN. Elevation 3500 ft.
Actaea	*racemosa*	PX	BC027	6/29/1999	Sevier County, TN. Elevation 3300
Actaea	*racemosa*	PX	BC624	6/16/2009	Atkins Garden, Cook County, IL
Actaea	*racemosa*	PX	BC404	10/1/2008	Atkins Garden, Cook County, IL
Actaea	*racemosa*	PX	BC582	10/7/2009	Atkins Garden, Cook County, IL
Actaea	*racemosa*	PX	BC025	6/29/1999	Rockbridge County, VA. Elevation 2481 ft.
Actaea	*racemosa*	RR	BC036	10/16/1999	Elk township, Chester County, PA
Actaea	*racemosa*	RR	BC007	6/30/1999	Sevier County, TN. Elevation 3300 ft.
Actaea	*racemosa*	RR	BC094	11/7/2000	Forbes State Forest, Somerset County. PA
Actaea	*racemosa*	RR	BC629	6/3/2010	Chillicothe, Ross County, OH
Actaea	*racemosa*	RR	BC626	6/16/2009	Atkins Garden, Cook County, IL
Actaea	*racemosa*	RR	BC581	10/6/2009	Atkins Garden, Cook County, IL
Actaea	*podocarpa*	PX	BC029	6/30/1999	Sevier County, TN. Elevation 3300 ft.
Actaea	*podocarpa*	PX	BC020	8/26/1999	Swain County, NC. Elevation 2875 ft.
Actaea	*podocarpa*	PX	BC028	6/28/1999	Rockbridge County, VA. Elevation 3150 ft.
Actaea	*podocarpa*	RR	BC006	6/30/1999	Sevier County, TN. Elevation 3300 ft.
Actaea	*podocarpa*	RR	BC008	6/30/1999	Swain County, NC. Elevation 2875 ft.
Actaea	*podocarpa*	RR	BC011	8/26/1999	Sevier County, TN. Elevation 3300 ft.
Actaea	*cordifolia*	PX	BC022	8/27/1999	Scott County, VA, Elevation 1800 ft.
Actaea	*cordifolia*	PX	BC023	6/29/1999	Scott County, VA. Elevation 1800 ft.
Actaea	*cordifolia*	RR	BC015	8/27/1999	Scott County, VA, Elevation 1800 ft.
Actaea	*cordifolia*	RR	BC003	6/29/1999	Scott County, VA. Elevation 1800 ft.
Actaea	*cordifolia*	RR	BC016	8/27/1999	Scott County, VA, Elevation 1800 ft.

[a] Plant Parts codes are as follows: PX aerial parts, RR roots/rhizomes.

[b] The BC number is a sole number assigned to each collected plant part at the University of Illinois at Chicago Botanical Center.

Table 2.

Calculated triterpene content in 70% methanolic extract of *Actaea* species.

Plant code	BC Number	Sample weight [mg]	Integral Value [a]	Cycloartane Triterpene Weight [mg]	Cycloartane Triterpene Content	Content Range ± St. Dev.
ARPX	BC026	17.66	3.3%	1.03	5.9%	
ARPX	BC027	18.36	3.3%	1.03	5.6%	
ARPX	BC624	25.74	3.2%	0.99	3.8%	3.8 – 20.8%
ARPX	BC404	17.74	2.5%	0.77	4.3%	± 8.2%
ARPX	BC582	8.48	5.6%	1.75	20.7%	
ARPX	BC025	18.67	12.5%	3.89	20.8%	
ARRR	BC626	18.46	6.4%	1.98	10.7%	
ARRR	BC036	12.60	4.3%	1.34	10.7%	
ARRR	BC007	16.04	9.9%	3.10	19.3%	7.2 – 19.3%
ARRR	BC094	11.01	2.5%	0.79	7.2%	± 4.0%
ARRR	BC629	16.31	6.9%	1.99	13.2%	
ARRR	BC581	14.85	5.3%	1.66	11.2%	
APPX	BC029	14.72	1.5%	0.47	3.2%	
APPX	BC020	14.10	0.9%	0.29	2.1%	2.1 – 3.3%
APPX	BC028	10.21	1.1%	0.32	3.3%	± 0.7%
APRR	BC006	20.44	0.7%	0.22	1.1%	
APRR	BC008	19.78	2.5%	0.78	4.0%	1.1 – 4.0%
APRR	BC011	18.97	2.1%	0.65	3.4%	± 1.5%
ACPX	BC022	12.99	3.6%	1.13	8.7%	7.5 – 8.7%
ACPX	BC023	15.37	3.7%	1.15	7.5%	± 0.8%
ACRR	BC015	16.78	15.3%	4.78	28.5%	
ACRR	BC003	19.44	8.7%	2.71	13.9%	13.9 – 28.5%
ACRR	BC016	18.28	15.2%	4.73	26.0%	± 7.3%

[a]Integral value was obtained relative to that of DMSO-*d5*; calculated for 600 μL solvent volume and a cycloartane triterpene molecular weight of 660.8 amu.

Table 3.

The ^{1}H and ^{13}C NMR assignments of **1** and **3/4** in DMSO-d_6.

position	Compound 1			Compounds 3/4		
	δ_C [a]	δ_H	J_{H-H} (Hz); multiplicity [b]	δ_C [a]	δ_H	J_{H-H} (Hz); multiplicity [b]
1	30.8	1.091	overlapped	30.8	1.105	overlapped
		1.472	overlapped		1.493	overlapped
2	28.6	1.493	overlapped	28.6	1.508	overlapped
		1.782	overlapped		1.796	overlapped
3	87.3	3.087	overlapped	87.3	3.086	overlapped
4	40.9	-		40.9	-	
5	46.2	1.233	4.0, 12.1; dd	46.2	1.232	4.0, 12.1; dd
6	19.4	0.789	overlapped	19.4	0.780	overlapped
		1.494	overlapped		1.508	overlapped
7	24.8	0.983	overlapped	24.8	0.988	overlapped
		1.331	m		1.332	m
8	45.2	1.575	5.2, 12.1; dd	45.2	1.598	5.3, 12.1; dd
9	19.5	-		19.5	-	
10	25.9	-		25.9	-	
11	35.6	0.946	overlapped	35.6	0.958	overlapped
		2.481	overlapped		2.481	overlapped
12	75.3	4.725	3.4, 8.8; dd,	76.5	4.737	3.4, 8.8; dd
13	47.7	-		47.7	-	
14	47.2	-		47.7	-	
15	43.0	1.407	overlapped	43.0	1.423	overlapped
		1.851	7.5, 13.2; dd		1.786	7.6, 12.8; dd
16	73.6	4.097	7.5, 14.7; dd	71.9	4.257	7.1, 14.6; q
17	55.2	1.683	8.9; t	55.2	1.711	overlapped
18	12.2	1.116	s	13,4	1.128	s
19	28.8	0.323	4.2; d	29.5	0.330	4.0, d
		0.604	4.2; d		0.593	4.0, d
20	21.9	1.762	m	20.4	1.593	m
21	20.1	0.858	6.5; d	21.1	0.865	6.3; d
22	36.1	1.410	overlapped	37.3	1.290	13.5; t
		1.410	overlapped		1.697	overlapped
23	105.2	-		104.7	-	
24	60.8	3.509	s	61.8	3.556	s
25	61.9	-		64.8	-	
26	67.1	3.449	10.0; d	96.5	4.996	5.7, d
		3.710	10.0; d			
27	13.3	1.388	s	12.9	1.423	s
28	18.8	0.831	s	18.8	0.833	s
29	24.5	0.957	s	24.5	0.958	s

	Compound 1			Compounds 3/4		
position	**δ_C** [a]	**δ_H**	**J_{H-H} (Hz); multiplicity** [b]	**δ_C** [a]	**δ_H**	**J_{H-H} (Hz); multiplicity** [b]
30	14.2	0.768	s	14.2	0.770	s
1’	106.7	4.125	7.8; d	106.7	4.125	7.8; d
2’	74.0	2.946	m	74.0	2.946	m
3’	77.0	3.062	overlapped	77.0	3.060	overlapped
4’	69.8	3.240	m	69.8	3.239	m
5’	65.7	3.005	10.6; t	65.7	3.004	10.6; t
		3.640	5.5, 11.5; d,		3.640	5.5, 11.5; dd
Oac	21.0/170.4	1.967	s	21.0/170.4	1.969	s

[a] The ^{13}C chemical shifts were extracted from the HSQC and HMBC data.

[b] Multiplicities: d doublet; dd double doublet; m multiplet; t triplet.