# Chlorination Diversifies *Cimicifuga racemosa* Triterpene Glycosides

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Extracts from the roots and rhizomes of black cohosh (*Cimicifuga racemosa*) are widely used as dietary supplements to alleviate menopausal symptoms. State-of-the-art quality control measures involve phytochemical fingerprinting of the triterpene glycosides for species identification and chemical standardization by HPLC. In the course of developing materials and methods for standardization procedures, the major *C. racemosa* triterpene glycoside (**1**) was isolated and initially thought to be cimicifugoside (**2**). Detailed HR-LC-MS and 1D and 2D NMR analysis of **1** and **2** unambiguously revealed that **1** is the chlorine-containing derivative of **2**, namely, 25-chlorodeoxycimigenol-3-*O*-*β*-D-xyloside. Accordingly, HPLC profiles of black cohosh preparations require revision of the assignments of the chlorinated (**1**) and nonchlorinated (**2**) pair. Besides explaining the substantial shift in polarity ($\Delta t_R$[RP-18] ca. 20 min), 25-deoxychlorination opens a new pathway of structural diversification in triterpene glycoside chemistry. As chemical conversion of **2** into **1** could be demonstrated, deoxychlorination may be interpreted as artifact formation. Simultaneously, however, it is a potentially significant pathway for the gastric *in vivo* conversion ("nature's prodrug") of the relatively polar triterpene glycosides into significantly less polar chlorinated derivatives with altered pharmacological properties.

*Cimicifuga racemosa* (L.) Nutt. (synonym *Actaea racemosa* L.) (Ranunculaceae), commonly referred to as black cohosh, is native to North America and has had a history of use by Native American Indians.[1] The roots/rhizomes have traditionally been used to treat a variety of medical conditions including colds, kidney ailments, malaise, rheumatism, and women's conditions such as uterine disorders and menstrual complaints.[2] From the results of a recent clinical trial,[3] a water−ethanol extract was demonstrated to relieve climacteric symptoms of menopause, such as hot flashes. No toxicity was observed even at high doses (127 mg/day, recommended daily dose: 40−80 mg/day).[4] Further exemplary cases of recent studies regarding clinical efficacy and safety of black cohosh are documented in the literature.[5−10] While virtually none of the *C. racemosa* preparations used in any reported clinical study have been chemically *and* biologically standardized, the dual standardization using both phytochemical and biological parameters has been a core aspect of the recent botanical research in the UIC/NIH Botanical Center.

For many years, investigations of *C. racemosa* have led to the isolation of phytochemicals that constitute, or are part of, the underlying bioactive principles of the plant.[11−13] In a quantitative context, however, and as typically observed with botanical dietary supplements, the constituents characterized thus far do not account for all of the biological activity observed and relative to the biological end points of similar clinical trials. Accordingly, there is continued demand for the identification of new marker compounds for chemical standardization of black cohosh.

In addition, because *C. racemosa* has been associated with more than 20 common names, this botanical has often been confused with other *Cimicifuga* species, e.g., the Asian varieties such as Rhizoma Cimicifugae from *C. foetida* L., or *C. dahurica* Maxim, which is typically marketed in China, as well as rhizomes and roots of *C. simplex* Warm., which is typically marketed in Japan. There is no recent literature indicating that Asian *Cimicifuga* rhizomes (Sheng Ma) have been used to treat women's disorders during menopause, although there have been earlier reports relative to the treatment of uterine disorders and menorrhagia.[14] In addition to a significant number of case reports from Europe, Australia, Canada, and the United States, which often lack verification of botanical identity of *C. racemosa*, two adverse medical events that recently cited the use of black cohosh as inducing hepatitis or liver damage were reported from both Australia[15] and the United States (black cohosh tablets, unknown brand or dose).[16] Although there is ongoing discussion as to whether or not black cohosh was the common causative agent noted in these two reports, there was no analytical documentation that could unequivocally identify the consumed supplement as black cohosh. HPLC analysis of black cohosh exhibits a distinct fingerprint, characteristic of its chemical constituency with the major constituents being the 9,19-cyclolanostane triterpene glycosides present in all *Cimicifuga* species. The triterpene glycoside HPLC fingerprint is useful for differentiating *C. racemosa* (black cohosh) from the Asian *Cimicifuga* species and other related plants possessing the same common name.[17]

As representative of one of the plants presently under investigation in the UIC/NIH Botanical Center, the phytochemical study of *C. racemosa* has previously led to the identification of a number of new triterpene glycosides with the total assignment of 26 triterpene glycoside structures.[18,19] In the process of standardizing the root extracts, using a previously established HPLC method,[20] a high abundance peak was observed ($t_R$ = 42 min) (Figure 1) that had not been previously identified. Isolation of the compound underlying this chromatographic peak and preliminary survey spectroscopic analysis (NMR, 1D $^1H/^{13}C$) of the isolate suggested that it possessed nearly identical spectroscopic data to that obtained for cimicifugoside, i.e., cimigenol-3-*O*-*β*-D-xyloside (**2**). The chromatographic peak originally assigned to **2**, however, was reported to have a considerably longer retention time ($t_R$ = 62 min, S1).[20] As a result of this observation, it was clear that an ambiguity existed with respect to the assignment of the respective chromatographic peaks, which prompted a more detailed NMR and LC-MS investigation (see Supporting Information for the HMBC spectra of **1** [S2] and **2** [S3]). The results revealed that the unassigned HPLC peak ($t_R$ = 42 min) is in fact the known triterpene glycoside **2** and that the later eluting peak ($t_R$ = 62 min), which had originally been

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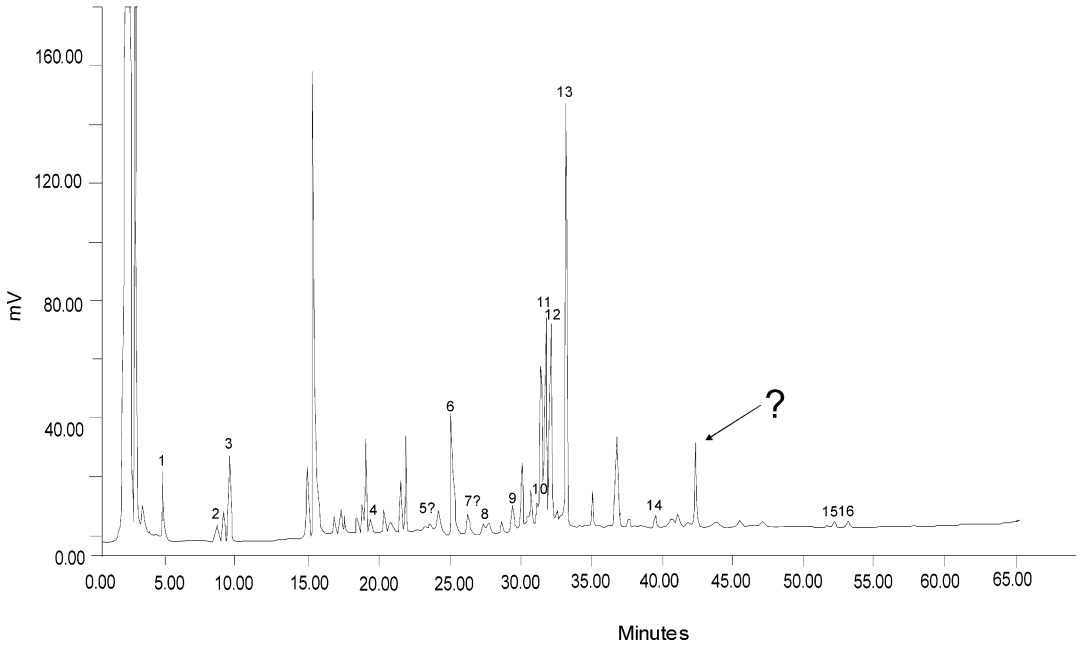
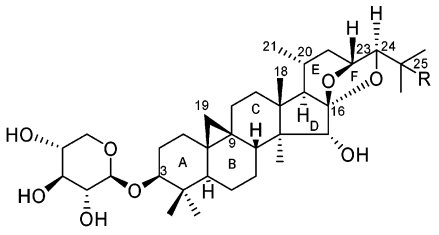



**Figure 1.** HPLC profile of a hydroalcoholic *C. racemosa* extract with ELSD detection using a previously reported method (briefly: RP-18 [YMC ODS-AQ, 5 um, 120 Å, 4.6 mm × 250 mm] with 0.05% aqueous TFA−MeCN gradient).[20] Of the several unassigned chromatographic peaks, the arrow indicates a major constituent ("?") that was originally isolated as being new, but was later identified as the known **2**. The latter had previously been assigned to a peak eluting at 62 min (see HPLC of reference standards in Supporting Information, S1), which as a result of this study corresponds to the new chlorinated analogue **1**.

assigned structure **2**, is in fact the chlorinated triterpene glycoside, chlorodeoxycimigenol-3-*O*-*β*-D-xyloside, a new chemical entity possessing structure **1**. While **1** and **2** are structurally almost indistinguishable, they have distinctly different HPLC retention times with a $\Delta t_R$ of 20 min. Consistent with replacement of the C-25 hydroxyl substituent with a chlorine atom, the $^1$H and $^{13}$C data for the two triterpene glycosides are *almost indistinguishable*, even for $^1$H data recorded at 900 MHz (Supporting Information S4 and S5). Herein, we describe the details of the isolation and structure elucidation of **1**, report on the chemical conversion of triterpenes into chlorinated analogues, and discuss the broader implications of this exchange with regard to chemical diversification and biological impact.




**1** R = Cl Chlorodeoxycimigenol-3-O-β-D-xyloside
**2** R = OH Cimigenol-3-O-β-D-xyloside

## Results and Discussion

Pure 25-chlorodeoxycimigenol-3-*O*-*β*-D-xyloside (**1**) was obtained from the MeOH extract of roots/rhizomes of *C. racemosa* as a pale yellow powder after repeated chromatography on silica gel followed by reversed-phase silica gel LPLC (RP-18, Lobar). During positive ion electrospray, **1** exhibited a protonated molecule of *m*/*z* 639.3666 and the typical isotope pattern of chlorinated compounds, consistent with the molecular formula $C_{35}H_{55}O_8Cl$ (calcd 639.3658; 1.3 ppm error). Compound **1** was clearly recognized as a highly oxygenated 9,19-cycloartane triterpene monoglycoside from its proton NMR data (Table 1). The characteristic high-field methylene signals of **1** appearing at $\delta$ 0.297 and 0.536 (each 1H, d, $J$ = 4.2 Hz) were diagnostic for the presence of the cyclopropane ring present in the 9,19-cyclolanostane triterpene ring skeleton. The presence of seven methyl groups was indicated from the $^1$H NMR data (pyridine-$d_5$) appearing at $\delta_H$ 0.867 (d, $J$ = 6.3 Hz, Me-21), 1.077 (s), 1.141 (s), 1.189 (s), 1.336 (s), 1.710 (s), 1.720 (s). A single *β*-anomeric glycoside proton resonance was observed at $\delta_H$ 4.889 (d, $J$ = 7.6 Hz) and confirmed the presence of a single carbohydrate moiety. The $^{13}$C and DEPT-135 NMR data (Table 2) for **1** showed signals that were ascribed to four oxygen-bearing methine carbons appearing at $\delta_C$ 89.44 (C-24), 88.28 (C-3), 80.25 (C-15), and 72.48 (C-23) and quaternary carbon resonances appearing at $\delta_C$ 113.16 (C-16) and 71.56 (C-25) corresponding to the spiro-ketal carbon and chlorine-bearing carbons, respectively. The spectra for **1** also exhibited signals consistent with the presence of five oxygenated carbon signals associated with the glycoside residue, and the $^{13}$C chemical shifts were found to be identical to those reported for the *β*-D-xylopyranose moiety [$\delta_C$ 107.67 (C-1′), 75.62 (C-2′), 78.68 (C-3′), 71.26 (C-4′), 67.17 (C-5′)] found in 26-deoxyactein and 23-*epi*-26-deoxyactein; their structures have been previously confirmed by single-crystal X-ray analysis.[18]

Detailed analysis of the $^1$H, $^{13}$C, HMQC, HMBC, and COSY spectra for **1** established the structural elements for rings A to D. The partial structure, −CHCH($CH_3$)$CH_2$CH− (for H-17, H-20, to H-23), present in both **1** and **2** displayed a clearly observable spin pattern network in the COSY spectrum. The HMBC spectrum, in addition to revealing the long-range correlation information associated with rings A to D, showed $^3J$ correlations between the methine carbon signal at $\delta_C$ 59.48 (C-17) and the protons of the two methyl signals at $\delta_H$ 0.867 (Me-21) and 1.141 (Me-18). The pair of methyl groups at $\delta_H$ 1.710 and 1.720 (Me-26, and Me-27) showed $^2J$ correlations to the quaternary chlorine-bearing carbon C-25 ($\delta_C$ 71.56) and a $^3J$ correlation to the C-24 methine carbon ($\delta_C$ 89.44), which is consistent with the acyclic partial structure present in both **1** and **2**. Finally, both rings E and F were established on the basis of HMBC correlations observed between the quaternary ketal carbon C-16 ($\delta_C$ 113.16) and H-23 ($\delta_H$ 4.699), as well as with H-24 ($\delta_H$ 3.885). This confirmed a prior suspicion that compound **1** must be structurally very similar to the known **2**.[21]

The only significant differences that were observed in the proton NMR spectra of **1** and **2** were associated with the chemical shifts

**Table 1.** $^1$H NMR Data of Compounds **1** and **2**

| proton | **1**[a] | **1**[b] | **2**[a] | **2**[b] |
|---|---|---|---|---|
| 1 | 1.24 m | 1.248 (3.5, 4.3, 13.3) | 1.24 | 1.250 (3.5, 4.3, 13.3) |
| | 1.61 m | 1.613 (1.3, 4.0, 12.9, 13.3) | 1.62 | 1.616 (4.0, 12.9, 13.3) |
| 2 | 1.981 (4.2, 11.8, 12.0, 13.0) | 1.975 (4.3, 12.9, 11.7, 12.9) | 1.983 (4.0, 12.5, 16.5) | 1.977 (4.3, 12.9, 11.7, 12.9) |
| | 2.392 (3.0, 3.5, 4.5, 13.0) | 2.382 (3.5, 4.0, 4.5, 12.9) | 2.395 (4.0, 7.8, 12.5) | 2.385 (3.5, 4.0, 4.5, 12.9) |
| 3 | 3.536 (4.5, 11.8) | 3.530 (4.5, 11.7) | 3.539 (4.3, 11.8) | 3.530 (4.5, 11.7) |
| 5 | 1.341 (4.5, 11.2) | 1.348 (4.5, 12.4) | 1.347 (4.3, 13.2) | 1.352 (4.5, 12.4) |
| 6 | 0.731 (1.7, 12.6, 12.3, 12.3) | 0.735 (2.5, 12.4, 12.7, 12.7) | 0.729 (2.0, 13.0, 14.2) | 0.729 (2.0, 13.0, 14.2) |
| | 1.58 m | 1.546 (2.5, 2.5, 4.5, 12.7) | 1.50 | 1.537 m |
| 7 | 1.17 m | 1.177 (2.5, 12.5, 12.5, 12.7) | 1.12 | 1.179 (2.5, 12.5, 12.5, 12.7) |
| | 2.14 m | 2.125 (2.5, 4.5, 4.5, 12.7) | 2.09 | 2.090 overlapped |
| 8 | 1.681 (4.2, 11.8) | 1.698 (4.5, 12.5) | 1.687 (4.4, 12.5) | 1.707 (4.5, 12.5) |
| 11 | 1.07 m | 1.085 (3.9, 10.7, 13.8) | 1.10 | 1.088 (3.9, 10.7, 13.8) |
| | 2.12 m | 2.090 (5.5, 10.7, 15.2) | 2.13 | 2.090 overlapped |
| 12 | 1.67 m, | 1.554 (5.5, 11.0, 13.8) | 1.52, 1.70 | 1.562 m |
| | 1.52 m | 1.675 (0.8, 3.9, 11.0, 15.2) | | 1.675 overlapped |
| 15 | 4.263 (9.4) | 4.257 (9.4) | 4.281 (8.9) | 4.276 (9.0) |
| OH-15 | 4.755 (9.4) | 4.703 (9.4) | 4.488 (8.9) | 4.460 (9.0) |
| 17 | 1.464 (11.0), | 1.466 (11.3) | 1.513 (10.8) | 1.518 (10.9) |
| 18 | 1.141 s | 1.141 (0.8) | 1.161 s | 1.163 (0.8) |
| 19 | 0.297 (4.2) | 0.299 (4.2) | 0.294 (4.2) | 0.299 (4.2) |
| | 0.536 (4.2) | 0.536 (1.8, 4.2) | 0.538 (4.2) | 0.536 (1.8, 4.2) |
| 20 | 1.65 m | 1.642 (6.7, 6.9, 11.1, 11.3) | 1.65 m | 1.675 overlapped |
| 21 | 0.867 (6.3) | 0.869 (6.7) | 0.863 (6.5) | 0.866 (6.5) |
| 22 | 1.011 (1.7, 11.2,13.2) | 1.010 (2.0, 11.3, 13.3) | 1.039 (1.8, 11.4, 13.2) | 1.035 (2.1, 11.9, 13.3) |
| | 2.268 (6.9, 9.5, 13.2) | 2.268 (6.9, 9.4, 13.3) | 2.283 (6.7, 9.7, 13.2) | 2.281 (6.9, 9.4, 13.3) |
| 23 | 4.699 (0.9, 1.8, 9.5) | 4.693 (0.8, 2.0, 9.4) | 4.780 (brd 8.5) | 4.727 (1.1, 1.7, 9.5) |
| 24 | 3.885 (0.9) | 3.880 (0.9, 0.9) | 3.788 brs | 4.785 (1.0, 1.3) |
| 26 | 1.710 s | 1.697 s | 1.488 s | 1.480 s |
| 27 | 1.720 s | 1.714 s | 1.509 s | 1.504 s |
| 28 | 1.189 s | 1.188 s | 1.197 s | 1.197 s |
| 29 | 1.336 s | 1.330 s | 1.335 s | 1.329 s |
| 30 | 1.077 s | 1.073 s | 1.077 s | 1.074 s |
| 1′ | 4.889 (7.6) | 4.881 (7.5) | 4.891 (7.6) | 4.881 (7.5) |
| 2′ | 4.061 (7.6, 8.6) | 4.054 (7.5, 8.9) | 4.061 (7.6, 8.5) | 4.052 (7.5, 8.9) |
| 3′ | 4.187 (8.6, 8.9) | 4.248 (8.7, 8.9) | 4.185 (8.5, 8.7) | 4.175 (8.7, 8.9) |
| 4′ | 4.260 (5.0, 8.9, 10) | 4.248 (5.2, 8.7, 10.0) | 4.260 (5.1, 8.7, 10.2) | 4.246 (5.2, 8.7, 10.0) |
| 5′ | 3.758 (10.0, 11.2, b) | 3.751 (10.0, 11.5, b) | 3.760 (10.2, 11.3, b) | 3.752 (10.0, 11.5, b) |
| | 4.384 (5.0, 11.2, a) | 4.375 (5.2, 11.5, a) | 4.386 (5.0, 11.3, a) | 4.376 (5.2, 11.5, a) |

[a] Recorded at 360 MHz in pyridine-$d_5$. [b] Recorded at 900 MHz in pyridine-$d_5$.

of H-24 ($\delta_H$ 3.788 in **2**), which appeared to be shifted downfield ($\Delta\delta$ = 0.107 ppm) as compared to **1**; H-23 ($\delta_H$ 4.780 in **2**), which appeared shifted upfield ($\Delta\delta$ = 0.081 ppm) in **1** (Figure 2); and the two methyl signals for Me-26 ($\delta_H$ 1.509 in **2**) and Me-27 ($\delta_H$ 1.488 in **2**), which appeared shifted downfield to $\delta_H$ 1.720 and 1.710 ($\Delta\delta$ = 0.211 and 0.222 ppm) in **1** (Figure 3), respectively. Comparative analysis of the $^{13}$C NMR spectra of **1** and **2** demonstrated the fact that most of the carbon resonances for rings A−E were also essentially identical. In the $^{13}$C domain, the only signals exhibiting a significant but still small deviation in chemical shift values were C-16 and C-22 to C-27 (Table 2). The strongest substituent chemical shifts (SCSs) of $^{13}$C NMR signals were observed for the ketal carbon C-16 [$\delta_C$ 112.00 (s)] in **2**, which is subject to a slight downfield shift ($\Delta\delta$ = 1.16 ppm) to $\delta_C$ 113.16 (s) as compared to **1**. At the same time, the signals due to C-26 and C-27 [$\delta_C$ 27.25 q and 25.41 q] in **2** were also shifted downfield as compared to **1** ($\Delta\delta$ = 2.95 and 0.95 ppm, respectively). The proton signals for Me-26 and Me-27 were shown to correlate (HMBC) with the carbon signals for C-26 and C-27 in **2**. A strong $^3J$ HMBC correlation was observed between H-24 and C-16 in **1** and parallels a similar observation present in the HMBC spectrum of **2**. Evidence for homonuclear correlation between H-24 and Me-27 in **1** was observed in the COSY data as well. Proton H-24 in **1** showed spin−spin coupling with only *one* of the methyls, i.e., Me-27, through four single bonds (W or zigzag effect).[22]

Two-dimensional NOESY experiments, carried out at 500 MHz, provided information regarding the relative stereochemistry of **1** versus **2** (Table 3). In addition to the NOEs expected for and associated with rings A to D of the 9,19-cyclolanostane basic skeleton, NOEs were observed between H-24 and protons H-22α and Me-26 in **1**, which is consistent with the assignment of H-24 to an α-orientation. Another key NOE was observed between H-23 and Me-27 in **1**, which served to confirm the stereochemistry of H-23 as possessing a *β*-orientation. In **2**, NOEs were observed from H-23 to H-22*β* and Me-26 and from H-24 to H-22α and Me-26, thus confirming the partial structure C-25 to C-27 as not being constrained in a ring, but rather reflecting an acyclic substructural element in both **1** and **2**. The observed NOEs for the side-chain protons in **1** and **2** are essentially identical. The structural relationship of protons H-23 and H-24 places the dihedral angle at or near 90°, and thus, coupling between these two protons is expected to be zero. No coupling between H-23 and H-24 is observed in either **1** or **2**. The "extra" NOE between H-23 and H-22α in **2**, but not observed in the $^1$H NMR spectrum of **1**, may reflect a subtle difference in the average side-chain conformational populations of **1** versus **2**. All spectroscopic evidence for compound **1** is consistent with the structure of 25-chloro-15*β*-hydroxyl-16*β*,-23*β*:16α,25-diepoxy-24*β*-hydroxyl-9,19-cyclolanostane-3-*O*-*β*-D-xylopyranoside, which represents the first chlorine-containing triterpene glycoside isolated from *C. racemosa*. In analogy with the nomenclature of halogenated sugars, the aglycone of **1** is named 25-chlorodeoxycimigenol, assigning **1** to 25-chlorodeoxycimigenol-3-*O*-*β*-D-xylopyranoside.

The $^1$H and $^{13}$C NMR data for **1** and **2** were practically identical at the field strengths that are typically used for routine analysis of natural products (300−500 MHz $^1$H, data not shown) and are often employed for preliminary examination of isolates. Only the $^1$H NMR data subsequently recorded at 900 MHz NMR allowed for the resolution of important details and led to the unambiguous assignment of all $^1$H resonances. The proton NMR data obtained

**Table 2.** $^{13}C$ NMR Data[a] of Compounds **1** and **2** and Calculated $^{13}C$ Chemical Shift Differences Indicating the Substituent Chemical Shift Effect of Deoxychlorination

| carbon | **1** | **2** | $\Delta\delta$[**1**−**2**][b] |
|---|---|---|---|
| 1 | 32.46 t | 32.44 t | Ø |
| 2 | 30.02 t | 30.20 t | Ø |
| 3 | 88.28 d | 88.58 d | Ø |
| 4 | 41.39 s | 41.38 s | Ø |
| 5 | 47.58 d | 47.61 d | Ø |
| 6 | 20.08 t | 21.07 t | Ø |
| 7 | 26.33 t | 26.37 t | Ø |
| 8 | 48.67 d | 48.65 d | Ø |
| 9 | 20.00 s | 20.03 s | Ø |
| 10 | 26.66 s | 26.67 s | Ø |
| 11 | 26.44 t | 26.47 t | Ø |
| 12 | 34.00 t | 34.08 t | Ø |
| 13 | 41.72 s | 41.86 s | Ø |
| 14 | 47.16 s | 47.30 s | Ø |
| 15 | 80.25 d | 80.24 d | Ø |
| 16 | 113.16 s | 112.00 s | +1.16 |
| 17 | 59.48 d | 59.58 d | Ø |
| 18 | 19.52 q | 19.53 q | Ø |
| 19 | 30.92 t | 30.90 t | Ø |
| 20 | 23.96 d | 24.12 d | Ø |
| 21 | 19.48 q | 19.60 q | Ø |
| 22 | 37.49 t | 38.15 t | −0.66 |
| 23 | 72.48 d | 71.83 d | +0.65 |
| 24 | 89.44 d | 90.20 d | −0.76 |
| 25 | 71.56 s | 70.97 s | +0.59 |
| 26 | 30.20 q | 27.25 q | +2.95 |
| 27 | 26.36 q | 25.41 q | +0.95 |
| 28 | 11.81 q | 11.84 q | Ø |
| 29 | 15.47 q | 15.47 q | Ø |
| 30 | 25.74 q | 25.75 q | Ø |
| 1′ | 107.67 d | 107.67 d | Ø |
| 2′ | 75.62 d | 75.62 d | Ø |
| 3′ | 78.68 d | 78.68 d | Ø |
| 4′ | 71.26 d | 71.27 d | Ø |
| 5′ | 67.17 t | 67.18 t | Ø |

[a] At 90 MHz in pyridine-$d_5$. [b] $\Delta\delta = \delta\mathbf{1} - \delta\mathbf{2}$ in ppm; Ø indicates $|\Delta\delta| < 0.30$ ppm.

at both 360 and 900 are summarized in Table 1, and a detailed comparison of various regions of the 900 MHz $^1H$ NMR spectra of **1** and **2** is shown in Figure 2 and Figure 3 to illustrate the subtleties of the differences and clarify the signal assignments. Although much attention must be paid to minute detail, even when performing $^1H$ NMR analysis at high magnetic field strengths, the distinction between **1** and **2** *can* in principle be made by means of NMR. However, the contribution of MS in resolving what otherwise represented a structural ambiguity needs to be emphasized, with the recognition of the chlorine isotope pattern being a key aspect. At the same time, it must be recognized that, especially for polyhydroxylated natural products (glycosides), and depending on ionization conditions and other MS parameters, frequently observed dehydrated species ([M* − *n*·18] ions) may provide a challenge to spectrum interpretation. Considering the 17 amu mass difference between a chlorodeoxy derivative and its parent compound, the recognition of a chlorine substitution can escape routine examination, especially in view of the very close similarity of their NMR spectra.

The occurrence of the chlorinated triterpene **1** not only expands the structural diversity of 9,19-cycloartane-type triterpenes, but also provides valuable information pertaining to the relationship of chromatographic trends and the structure of chemically related triterpene glycosides. During the analysis of triterpene glycosides present in black cohosh by HPLC-ELSD and LC-MS, it was observed that, for a pair of epimers only involving one stereocenter, the difference in the retention times was 7.3 min.[18] In contrast, the HPLC profile of the extract of black cohosh shown in Figure 1 displays a considerably larger difference in the retention times between compounds **1** and **2** ($\Delta t_R \approx 20$ min). However, in this instance, the compounds are *not* isomeric but differ because of the replacement of the 25-OH found in **2** with a chlorine atom in **1**. Clearly, introducing significant changes in the substructural elements by atom replacement serves to alter the inter- and intramolecular hydrogen-bonding networks associated with **1** versus **2** (also refer to discussion of SCSs of exchangeable proton signals in the caption of Figure 2). The chromatographic trends associated with **1** and **2**, as well as their congeners, may serve as useful indicators to assist in subsequent structural characterizations.

A question immediately arises as to whether **1** occurs naturally or whether its presence is an artifact of the isolation protocol. During the isolation procedure (see Experimental Section), the initial methanol extract was partitioned using $CHCl_3$ and *n*-BuOH. Chloroform, depending on purity, age of the solvent, and length of time exposed to light, is known to break down to phosgene and HCl. The HCl could react with the hydroxyl function at C-25 (a quaternary carbon center) and replace the 25-OH with 25-Cl. Experiments were performed to answer this question. First LC-MS was run on a crude botanical extract of black cohosh, prior to the $CHCl_3$−*n*-BuOH partition, to look for **1**. None could be detected. Second, a series of experiments was conducted in which a dilute chloroform solution of **2** was allowed to come in contact with HCl vapor for a period of time. The result was that **2** chemically converted into **1**, suggesting that the origin of **1** may indeed be, at least in part, an isolation artifact. Since naturally occurring chlorinated compounds derived from plants are rare, in this instance, experimental verification of its possible source was clearly justified. In an effort to simulate the interaction of **2** with chloroform, which is known to contain varying levels of HCl from decomposition, chemical conversion studies were carried out with more concentrated $CHCl_3$ solutions containing HCl gas. The reactions were monitored by LC-MS. As a result, it was shown that **2** was readily but not quantitatively converted into **1**.

The occurrence of organohalogen natural products in terrestrial plants, marine organisms, and animals is typically underrated.[23,24] In addition to organochlorines, organobromines have been detected across all phyla, including plants.[25,26] Specific biosynthetic pathways exist for enzymatic halogenation through haloperoxidases (e.g., haloperoxidases [CPO] and bromoperoxidases [BPO]),[27,28] $FADH_2$-dependent halogenases, *S*-adenosylmethionine fluorinating enzymes, and non-heme $Fe^{2+}$, α-ketoglutarate, and $O_2$-dependent halogenases.[29−32] More recently, biogenetic conversion by diatomaceous microorganisms has been shown to generate halogenated medium-chain hydrocarbons from $C_{20}$ fatty acids.[33] These specific pathways add to the possibly nonspecific or spontaneous formation of halohydrins from epoxide precursors. It should be noted that numerous *Cimicifuga* triterpenes contain the epoxide motif and that the halide/peroxidase/hydrogen peroxide chemical system is well established in mammals.[34]

Regardless of whether it represents an acid-catalyzed "artifact" formation or an enzyme-controlled reaction, the demonstration of the likelihood of a straightforward chemical exchange of a tertiary OH for a Cl atom opens a new pathway of structural diversification in triterpene glycoside chemistry. Moreover, deoxychlorination converts a relatively *polar* triterpene alcohol (**2**) into a much more *lipophilic* chlorohydrin derivative (**1**). It might be reasonable to expect that deoxychlorination is a universal reaction and can produce new chemical entities that are equally complex assortments as their precursor triterpenes. Given the reactivity of tertiary alcohols, the numerous *Cimicifuga* triterpenes with an oxirane partial structure, e.g., the whole acetein series, are even more likely to undergo reactions that eventually yield chlorohydrin derivatives. Although different in basic skeleton, the very rare finding of the chlorinated cycloartane-type glycoside **1** shares an interesting relationship with the chlorinate oleanane-type triterpene aglycone known from *Mentha villosa*.[35]

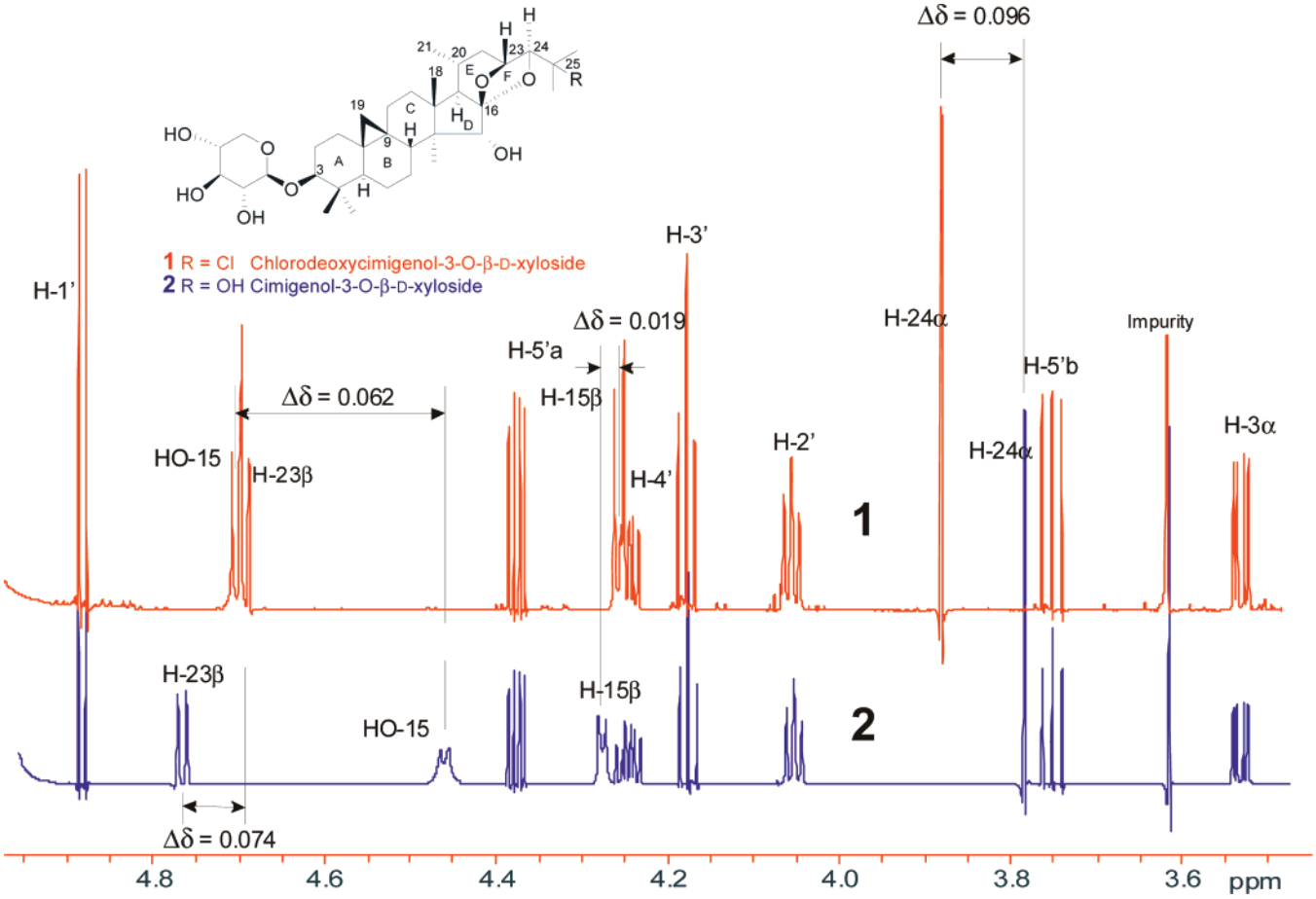



**Figure 2.** Comparison of the downfield signal pattern (3.45−4.95 ppm) of the 900 MHz $^1$H NMR spectra of the new chlorodeoxycimigenol-3-*O*-*β*-D-xyloside (**1**, above/red) and cimicifugoside (**2**, below/blue, both recorded in pyridine-$d_5$). In contrast to the minor chemical shift differences of the spiroketal protons of H-23 and H-24, only the exchangeable OH-15 proton showed a prominent change of chemical shift, most likely due to a change in intramolecular hydrogen bonding. Accordingly, extreme care needs to be taken when comparing $^1$H NMR spectra of triterpene glycosides, especially when analyzing closely related structures and "isomers" and when working in protic solvents, because the signals of the significant exchangeable protons are lost under these conditions. The fact that solvent-induced shifts and concentration effects may even exceed the $^1$H-induced substituent chemical shift (SCS) differences observed between chlorodeoxy and hydroxy compounds and/or potential other (stereo)isomers further complicates the unambiguous structural identification of triterpene glycosides.

As conversion of **2** into **1** was experimentally demonstrated to occur *in vitro*, deoxychlorination may at first sight be interpreted as artifact formation. At the same time, however, it represents a potentially significant pathway for the gastric (pH 2 HCl) *in vivo* conversion of the triterpene glycosides to afford pharmacologically enhanced agents. Another plausible spontaneous pathway leading to the formation of organochlorine compounds has been reported for withanolides, in which the ubiquitous NaCl serves as the halogen source.[36] Under all of these considerations, when orally administered, the relatively polar glycosides would actually represent "nature's prodrugs" that are bioconverted into significantly more lipophilic chlorinated derivatives. These bioconversion products can be expected to possess altered pharmacodynamic and, almost certainly, improved pharmacokinetic properties, particularly with regard to their otherwise poor oral absorption. Therefore, the question of whether chlorohydrin (ketal derivative) artifact formation in *C. racemosa* and related botanical preparations is desirable, or undesirable, is only one facet of the interpretation of this finding: The concept of the potential gastric *in situ* bioconversion (bioactivation?) of a large variety of *Cimicifuga* triterpenes may in fact decrease the need for stringent control of the "genuine" triterpene spectrum.

An aspect with potentially much broader impact is the new chemical space opened by deoxychlorination of the structural class of triterpenes and related terpenes. Exploration of this space is in need of further study and a prerequisite for determining the *biological* impact of deoxychlorination *in vitro* and *in vivo*. The fact that (bioconverted) chlorohydrin derivatives can potentially be (part of the) active principle(s) underlines the need for *parallel* chemical and biological standardization of dietary supplements and evaluation of other natural products, respectively. At the same time, considering this form of bioconversion, it must be borne in mind that correlations between phytochemical profiles and *in vitro* or clinical biological end points might be less straightforward than they appear. While this report establishes basic evidence for this relationship in black cohosh preparations, it is reasonable to predict that similar mechanisms apply to other natural products containing a similar (tri)terpene profile. Finally, it is perceivable that, besides chlorination of hydroxylated triterpenes, highly functionalized and hydroxylated natural products present in almost all dietary supplements have a similar potential for *in vivo* bioconversion when taken orally.

While it remains to be demonstrated to what extent chlorodeoxygenation might even be a universal mechanism that equally affects biologically active and (genuinely) inactive natural products, the phenomenon of chlorodeoxygenation deserves added attention from all relevant biological perspectives (*in vitro*, QSAR, *in vivo*, clinical). Regarding *C. racemosa*, we are currently pursuing the possibility of identifying other chlorinated derivatives of the

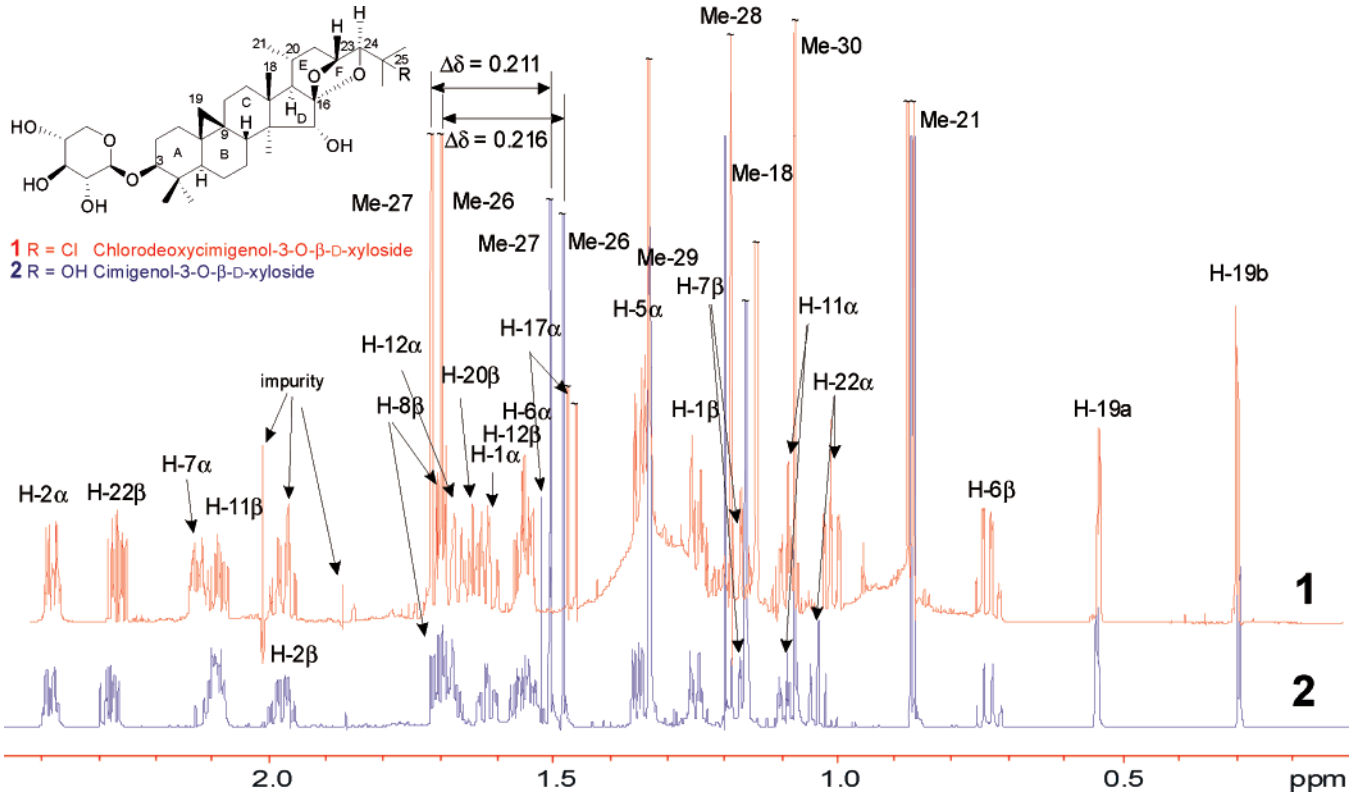



**Figure 3.** Close similarities are also observed in the upper field signal pattern (0.20−2.60 ppm) of the 900 MHz $^1$H NMR spectra of chlorodeoxycimigenol-3-*O*-*β*-D-xyloside (**1**) (above/red) and cimicifugoside (**2**) (below/blue; both in pyrindine-$d_5$). Interestingly, the methylene and methine protons of the rings A, B, and C remain largely unaffected by the alteration of the isopropyl substituent attached to C-24 of the five-membered ketal (ring F). On the other hand, there are small but defined changes in the chemical shift of the methyl groups (Me-26 and Me-27). This feature exemplifies the importance of standardized NMR conditions, in particular solvent choice, when identifying/elucidating the structure of *Cimicifuga* triterpene glycosides.

**Table 3.** Summary of Major NOEs[a] Observed for **1** and **2**

| | NOE effects | |
|---|---|---|
| proton | **1** | **2** |
| H-2*β* | Me-30 (1.077) | Me-30 (1.077) |
| 15 | Me-18 (1.141) | H-8 (1.687), Me-18 (1.161) |
| 17 | Me-21 (0.867), Me-28 (1.189) | no NOE was observed |
| 18 | H-8*β* (1.861), H-12*β* (1.52) | H-8*β* (1.687) |
| 21 | H-22α (1.011) | H-20 (1.65) |
| 23 | Me-27 (1.720), H-22*β* (2.268), H-22α (1.011 w) | H-22*β* (2.268), Me-26 (1.710) |
| 24 | H-22α (1.011), Me-26 (1.710) | H-22α (1.011), Me-26 (1.710) |
| 28 | H-11α (1.07), H-7α (2.14), H-17α (1.464) | H-11α (1.10) |
| 30 | H-19b (0.297), Me-29 (1.336) | H-19b (0.294) |

[a] Obtained from 2D NOESY at 500 MHz in pyridine-$d_5$, mixing time 250 ms; $\delta$ of NOE enhanced signals in ppm.

cimigenol and perhaps other (epoxy) series of triterpene glycosides and of further characterizing the conditions of such bioconversion processes.

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Fisher-Johns melting point apparatus. Optical rotations were obtained with a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Inc., MA). $^1$H NMR spectra were measured at 360, 500, and 900 MHz and $^{13}$C NMR spectra were measured at 90 MHz on Bruker Avance spectrometers (Bruker, Zurich, Switzerland) in pyridine-$d_5$ as the solvent. The chemical shifts were referenced to tetramethylsilane (TMS) using the signals from the solvent ($\delta_H$ 8.737, $\delta_C$ 149.911 relative to TMS) as internal standard. NMR data were processed with NUTS (Pro version for Microsoft Windows, Acorn NMR Co., Livermore, CA) and MestRe-C (version 3.6.3 for Microsoft Windows, MestRe-C Co., Spain). $^1$H NMR spectra were acquired with 64 K data points and zero-filled to 256 K data points after Fourier transformation. HR-ESI data were recorded on a Micromass (Manchester, UK) Q-TOF-2 system. Infrared spectra were recorded on an ATI Mattson Genesis Series FTIR (ATI Mattson Instruments Inc., WI) by using a liquid layer on NaCl. Thin-layer chromatography was performed on precoated TLC plates (250 $\mu$m thickness, K6F Si gel 60 and K6F RP-18 Si gel 60, EM Science, Germany) with compounds visualized by spraying the dried plates with 5% $H_2SO_4$ in EtOH followed by heating until dryness. Semipreparative HPLC was carried out on a Waters Delta 600 system with a Waters 996 photodiode array detector, Waters 717 plus autosampler, and Millennium$^{32}$ chromatography manager (Waters Corp., Milford, MA) on a GROM-Sil 120 ODS-4 HE (Watrex-International, Inc., NY) semipreparative column (5 $\mu$m, 300 × 20 mm) with a flow rate of 6 mL/min. Reversed-phase column chromatography was carried out on Merck Lobar LiChroprep RP-18 columns (EM Science, Germany) with a fluid pump (FMI, Fluid Metering, Inc., Syossest, NY) and fraction collector (Spectrum, Spectrum Chromatography, Houston, TX). Silica gel (230−400 mesh, Davisil, W.R. Grace & Co., Columbia, MD) was used for column chromatography.

**Plant Material.** *Cimicifuga racemosa* (L.) Nutt. roots/rhizomes were collected in Rockbridge County, Virginia (June 1999), GPS coordinates

37 48.27 N × 79 18.67 W, and identified by Dr. G. Ramsey, Department of Biology, Lynchburg College, Lynchburg, VA. Voucher specimens have been deposited at the Ramsey-Freer Herbarium at Lynchburg College, Lynchburg, VA, and at the Field Museum of Natural History Herbarium, Chicago, IL.

**Extraction and Isolation.** The dried, milled roots/rhizomes of *C. racemosa* (6 kg) were extracted with MeOH and fractionated by successive partitions with $CHCl_3$ and *n*-BuOH. The $CHCl_3$-soluble fraction (160 g) was chromatographed on a silica gel (1.8 kg) column and eluted with $CHCl_3$−MeOH (20:1, 500 mL), $CHCl_3$−MeOH (10:1, 8 × 500 mL), $CHCl_3$−MeOH (2:1, 3 × 500 mL), $CHCl_3$−MeOH (1:1, 6 × 1500 mL), and MeOH (3 × 1500 mL) to give a total of 22 fractions. Fractions 21−22 (34.19 g) were further fractionated by normal-phase silica gel column chromatography (330 g) eluting with a gradient $CHCl_3$−MeOH (10:1 in increasing polarity to 0:1) solvent system to afford six subfractions (SF-I to SF-VI). The fraction SF-I (3.47 g) was subjected to a RP-18 open column eluted with MeOH−$H_2O$ (2:1, 2 × 250 mL), MeOH−$H_2O$ (4:1, 3 × 250 mL), and MeOH to yield the fractions SF-I-A to SF-I-F. After combining fractions SF-II and SF-I-B (2.40 g), the material was further fractionated on a LiChroprep RP-18 column (Lobar, 310 × 25 mm, E. Merck) and finally purified by a semipreparative HPLC column (gradient, from 50% to 80% MeCN in water in 30 min) to yield pure **1** (5.4 mg).

For the isolation and characterization of cimicifugoside (**2**), see previous report[19] and S5 for $^1$H and S3 for HMBC NMR spectra.

**25-Chlorodeoxycimigenol-3-*O*-$\beta$-D-xylopyranoside (1):** pale yellow powder, mp 241−243 °C, $[\alpha]^{25}_D$ +4.8 (*c* 0.003, $CHCl_3$); $^1$H and $^{13}$C NMR see Table 1 and Table 2, respectively, as well as S4 for $^1$H and S2 for HMBC NMR spectra; IR max ($cm^{-1}$) 3425, 2935, 2869, 1376, 1233, 1042, 760; HRESIMS *m*/*z* 639.3666 (calcd 639.3658 for $C_{35}H_{56}O_8Cl$, $[M + H]^+$).

**Chlorination of 2.** The following experiments were conducted in order to attempt to "simulate" approximate isolation conditions under which the formation of **1** might have occurred. Two 10 $\mu$L samples of **2** were removed from a pyridine-$d_5$ NMR solution (conc 3.3 mg/mL) and transferred into two 1 mL vials, respectively. After evaporating the pyridine under vacuum, to one vial was added 0.5 mL of distilled $CHCl_3$, and to the other vial was added 0.35 mL of $CHCl_3$, 0.15 mL of HPLC-grade acetone, and 0.1 g of Si gel. These two samples were stored on a lab bench at ambient temperature for 3 weeks. The solvent and Si gel were removed and the samples were analyzed by LC-MS. The chlorodeoxy compound **1** could not be detected in either of the two samples.

Two 60 $\mu$L samples of **2**, obtained from a pyridine-$d_5$ NMR solution (see above), were placed in two 1 mL vials, respectively. After removing the pyridine under vacuum, to one vial was added 0.5 mL of distilled $CHCl_3$, and to the other vial was added 0.40 mL of $CHCl_3$, 0.10 mL of HPLC-grade acetone, and 0.1 g of Si gel. The $CHCl_3$ was pretreated with the gaseous vapor from 37% hydrochloric acid by removing 5 × 1 mL of HCl gas vapor from the surface of the HCl solution with a disposable glass Pasteur pipet, bubbling the gas into 2 mL of freshly distilled $CHCl_3$. Both vials were kept on a lab bench at ambient temperature for 1 week. After removal of the solvent and the Si gel, the samples were analyzed by LC-MS. The chlorodeoxy compound **1** was detected in both samples. This clearly indicates that small amounts of HCl present in the chloroform solution can lead to 25-deoxychlorination of **2** to form **1**.

**Repetition of the Isolation Procedure.** A 50 g sample of *C. racemosa* (L.) Nutt. roots/rhizomes (identical to raw material previously used) was ground and exhaustively extracted with MeOH (4000 mL total). The MeOH extract was divided into two portions, and each evaporated to afford 4.38 and 4.53 g portions of crude extract. The extract portions were dissolved in 90% MeOH and partitioned with EtOAc (4 × 100 mL). The EtOAc-soluble fractions yielded 1.48 and 1.53 g after removal of the EtOAc. The 1.53 g EtOAc portion was redissolved in MeOH, mixed with Si gel (100−230 mesh, 2 g), and loaded on a vacuum column of Si gel (46 g). Elution with 600 mL of $CHCl_3$, $CHCl_3$−acetone (10%), $CHCl_3$−acetone (20%), $CHCl_3$−acetone (30%), $CHCl_3$−MeOH (10%), $CHCl_3$−MeOH (50%), and MeOH, provided nine fractions based on TLC monitoring: F1 ($CHCl_3$), F2 ($CHCl_3$ + 10% acetone), F3 (400 mL of $CHCl_3$ + 20% acetone), F4 (200 mL of $CHCl_3$ + 20% acetone and 300 mL of $CHCl_3$ + 30% acetone), F5 (300 mL of $CHCl_3$ + 30% acetone and 100 mL of 10% MeOH), F6 (150 mL of 10% MeOH), F7 (400 mL of 10% MeOH), F8 (600 mL of 50% MeOH), and F9 (600 mL of MeOH). Fractions F2 to F6, the EtOAc-soluble fraction, and the crude MeOH extract were analyzed by LC-MS. The chlorodeoxy compound **1** could not be detected in the MeOH extract nor in the EtOAc fraction, while a small amount (near the LOD) of **1** was detected in fraction F3 by LC-MS.

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**Supporting Information Available:** This material is available free of charge via the Internet at http://pubs.acs.org and includes an HPLC chromatogram of *C. racemosa* reference compounds as well as $^1$H and 2D HMBC spectra of **1** and **2**.

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