Stereochemistry

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Stereochemistry Determination by Powder X-Ray Diffraction Analysis and NMR Spectroscopy Residual Dipolar Couplings**

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Traditional techniques for stereochemistry analysis have limitations; for example solution NMR spectroscopy has spatial limitation for the transference of stereochemical information, and suitable single crystals for X-ray analysis may not be available. Residual dipolar coupling (RDC) and powder X-ray diffraction (PXRD) are both techniques whose use is not yet widespread. We report a double-blind solution of the structure of jaborosalactol 24 (**1**), a new withanolide isolated from *Jaborosa parviflora* (Phil.) A. T. Hunziker et Barboza, by RDCs and PXRD, since conventional NMR spectroscopy experiments such as nuclear Overhauser effect (NOE) and ^3J analysis did not provide a unique solution.

NOE,[1] frequently combined with ^3J coupling constants analysis,[2] is one of the most powerful tools for structural and conformational analysis by solution NMR spectroscopy. Unfortunately, the determination of relative stereochemistry between remotely located stereocenters becomes problematic arising from the limitations imposed by the $1/r^6$ dependence of NOE (where r = internuclear distance). However, when molecules are forced to adopt a minor degree of alignment in solution and no longer tumble isotropically, a measurable fraction of the dipolar coupling (0.01–0.1 %) can be observed in the NMR spectrum. These so called residual dipolar couplings[3–7] (RDCs) contain important structural information of non-local character, since their values depend not only on the internuclear distances but also on the angles between the internuclear vectors and the external magnetic field. Hence, they provide information about the relative orientation between the internuclear vectors (e.g., H-H, H-C, H-N, C-C, etc.), regardless of the distance between them. Since the pioneering work by Courtieu and co-workers on the determination of relative configuration by RDCs using poly-γ-benzyl-L-glutamate (PBLG)/$CDCl_3$,[8,9] more alignment media compatible with organic solvents are now available.[10–22] In natural products in particular, the power of RDCs for the resolution of configurational problems has been tested against known molecules such as menthol,[23] cyclosporin,[15] ludartin,[22] strychnine,[21,24] sphaeropsidin A,[11] sagittamide A,[25] archazolid A,[26] sodium cholate,[27] and even used, in the case of the novel glycoside sucro-neo-lambertellin[28] for the determination of the unknown configuration in several stereocenters. Another case is the configuration of a synthetic α-methylene-γ-butyrolactone that could not be resolved by conventional means.[29] At present, PXRD analysis can be used to determine the crystal structures of small organic molecules.[30] One fruitful technique is real-space simulated annealing, in which trial structures are generated and optimized using a set of structural parameters that define the location, orientation, and conformation of the molecules in the solid, consistent with bond lengths and angles from the known (or hypothesized) molecular structure.

RDCs and PXRD can only determine relative stereochemistry, and the absolute configuration of a given molecule can be determined only if at least the absolute configuration of one chiral center is known.

The withanolides[31] are a group of naturally occurring C_{28} steroids which are built on an ergostane skeleton functional-

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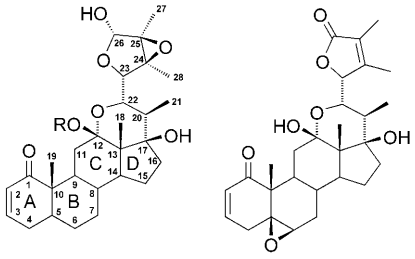
ized at carbons 1, 22, and 26, and have a high occurrence in genera of the Solanacea family. Six new withanolides (**1**–**6**) of the trechonolide type were isolated from *Jaborosa parviflora*.



1 5β,6β-epoxy; R=H
2 5β,6β-epoxy; R=Ethyl
3 5α-Cl,6β-OH; R=H
4 5α-Cl,6β-OH; R=Ethyl
5 5α-Cl,6β-OH-2,3-dihydro; R=H
6 5α,6β-diOH; R=H

7

The ^{1}H and ^{13}C NMR spectra of **1**–**6** were closely related, showing patterns typical of the hemiketal or ketal ring system and a γ-lactol ring. Compounds **1**–**6** showed almost identical ^{13}C NMR spectroscopic data for rings C, D, and the side chain, indicating that their structural differences were restricted to the substitution pattern in rings A and B. For structures such as **7**, the absolute configuration at C23 can be straightforwardly determined by circular dichroism (CD).[32] However, because of the lack of the γ-lactone carbonyl group in **1**–**6**, it is no longer possible to use CD for this purpose. In addition, there are three more chiral carbon centers with unknown configuration (C24, C25, and C26). Apart from the *R* or *S* configuration at C23 and C26, there are only two possible orientations for the epoxide ring at C24,C25, leading to a total of eight configurations in the side chain, named *RRRR*, *RRRS*, *SRRS*, *SRRR*, *RSSR*, *RSSS*, *SSSR*, and *SSSS* after the configurations at carbons C23, C24, C25, and C26, respectively. The free rotation around the C22–C23 bond leads to three possible rotamers per configuration, increasing the number of possible structures to 24. This set of 24 structures can be divided into three subsets based on the dihedral angle between H22 and H23. There are two subsets of *gauche* rotamers named G− for a negative dihedral of the H22-C22-C23-H23 torsion and G+ for a positive dihedral angle. The third subset corresponds to the *anti* relationship between H22 and H23. For example, the three rotamers of the 23*R*,24*R*,25*R*,26*R* will be referred as *RRRR*-G+, *RRRR*-G−, and *RRRR-anti*. For all of these conformers, geometries were generated at the density functional theory (DFT) B3LYP/6-31G* level. Table 1 shows the relative enthalpies with respect to the most stable conformer for all the computed structures.

In compounds **1**–**6** the ^{1}H NMR signal of H23 appears as a broad signal with a half line width of approximately 3.5 Hz owing to scalar coupling with H22, H26, and Me28 as determined from a COSY experiment. The very small value, 1.5 Hz, of the ^{3}J between H22 and H23 rules out the subset of *anti* conformations narrowing the number of possible structures down to 16 (eight G+ and eight G− rotamers).

Table 1: DFT B3LYP/6-31G* ΔH_o relative enthalpies with respect to the lowest energy conformer for all computed structures.

Rotamer	G+	G−	*anti*	Rotamer	G+	G−	*anti*
RRRR	0.0	10.2	7.8	*RSSR*	0.0	1.7	10.2
RRRS	0.0	6.9	[a]	*RSSS*	1.2	0.0	6.8
SRRS	1.9	3.2	0.0	*SSSR*	0.0	3.1	2.2
SRRR	4.4	3.0	0.0	*SSSS*	0.0	5.7	3.8

[a] Not a stationary point.

By comparison with well defined proton–proton distances of the steroidal skeleton, a 900 ms NOESY experiment of **1** showed strong cross-peaks for protons within 2–3 Å, moderate to weak cross-peaks for protons within 3–3.5 Å, and very weak cross-peaks for protons within 3.5–4 Å, approximately. Strong to moderate cross-peaks were observed for the pairs H21/H22, H21/H23, H22/H23, H28/20, and H28/H14. A very weak cross-peak was observed for H23/H26 and no cross-peak for H22/H28. In all of the computer-generated structures it is observed that regardless of the stereochemistry at C23, C24, C25, and C26, NOE cross-peaks will be always observed for the pairs H26/Me27 and H23/Me28, and a very weak NOE cross-peak should be observed for the pair H23/H26. Out of the eight lowest energy conformations/configurations, three structures satisfy the above NOE constraints, namely, *SSSS*-G+, *SSSR*-G+, and *RSSS*-G−. This is a clear case in which NOE and ^{3}J analysis show limitations in the solution of stereochemical problems in small molecules.

The structure of compound **1** was partially aligned in poly(methylmethacrylate) (PMMA)/$CDCl_3$. A total of 15 one-bond ^{1}H-^{13}C RDCs for pairs C2-H2, C3-H3, C4-H4α, C4-H4β, C6-H6, C7-H7α, C7-H7β, C8-H8, C9-H9, C11-H11α, C11-H11β, C14-H14, C22-H22, C23-H23, and C26-H26 were measured using proton-coupled standard HSQC NMR experiments in isotropic ($CDCl_3$ only) and anisotropic (PMMA/$CDCl_3$) conditions, as described for ludartin.[22]

To determine the correct structure of compound **1** by RDCs, the alignment tensor was calculated using the singular value decomposition method (SVD)[5] as implemented in the program MSpin,[33] fitting the experimental RDCs on each of the structures that met the energy and NOE restraints criteria (*SSSS*-G+, *SSSR*-G+, and *RSSS*-G−). Agreement between experimental and back-computed values was expressed in terms of the quality factor Q.[34] Isomer *SSSS*-G+ showed the best quality factor ($Q = 0.213$), while *SSSR*-G+ and *RSSS*-G− showed much higher Q values of 0.451 and 0.510, respectively. To estimate the impact of errors in the measurements, Monte Carlo bootstrapping analysis[5] by generating random Gaussian distributions centered on measured values and with standard deviations of 1, 2, and 5 Hz was performed.[35] Results are summarized in Figure 1 and it can be seen that even large error bars have only a minor effect on the assessment of the configuration; no overlapping of error bars are observed. The experimental error for RDCs measurement was approximately 0.5 Hz. These results show that the method is quite robust, since changes in stereochemistry lead to significant changes in the quality factors and even high errors will have only a low impact in the determination of the correct stereochemistry.

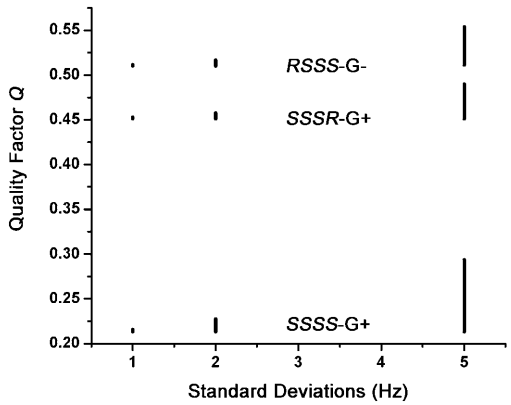


Figure 1. Representation of lowest, highest, and mean quality factors *Q* obtained by bootstrapping error analysis at standard deviation values of 1, 2, and 5 Hz on selected structures (*SSSS*-G+, *SSSR*-G+ and *RSSS*-G−).

To verify that there are no conformation mobility effects resulting from rotation around the C22–C23 bond, only the RDCs of the rigid steroidal skeleton were used to calculate the alignment tensor of **1**. In turn, using the calculated tensor, the RDCs values for the C–H bonds at C23 and C26 were calculated for isomers *SSSS*-G+, *SSSR*-G+ and *RSSS*-G−. We also included in this case the measured values for the methyl groups C27 and C28. As a result of their three-fold symmetry, methyl groups average approximately as −1/3 with respect to an equivalent vector pointing in the direction of the symmetry axis. In our case, we implemented the averaging by automatically recognizing the equivalent C–H bonds and averaging director cosine products inside the SVD procedure in MSpin (See Table 2).[36]

Table 2: Calculated versus experimental (D_{exp}) RDCs (Hz) for the C–H bonds at C23 and C26, Me27, and Me28.

Bond	D_{exp}(Hz)	*SSSS*-G+	*SSSR*-G+	*RSSS*-G−
C23-H23	−46	−47	−47	22
C26-H26	5	11	−49	−31
Me27	2	0	1	−2
Me28	−19	−13	−14	7

Consistent with the result from the fitting of the whole set of RDCs to the best alignment tensor, *SSSS*-G+ shows the best agreement between experimental and calculated results. Noteworthy is the dramatic change in the calculated RDC value of the bond C26–H26 when the stereochemistry is inverted from *S* to *R* in the isomers *SSSS* and *SSSR*. NOE is not able to differentiate 26*S* from 26*R* since there is a small change in the H23/H26 proton–proton distance from one isomer to the other (3.83 Å in *SSSS*-G+; 3.95 Å in *SSSR*-G+). This is again another demonstration of the power of RDCs, which can determine the relative orientation of any spin pair of nuclei irrespective of their location in the molecule. Thus, since the absolute stereochemistry of the steroidal skeleton is well known, the stereochemistry of the epoxy γ-lactol ring in **1** is determined to be 23*S*,24*S*,25*S*,26*S*, by NMR spectroscopy RDCs. By comparison of the ^{13}C NMR spectroscopy data the same stereochemistry was also determined for compounds **2**–**6**.

Independently, a powder sample of compound **1** was subjected to PXRD analysis. The high-resolution PXRD pattern was indexed with the program DICVOL04,[37] and lattice parameters (after Rietveld refinement) are $a = 10.6339(2)$, $b = 7.3448(1)$, $c = 16.0834(3)$ Å, $\alpha = 90$, $\beta = 93.493(2)$, $\gamma = 90°$, $V = 1253.84(4)$ Å^3. A Le Bail fit[38] performed with the program GSAS,[39] had $R_{wp} = 4.38\,\%$ and $\chi^2 = 1.157$. The systematic absences suggested the space group $P2_1$, which is compatible with an enantiopure compound, and the estimated density of approximately 1.3 g cm^{-3} suggested $Z = 2$.

The crystal structure of **1** was solved with direct-space methods using the simulated annealing global optimization algorithm. Molecular models of the various configurations were constructed with the program ARGUSLAB,[40] using the molecular geometries of the Cambridge Structural Database[41] entries JELPUM ((+)-trechonolide A)[42] and GAKTOB (jaborosalactone 32 (**7**) monohydrate),[31] and omitting the hydroxy and epoxide oxygen atoms of the epoxy γ-lactol ring. The molecules so defined were located in the unit cell using the program PSSP,[43] based on the agreement between the first 230 integrated intensities from the Le Bail[38] fit and the ones calculated with trial models. The structure solution was parameterized with 7 variables: 3 molecular positional parameters, 3 Eulerian angles, and 1 torsion (determined by rotating the epoxy γ-lactol ring around the C22–C23 bond). The structure solution runs where C23 had *S* absolute configuration yielded an agreement factor of S = 0.25, whereas the ones with C23*R* had S = 0.35, suggesting an *S* absolute configuration at C23. The remaining hydroxy and epoxide oxygen atoms of the epoxy γ-lactol substituent, as well as an additional atom that was assigned to an oxygen atom from water of hydration, were found by Fourier difference as implemented in GSAS,[39] leading to the *SSSS* configuration. Fourier maps of the candidate solution with C23*R* did not lead to an additional valid solution with reasonable atom connectivity and fit of the PXRD pattern. The final Rietveld refinement has $R_{wp} = 5.12\,\%$, $\chi^2 = 1.58$ and $R_I = 5.68\,\%$. Minimum and maximum Fourier difference residuals were −0.259 and +0.186 e Å^{-3}, respectively. Additional details of the refinement are included in the Supporting Information. The stereo view of the molecular structure of **1** monohydrate obtained by PXRD is represented in Figure 2.

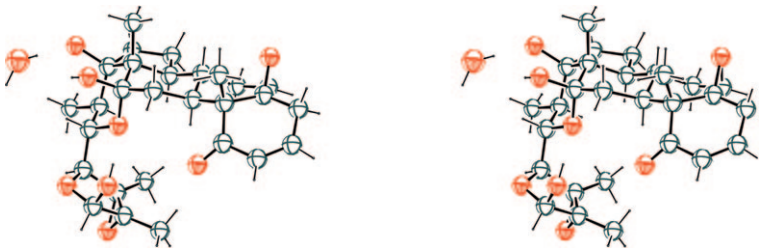


Figure 2. Stereoview of the molecular structure of **1** monohydrate in its solid-state conformation. Oxygen atoms are in red.

To determine the hydrogen-bonding scheme around the water molecule, to better locate its hydrogen atoms, and to independently validate the crystal-structure results, first-principles density-functional theory (DFT) total energy and force calculations in the periodic lattice environment were performed using the QUANTUM-ESPRESSO PWSCF computer code.[44]

Starting with the atomic positions determined from PXRD, all the atoms were allowed to relax. The averaged magnitudes of the displacements from the experimental positions were 0.059, 0.106, and 0.273 Å, for C, O, and H, respectively. These results also validate the proposed structure.

Based on our results, the structure of a similar epoxy γ-lactol from *Jaborosa lanigera* should be revised.[45] Different from compound **1**, this structure has a double bond at C5–C6. The ^{13}C NMR spectra of the jaborosalactol from *J. lanigera* and our jaborosalactol 24 (**1**) can be superimposed in the regions corresponding to carbons C12, C18, and C20 to C28, suggesting that both compounds have the same stereochemistry in the five-membered ring. However, the jaborosalactol from *J. lanigera* has been assigned an *SRRS*-G+ structure based only on semiempirical calculations and NOE measurements.

In conclusion, we showed that the application of NMR spectroscopy RDCs and PXRD, independently led to the same relative stereochemistry and conformation for the epoxy γ-lactol side chain of the new withanolide jaborosalactol 24 (**1**). The final absolute stereochemistry of **1** is determined relative to the established absolute configuration of the steroidal skeleton. The use of only conventional NMR spectroscopy experiments (NOE and ^{3}J analysis) led to three ambiguous solutions for the structure of **1**. Without the aid of RDCs and PXRD it would have posed great difficulty to complete the phytochemical study of *Jaborosa parviflora* and to unambiguously determine the stereochemistry of its isolates. Monte Carlo bootstrapping analysis on the RDC data showed that even with larger error bars, the same stereochemistry is obtained, showing the robustness of the method when applied to small molecules.

Experimental Section

General and more specific experimental procedures regarding isolation of compound **1**–**6**, ^{1}H and ^{13}C NMR assignments, additional physical data (MS, IR, UV, and $[\alpha]_D^{21}$), NMR measurements, preparation of alignment, experimental and computed RDCs values for the isomers *SSSS*-G+, *SSSR*-G+ and *RSSS*-G−, DFT calculations and a Rietveld refinement plot are provided with the Supporting Information. Additional details of the Rietveld refinement and atomic coordinates of jaborosalactol 24 (**1**) are included in the crystallographic information file CCDC 714513. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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