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Structure Elucidation of a Plant Metabolite of 4-Desoxynivalenol

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Abstract: 3-β-D-Glucopyranosyl-4-desoxynivalenol, a glucoconjugate of the trichothecene 4-desoxynivalenol was isolated as main metabolite of DON from *Zea mays* suspension cultures. The structure was elucidated by two-dimensional NMR spectroscopy and electrospray mass spectroscopy.

4-Desoxynivalenol **1** (DON, vomitoxin) is an important mycotoxin produced by *Fusarium* species. It has been found worldwide in cereals[1,2]. *Fusarium graminearum* and *Fusarium culmorum*, the main producers of DON are causative agents of the *Fusarium* head blight of wheat and ear rot of maize. Upon natural and artificial infection of wheat with *Fusarium graminearum* DON concentration reaches a maximum and then declines until harvest[3]. This was attributed to plant enzymes being able to metabolize DON. There seems to be a correlation between resistance of the host and the ability to metabolize mycotoxins.

Mycotoxins are usually metabolized by plants and animals via two pathways: transformation (e.g. deepoxidation) and conjugation (e.g. glycosidation). However, 15-acetylscirpenol-4-glucoside is the only trichothecene glycoside reported to date[4]. Miller et al. found three metabolites of ^{14}C-labelled DON in suspension cultures of wheat [5a]. One of them had a higher molecular weight than DON and was considered to be a DON-glucoside[5b]. Recently Fujita et al.[6] also found three metabolites of DON in sweet potato root tissues in a similar radiolabel experiment, but in both cases no proof of the structure was given.

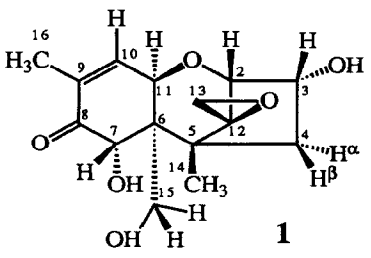
Results and Discussion

We report on the isolation and structure elucidation of the main metabolite of DON from *Zea mays* suspension cultures. ^{14}C-Labelled DON added to maize cell suspension cultures was converted into three labelled products with higher polarity than DON. The main metabolite **2** was extracted and purified in a three stage chromatography process.

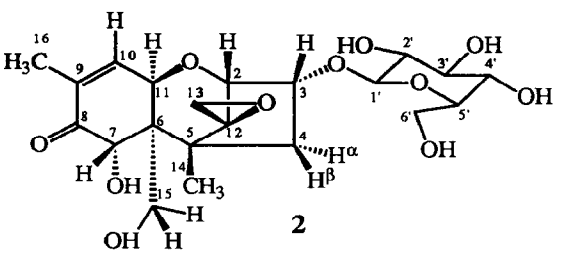
2 has a UV-spectrum identical to that of DON and was cleaved by hot alkali to give formaldehyde. This confirms the presence of the 7-hydroxyl-, the 8-keto-, and the 15-hydroxymethyl-moieties of DON. Unlike DON, **2** gave a colour reaction with the sugar-reagent aniline/diphenylamine/phosphoric acid. The epoxy group was proved to be intact in **2** by a positive reaction with nicotinamide/acetylpyridine/NaOH[7]. However, attempts to cleave the glycoside with α- or β-glucosidase were unsuccessful and hydrolysis with NaOH or acid yielded complicated mixtures.

Electrospray mass spectroscopy gave three peaks corresponding to the molecular ion: 459 $[M+H]^+$, 481 $[M+Na]^+$, and 497 $[M+K]^+$ in agreement with the postulated molecular formula $C_{21}H_{30}O_{11}$ of **2** being a glucoconjugate of DON. Upon addition of formic acid, the intensity of the peak at *m/e* = 459 increased significantly.

Although ^1H and ^{13}C NMR data on DON and its synthetically obtained 3- and 15-glucosides have already been published[8-10], these spectra were recorded either in $CDCl_3$ or in CD_3OD. Therefore, data for both compounds were collected in D_6-DMSO to provide a basis for the comparison of the spectra of **1** and **2**, as considerable solvent dependent shift differences can be registered.



1



2

In the H,H-COSY spectrum[11] of **2** the desoxynivalenol skeleton can be identified completely. Due to the presence of water in the sample of **2**, which could not be eliminated even after freeze-drying and subsequent drying over P_2O_5 for several weeks, no sharp signals for the hydroxyl groups could be observed.

The olefinic proton **H-10** resonates as a double quartet at 6.56 ppm. Two crosspeaks to proton **H-11** at 4.85 ppm and to the methyl protons **H-16** at 1.72 ppm prove the existence of the CH_3-C=CH-CH-O moiety. Proton **H-3**, resonating at 4.32 ppm, is supposed to give rise to the signal with the highest multiplicity. From there, crosspeaks can be found leading to the protons **H-2**, **H-4α**, and **H-4β** with the shift values 3.59 ppm, 2.61 ppm and 1.78 ppm; the latter two protons also correlating with each other. Their geminal coupling constant is 14.4 Hz. As the coupling constant ^3J(**H-2,H-3**) is 4.4 Hz, **2** has α-configuration at **C-3** [8]. Comparing compounds **1** and **2**, the protons **H-3** and the protons **H-4α** show the largest shift difference. In most trichothecene derivatives the coupling constant ^3J(**H-3,H-4β**) is about 10 Hz and ^3J(**H-3,H-4α**) can vary between 4.5 and 8 Hz due to the variation of the dihedral angle as predicted by the Karplus equation. The two doublets of the protons **H-13**, in the one-dimensional spectrum hidden below a group of multiplets belonging to the sugar moiety, can be localized in the H,H-COSY spectrum at 2.94 and 3.07 ppm. A similar situation applies to the protons **H-15**. Proton **H-7** gives rise to a broad singlet at 4.63 ppm in the one-dimensional spectrum due to the rapid exchange of the neighbouring hydroxyl proton. In the spectra of DON, all hydroxyl signals could be identified. The 7-OH trichothecenes reported to date have α-orientation[8].

The residual signals in the H,H-COSY spectrum of **2** give rise to a correlation pattern CH-CH-CH-CH-CH-CH_2 which corresponds to a sugar residue. Starting from a doublet at 4.23 ppm due to an anomeric proton with a coupling constant of 7.7 Hz, the whole correlation network can be established. All coupling constants of the methine groups, derived from a one-dimensional 600 MHz spectrum, are in the range from 7.7 to 10 Hz, indicating a pyranose chair with only axial protons, i.e. a β-D-glucopyranosyl residue.

The hydroxylic protons give rise to broad signals at 4.46 (1H), 4.63 (1H), 4.81 (1H), and 4.90- 5.05 ppm (3H). No correlations of these protons are visible in the two-dimensional spectra. As all hydroxylic protons in **2** exchange quickly on the NMR timescale, it proved to be impossible to locate the linkage between the sugar moiety and the DON aglycon by a one-dimensional NMR experiment.

Tab. 1: ^{1}H-NMR Assignment of DON (**1**), its Main Metabolite **2** and the Hexaacetyl Derivative **3**

	DON (**1**) [d_6-DMSO]		DON-Metabolite **2** [d_6-DMSO]		DON-Metabolite Hexaacetate **3** [CD_3OD]	
	δ(^{1}H) ppm	Multiplicity, J Hz	δ(^{1}H) ppm	Multiplicity, J Hz	δ(^{1}H) ppm	Multiplicity, J Hz
H-2	3.39	d, 4.3	3.59	d, 4.4	3.71	d, 4.5
H-3	4.16	dddd, 10.8, 4.6, 4.3, 3.8	4.32	ddd, 10.8,4.4,4.4	4.47	ddd, 11.1,4.5,4.1
H-3OH	5.10	d, 3.8				
H-4α	2.45	dd, 14.3, 4.6	2.61	dd, 14.4, 4.4	2.31	dd, 15.0, 4.1
H-4β	1.76	dd, 14.3,10.8	1.78	dd, 14.4, 10.8	2.10	dd, 15.0,11.1
H-7	4.64	d, 4.4	4.63	s	5.99	s
H-7OH	4.83	d, 4.4				
H-10	6.54	dq, 5.9,1.5	6.56	dq, 6.0. 1.3	6.63	dq, 5.9,1.5
H-11	4.90	d, 5.9	4.85	d, 6.0	4.74	d, br.
H-13A	2.90	d, 4.4	2.94	d, 4.5	2.79	d, 3.7
H-13B	3.03	d, 4.4	3.07	d, 4.5	3.07	d, 3.7
H-14	1.01	s	1.00	s	0.96	s
H-15A	3.40	dd (ABX), 11.7, 5.0	3.46	d (AB), 11.4	4.31	d(AB), 12.3
H-15B	3.63	dd (ABX), 11.7, 5.0	3.61	d (AB), 11.4	4.39	d(AB), 12.3
H-15OH	4.61	dd, 5.0,5.0				
H-16	1.72	s, br.	1.72	s, br.	1.83	s, br.
H-1'			4.23	d, 7.7	4.85	d, 7.8
H-2'			3.00	dd, 7.7,8.5	4.98	dd, 9.5,7.8
H-3'			3.14	dd, 8.5, 9.0	5.29	t, 9.5
H-4'			3.04	dd, 9.0,10.0	5.06	dd, 10.0,9.5
H-5'			3.09	m	3.90	ddd, 10.0,4.8,2.5
H-6A'			3.40	dd, 10.0, 5.0	4.16	dd, 12.3,2.5
H-6B'			3.65	dd, 10.0, br.	4.23	dd, 12.3,4.8
					1.89,1.99,2.00 2.04,2.07,2.18	s

In the ^{13}C NMR spectra of **2**, 21 carbon atoms can be found. According to the DEPT spectra, there are 2 methyl groups, 4 methylene groups, 10 methine groups and 5 quaternary carbon atoms present. The protonated carbon atoms can be assigned via a HMQC experiment[12]. The elucidation of the quaternary carbon atoms and long-range correlations in **2** was feasible via a HMBC experiment[13] (Tab. 2). The crosspeaks **C-1'/H-5'** and **C-5'/H-1'** confirm the postulated pyranose form of the carbohydrate residue. The long-range correlations also prove that the sugar residue is linked to **C-3.** This fact can be inferred by HMBC crosspeaks **H-1'/C-3** and **H-3/C-1'**. Comparison of the ^{13}C-NMR shift values of **1** and **2** (Tab. 2) emphasizes this finding. Significant differences are observed especially for the carbon atoms **C-3** (Δδ= +5.02 ppm), and **C-4** (Δδ= -2.84 ppm). 2

was acetylated to give the hexaacetyl derivative **3**. Comparison of the ^{1}H-, ^{13}C- and ^{13}C-DEPT- NMR data of **3** with literature data of the synthetically obtained hexaacetyl derivative of the DON-3-glucoside[10] did not reveal significant differences.

Tab. 2: ^{13}C-NMR Assignment of DON (**1**), its Main Metabolite **2**, and the Hexaacetyl Derivative **3**

	DON (**1**) [d_6-DMSO]		DON-Metabolite **2** [d_6-DMSO]		DON-Metabolite Hexaacetate **3** [CD_3OD]	DON-3-Glc-hexaacetate [CD_3OD] (lit.[10])
	$\delta(^{13}C)$ ppm	long-range coupling to H- atom Nr.	$\delta(^{13}C)$ ppm	long-range coupling to H- atom Nr.	$\delta(^{13}C)$ ppm	$\delta(^{13}C)$ ppm
C-2	80.14		79.78	4β,13A,13B	81.60	80.1
C-3	69.16	3OH,4β	74.18	4α,4β,1'	76.24	75.3
C-4	43.62	3OH,14	40.88	2,14	42.60	41.6
C-5	45.25	14,15A	45.00	3,4α,4β,7,11,13A,13B, 14,15A,15B	47.05	45.7
C-6	51.71	4β,7,7OH,10,14	51.96	4α,4β,7,10,11,14, 15A,15B	51.62	50.0
C-7	74.37	7OH,11	74.33	11,15A,15B	76.24	74.9
C-8	200.05	7,7OH,10,16	199.98	7,16	193.94	192.3
C-9	134.67	11,16	134.72	11,16	137.73	136.9
C-10	138.22	11,16	138.37	11,16	139.33	137.5
C-11	67.68		69.57	7,10,15A,15B	74.12	72.7
C-12	65.86	13A,13B,14	65.65	2,4α,13A,13B,14	65.91	64.9
C-13	46.60		46.93		48.30	47.8
C-14	14.34	4β	14.41	4β	14.19	13.7
C-15	60.00	15OH	59.99	7,11	63.27	62.4
C-16	15.00	10	15.04	10	15.41	15.2
C-1'			102.25	3,2',5'	101.01	100.3
C-2'			73.43	3'	71.54	70.2
C-3'			76.86	2',4'	72.91	71.4
C-4'			70.25	3',5'	69.96	68.5
C-5'			77.07	1',2',4'	72.94	72.0
C-6'			61.20	5'	63.01	61.9
Acetyl					20.54 (3x), 20.63,20.73,20.91	20.5, 20.6, 20.8
					171.19,171.27, 171.46,171.52, 171.62,172.33	169.3,169.5,169.8, 170.1,170.3,170.6

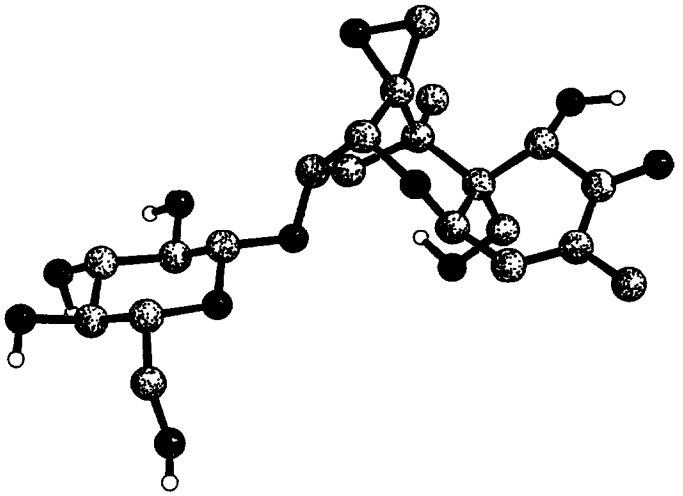
ROE-Crosspeaks between the signals of **H-2**, **H-3**, **H-4** and the anomeric proton **H-1'** of the glucosyl residue confirm the postulated structure. According to the ROE data, proton **H-13A** is the *pro*-R proton and **H-13B** is the *pro*-S proton. Distance information can be obtained from the ROESY experiments[14] by evaluation of the crosspeak intensities[15]. The method of restrained molecular dynamics (MD) simulations was employed to translate the interproton distances obtained from the ROESY experiments into a three-dimensional structure[16]. Both molecules, **1** and **2**, were simulated for 100 ps at 500 K to create a sufficient ensemble of molecular conformations. After analysis of the potential energy trajectories, low energy structures were selected and minimized. Mainly the restraints affecting the CH_2-OH- group (**C-15**) are violated. This can be assigned to a certain amount of rotational freedom of this substituent on the NMR timescale. Therefore, the analyzed conformation represents only a snapshot taken from the dynamics on a picosecond timescale and cannot fulfill all interproton relations involving this group at the same time.

A $^{12}C_{11}$ chair conformation was found[17] exclusively for the six-membered oxygen-containing ring (B-ring) as shown in Fig. 1. The atoms **O-1**, **C-2**, **C-5**, and **C-6** form a plane with **C-12** lying above and **C-11**

below. This is supported by a strong ROE between **H-11** and **H-4α**. It was not possible to induce a conformational transition under these conditions throughout the simulations. This indicates the stability of the analyzed ring conformation. A boat conformation of the B-ring, which also seemed to be possible[8] can be ruled out.

Fig. 1 shows the energy minimized equilibrium structure of the DON-glucoside **2**. A structure identical to the aglycon of **2** is obtained for **1**. The relative orientation of the glucosyl residue is determined by three sugar-aglycon ROEs. A more globular structure with the glucose ring folded under the DON skeleton can be excluded at an early stage. No further interproton contacts between the glucose protons and the aglycon moiety were observed by NMR. Furthermore, a systematic search investigation varying the two torsion angles of the bonds connecting the sugar residue with the aglycon supports that finding. The conformational space available for these torsions is severely restricted to exocyclic orientations.

Fig. 1 : Equilibrium Conformation after Restrained MD and Energy Minimization of **2**. Oxygen Atoms are Depicted as Filled Balls, Carbon Atoms are Stippled.



Experimental Part

UV spectra were recorded on a LCD 402 HPLC UV photometer. TLC was carried out on precoated silica gel plates (Merck), coating thickness 0.25 mm. Extracts were purified by Extrelut® (Merck), a charcoal/Al_2O_3/celite column according to Romer[18] and a preparative HPLC column (20 mm ID, 250 mm length, LiChrosorb® RP-18, 7 µm, Merck).

Electrospray mass spectra were measured on a VG BIO Q triple quadruple atmospheric pressure mass spectrometer equipped with an electrospray interface (VG Biotech, Tudor Road, Altrincham, Cheshire). Samples (10 µl) were injected into the electrospray source via a loop injector (Rheodyne 5717) as a solution, typically 50-100 $pmol\mu l^{-1}$ in water/acetonitrile (1:1) at a flow rate of 2 $\mu l min^{-1}$ (Applied Biosystems model 140A dual syringe pump).

All 2D NMR experiments were recorded on a BRUKER AM 360 spectrometer at 360 MHz (^{1}H) and 90 MHz (^{13}C). A ^{1}H-NMR spectrum of **2** was also recorded on a BRUKER AMX 600 spectrometer (600 MHz) and a ^{1}H-NMR spectrum of **3** was obtained using a BRUKER AM 500 spectrometer (500 MHz). Hetero decoupling in the HMQC experiment was achieved using a BRUKER BFX-5 hetero decoupler. ROESY, HMQC and HMBC spectra were edited using the BRUKER UXNMR software on a BRUKER X32 workstation. Quadrature detection in ω_1 of the DQF-COSY and ROESY was achieved by the TPPI method. All measurements were performed at 298 K. All spectra of DON were obtained using a 10 mg sample (WAKO Pure Chemical Industries Ltd., Japan) in 450 µl D_6-DMSO; the spectra of **2** were obtained with a 3 mg sample in 450 µl D_6-DMSO. The spectra of **3** were recorded using a 4 mg sample in 450 µl CD_3OD. The samples had been degassed in several freeze-thaw cycles; the sample tubes had been sealed. Chemical shifts are referenced to solvent peaks relative to TMS (^{1}H: D_6-DMSO, 2.49 ppm; CD_3OD, 3.35 ppm / ^{13}C: D_6-DMSO, 39.7 ppm; CD_3OD, 49.0 ppm).

Preparation and Cultivation of Maize Suspension Cultures.- Callus cultures of *Zea mays* (cv Black Mexican Sweet) were grown on agar solidified medium. For degradation studies the callus cultures were transferred to freshly prepared Murashige Skoog medium (about 2 g of callus material per 20 ml liquid medium in 100 ml Erlenmeyer flasks) and 100 µg [^{14}C]-DON (0.5 MBq/mmol) was added to the flasks to give a final DON concentration of 5 mgl^{-1}. The cultures were incubated in a rotary shaker for 14 days at 28 °C and 130 rpm.

Isolation and Purification of DON-Glucoside 2. -The freeze-dried cells were ground in a mortar and extracted with methanol/water 8:2 (v/v) and the methanol was removed by distillation. The residual water phase was saturated with NaCl and poured onto a column of Extrelut®. **2** was eluted with three portions of dichloromethane/isopropanol 1:1 (v/v). The eluate was evaporated to dryness and the residue reconstituted in acetonitrile/water 84:16 (v/v). The solution was purified by passage through a column of Darco® G-60 (Serva)/Al_2O_3 neutral/celite[5b]. The eluate was evaporated, the residue was dissolved in the HPLC eluent, and passed through a preparative HPLC column (LiChrosorb® RP 18). The eluent was acetonitrile/water 5:95 (v/v). The elution was monitored with a UV detector at 220 nm. Seven fractions were collected, evaporated, and dissolved in methanol. 5 µl were spotted on a TLC plate and developed with chloroform/methanol 9:1 (v/v). The plate was stained with $AlCl_3$ and **2** was detected in fraction 6 having an R_f value of 0.16. The yield of **2** was 1.89 mg (47 %). The radiolabelled metabolite was used only as reference material to study the chromatographic behaviour. For structure elucidation purposes about 10 mg of unlabelled **2** were isolated from preparative metabolism experiments.

Acetylation of 2. - The main metabolite **2** of DON (3.1 mg) was acetylated overnight with acetic anhydride/ 4-dimethylaminopyridine in CH_2Cl_2. The reaction mixture was extracted with water, the organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by preparative HPLC with methanol/water 7:3 (v/v) on a LiChrosorb® RP 18 column (16 mm ID, 250 mm length, 5 µm). The purity was confirmed by TLC using chloroform/methanol 9:1 (v/v) a eluent. Yield 4.1 mg of **3** (84 %).

Selected NMR Parameters for 2. - DQF-COSY: 128 scans, preceded by 2 dummy scans, were recorded into 1K data blocks for each of the 208 t_1 values with a relaxation delay of 1.5 s and spectral widths of 1369.86 Hz. The data matrix was zero-filled to 2Kx2K and apodized with shifted square sine bell window functions in both dimensions. Due to the small spectral width the signal of **H-10** was folded. Its connectivities had been established in a previous experiment. *ROESY:* The longitudinal relaxation time T_1 was determined in an inversion-recovery experiment. 80 scans, preceded by 2 dummy scans, were recorded into 512W data blocks

for each of the 239 t_1 values with a relaxation delay of 2.1 s, a spinlock time of 300 ms, and spectral widths of 2336.45 Hz. The data matrix was zero-filled to 2Kx2K and apodized with a Gaussian window function (LB= -2.5, GB= 0.13) in ω_2 and a shifted square sine bell window function in ω_1. After FT and phase correction a baseline correction in both dimensions was applied. *HMQC:* 64 scans were recorded into 2K data blocks for each of the 100t_1 values with a relaxation delay of 2.0 s and spectral widths of 2702.70 Hz in ω_2 and 10416.67 Hz in ω_1. The data matrix was zero-filled to 4Kx2K and apodized with a shifted square sine bell window function in ω_2 and a Gaussian window function (LB= -15, GB= 0.2) in ω_1. The data were then processed using the program AURELIA to eliminate t_1 noise. *HMBC:* 512 scans were recorded into 2K data blocks for each of the 152 t_1 values with a relaxation delay of 2.0 s and spectral widths of 2702.70 Hz in ω_2 and 10416.67 Hz in ω_1. The J-delay Δ_2 was set to 90 ms. The data matrix was zero-filled to 4Kx2K and apodized with a shifted square sine bell window function in ω_2 and a sine bell window function in ω_1. The data were then processed using the program AURELIA to eliminate t_1 noise.

Computational Details. - All molecular mechanics simulations were carried out on Silicon Graphics 4D/70 GTB and 4D/240 SX computers using the Consistent Valence Force Field (CVFF)[19] implemented in the DISCOVER software package (version 2.70) together with INSIGHT II as a graphic interface. The energy expression describing covalent bond stretching was represented by a harmonic potential and no cross terms were used. A skewed biharmonic function served as penalty term for the violation of the NMR derived distance restraints. A tolerance of +/- 5 % (+/- 10 % in the case of **2**) was imposed onto the ROE derived mean value for each constraint to allow the molecule more flexibility during the restrained dynamics simulations. All calculations were performed with a relative dielectric permittivity of 80 to avoid molecular distortions due to overemphasized long range interactions. The time step for integrating Newton's equations of motion was set to 1 fs, applying the Verlet integration algorithm. The starting conformation for the structure refinement procedure was constructed from building blocks available from the INSIGHT fragment library, all hydrogen atoms being treated explicitly. The manually built structure was minimized in 1000 steps of steepest descent energy minimization to remove any strain caused by the model building procedure. A high temperature molecular dynamics simulation was carried out over 5 ps at 1000 K to obtain a reasonable starting conformation meeting the experimental data, followed by 5 ps at 500 K and 20 ps at 300 K including all distance restraints. The resulting structure was then simulated for 100 ps at 500 K by restrained MD. This refinement protocol was applied to the structure elucidation of both **1** and **2**.

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[15] Interproton distances were derived from integration of crosspeaks. After offset correction the intensities were calibrated against a reference value using the two-spin approximation. In the spectrum of **1**, the distance between the geminal protons **H-4α** and **H-4β** (average 176 pm) was used as reference. In the ROESY spectrum of **2** the corresponding crosspeak was perturbed by TOCSY-transfer. Therefore, the distance between the *syn*-protons **H-3** and **H-4β** was approximated to be 224 pm and used as reference.

[16] The refinement procedure includes a high temperature molecular dynamics period in the initial phase to ensure that the starting system does not remain in a local minimum on the energy hypersurface, but is able to cross energy barriers due to the interconversion of kinetic and potential energy. Thus, the final refined conformations are mainly determined by the constraints derived experimentally and not by the initial starting conditions.

[17] Several high temperature dynamics calculations were performed to verify a possible conformational transition of the detected ring conformation. These simulations were carried out without any restraining potentials, each covering 100 ps at 500 K, 1000 K, and 2000 K.

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