Structure Elucidation of Secalosides A and B by NMR Spectroscopy

Bernhard Jaun,*,† Bruno Martinoni,† Fabrizio Marazza,‡ and Massimo Bertolin‡

Laboratory of Organic Chemistry, Eidgenössische Technische Hochschule, Universitätstrasse 16, CH-8092 Zürich, Switzerland, and Cernitin S.A., Barbengo, Switzerland

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The constitution of secalosides A and B was elucidated through NMR spectroscopy using fast 2D-NMR experiments with pulsed-field gradients for coherence selection. Secalosides A and B are epimers at center C-3′ of aglycon I and have the constitution shown as **1**. The presence of a saccharose unit was confirmed independently by mild basic hydrolysis and enzymatic quantification. The relative configuration of the stereogenic centers of aglycon II could be deduced from ROESY data. Assignments of the two epimers to the *R*- and *S*- configurations at C-3′ in aglycon I, as well as the absolute configuration of aglycon II remain to be determined.

The preceding paper in this issue[1] describes the isolation and biological activity as well as chemical and initial spectroscopic studies on the nature of secalosides A−D, four new glycosides shown to be primarily responsible for the antitumor activity of the Cernitin T-60 fraction of the commercially available pollen extract Cernilton. Herein we report the elucidation of the constitution of secalosides A and B by NMR methods and the subsequent verification of the presence of a saccharose unit by mild acid hydrolysis followed by quantitative enzymatic determination of saccharose in the hydrolysate. This study was done in the Zurich laboratories subsequent to and, in part, based on the work done in Geneva as described in the preceding paper.

Results and Discussion

Jaton, Burger, and co-workers[1] have observed equilibration between secalosides A and B in aqueous solution and, at a higher rate, in methanol/pyridine. This prompted us to search for conditions under which equilibration would be slow enough to allow fast acquisition of the most crucial 1D- and 2D-NMR spectra on a sample with a large excess of secaloside A (the major isomer at equilibrium). A kinetic study indicated that, in pure methanol, equilibration is much slower than in the presence of pyridine. Therefore, a sample with an isomer ratio of A:B > 10:1 was dissolved in CD_3OD and the 1D−^{1}H, gradient accelerated DQF-COSY, broadband-decoupled 1D−^{13}C and ^{1}H−^{13}C one-bond correlated (HSQC, ^{1}H-detected, gradient accelerated) NMR spectra were acquired within the first 3 h after dissolution. In all these spectra, the intensities of the subspectra of secalosides A and B were close to 10:1, such that they allowed unambiguous assignment of all ^{1}H- and ^{13}C-signals to either the A or B isomer. Within the next 48 h, the TOCSY, ^{1}H−^{13}C long-range correlation (HMBC, ^{1}H-detected, gradient accelerated), ROESY, NOESY, and broadband-decoupled ^{13}C/DEPT spectra were acquired, which exhibited a decreasing ratio of secalosides A and B with time as expected. The three ^{1}H control spectra taken at different times showed that proton OA-3′ of the aglycon I subunit was exchanged with deuterium from CD_3OD at a rate higher than that of the isomerization. Therefore, overlapping spin systems for three species were observed for protons OA-3′ and OA-2 in the second ^{1}H-NMR spectrum: the original secaloside A, its OA-3′ D-exchanged form, and the epimerized, D-exchanged form of secaloside B. In addition to the signals of the two secalosides, the ^{1}H- and ^{13}C-spectra showed several signals belonging to an impurity (see the Experimental Section).

In the following discussion, only the signals assigned to secaloside A will be interpreted. For reasons of clarity, the numbering system and abreviations for the individual subunits corresponding to the final structure proposal (see Chart 2) will be used throughout the discussion.

The proton spectrum (CD_3OD; Figure 1) showed signals of 35 ± 2 H according to the integrals observed. One signal, H(Frc-3), was partly covered by the solvent signal. The singlet at 3.71 ppm corresponding to 3H was *prima vista* assignable to a methoxy group.

Homonuclear correlations obtained from the COSY spectrum (and, in part, the TOCSY and HSQC spectra) allowed the identification of the spin systems **A**−**I** depicted in Chart 1. The proton spectrum contained one region, at ca. 3.45 ppm, where the signals of three protons strongly overlapped (β-Glc-5, β-Glc-2, and α-Glc-2). Using traces of the HSQC and TOCSY spectra, however, their shapes and couplings could be identified as given in Table 1.

The signals of proton-bearing carbons were assigned through the HSQC spectrum that, together with the broadband-decoupled ^{13}C-NMR spectrum, formed the basis for the deduction of the connectivity between the individual fragments **A**−**I** via the ^{1}H−^{13}C long-range correlation spectrum (HMBC). All long-range correlations (LRC) observed for secaloside A are listed in Table 1.

Starting with the aromatic spin system **I**, the long-range correlations confirmed the identity of aglycon I as a 2-oxindole-3-acetic acid unit, whereby the presence of one NH group (OA-1′) in aglycon I was deduced from MS data and the ^{1}H-NMR spectrum in DMSO-d_6 obtained by Jaton *et al.*[1] LRC from H(β-Glc-6) to C(OA-1) showed that C(OA-1) is connected to the end of spin system **C** via an ester linkage.

* To whom correspondence should be adressed. Phone: 0041-1-632 31 44. Fax: 0041-1-632 11 09. E-mail: jaun@org.chem.ethz.ch.
† Laboratory of Organic Chemistry, ETH Zürich.
‡ Cernitin S.A.
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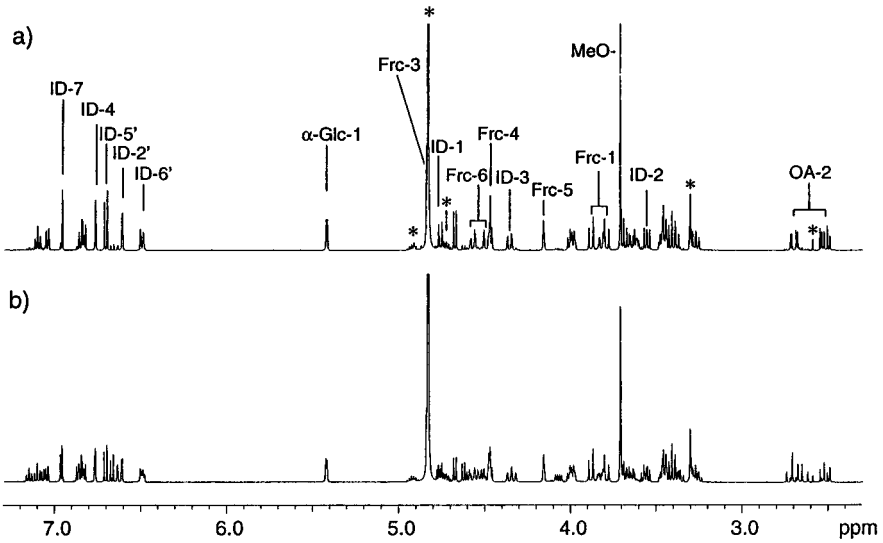
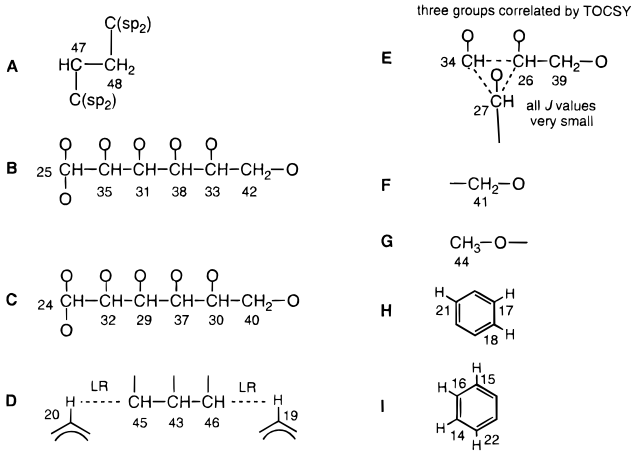


Figure 1. ^{1}H-NMR spectra of secalosides A and B in CD_3OD (a) 0.5 h after dissolution, A:B ca. 10:1; (b) 50 h after dissolution, A:B ca. 6:4. Signals of impurities and solvents are labeled with and asterisk.

Chart 1. Nine Spin Systems **A**−**I** of Protons Bound to Carbon[a]



[a] Carbon atoms are numbered in the order of chemical shifts of the corresponding ^{13}C-NMR signals (see column 1 and footnote b in Table 1).

The LRC's observed within spin system **C** confirmed its identity with *β*-glucose. The correlation from H(*β*-Glc-1) to C(ID-6), an aromatic quaternary carbon belonging to aglycon II, indicated that this carbon is linked to the anomeric center of the *β*-glucose unit via a glycosidic bond. The relative configuration of the sugar ring followed from the sequence of large coupling constants that were consistent with the axial positions of H(*β*-Glc-1), H(*β*-Glc-2), H(*β*-Glc-3), H(*β*-Glc-4), and H(*β*-Glc-5).

C(ID-5) showed an LRC to H(*β*-Glc-1) and to H(ID-7), which is part of spin system **D**. In fact, H(ID-7) and H(ID-4) showed a small coupling as deduced from TOCSY. According to their chemical shift values, C(ID-5) and C(ID-6) were both substituted by oxygen. Because two- and three-bond correlations cannot be distinguished in HMBC, the assignments of C(ID-5) and C(ID-6) and consequently C(ID-7a) and C(ID-3a) were at first ambiguous. The assignment shown in Chart 2 is based on an NOE observed between H(*β*-Glc-1) and H(ID-4) (see below).

H(ID-7) showed a long-range correlation to C(ID-1), and H(ID-4) showed one to C(ID-3). Interestingly, H(ID-1) correlated with C(ID-3), and both H(ID-1) and H(ID-3) showed LRC to C(ID-2), suggesting that C(ID-1), C(ID-2), and C(ID-3) are part of a five-membered benzannellated ring.

H(ID-3) was correlated to C(ID-1′), an aromatic quaternary carbon belonging to spin system **H**. LRC's from H(ID-6′) and H(ID-2′) to C(ID-3) showed that C(ID-1′) was directly attached to C(ID-3). The other carbons of this benzene ring were found from the interaromatic LRC's in the usual way, and the methoxy group showed a LRC to C(ID-3′). This was confirmed by an NOE between H(ID-2′) and the MeO- group. Both H(ID-2) and H(ID-1) correlated with the same two carboxyl functions: C(ID-1^1) and C(ID-2^1). However, H(ID-3) showed LRC to C(ID-2^1) only. This left the connectivity shown in Chart 2 as the only solution for the structure of aglycon II.

LRC between C(ID-2^1) and H(Frc-6) and between C(ID-1^1) and H(Frc-3) directly pointed to the carbon atoms linked via ester bonds to the two carboxyl functions. H(Frc-6) and H(Frc-3) both belong to spin system **E**, which was characterized by a series of very small homonuclear couplings. This pointed to the existence of a ring including the two ester carboxyls and at least a part of spin system **E**. Within spin system **E**, only H_2C(Frc-6) and H(Frc-5) showed resolved H-H couplings. Therefore, and despite the rather small coupling constants of 1.5 and 2.5 Hz, we assumed that they are vicinal to each other with a direct bond between C(Frc-6) and C(Frc-5).

The fructofuranose substructure shown in Chart 2 for spin system **E** was fully consistent with all LRC observations, as well as with the ^{13}C-NMR chemical shifts. In order to account for the observed coupling constants, however, protons H(Frc-3), H(Frc-4), and H(Frc-6) must all be in quasiequatorial positions (*trans*/*trans*), with the two ester linkages being in a quasiaxial *cis* arrangement. This is in contrast to the conformation of furanose found in free sucrose, where the corresponding protons are predominantly in a quasiaxial orientation and exhibit rather large couplings. The unusual coupling constants and the ambiguity of the HMBC data regarding the direct connection of C(Frc-6) and C(Frc-

Table 1. NMR Data of Secalosides A and B[a–d]

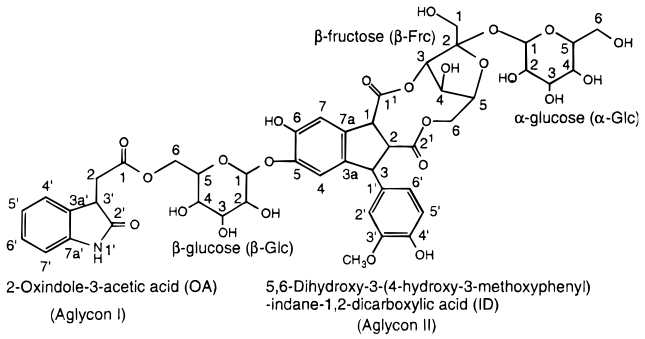
	secaloside A						secaloside B[d]	
no.	^{13}C assignment	δ_C	CH_n DEPT	δ_H of directly attached protons HSQC	^{1}H–^{1}H homonuclear scalar correlations COSY, TOCSY (J (Hz))	^{13}C–^{1}H scalar ($^{2,3}J_{CH}$) long-range correlations to protons HMBC	δ_C	δ_H
1	OA-1[1]	181.20	s			OA-2; OA-3′	181.25	
2	ID-2[1]	174.59	s			ID-1; ID-2; Frc-6	174.46	
3	OA-1	172.38	s			β-Glc-6; OA-2; OA-2′	172.33	
4	ID-1[1]	172.29	s			ID-1; ID-2; Frc-3	172.25	
5	ID-3′	149.24	s			CH_3O-(ID-3′); ID-2′; ID-5′	149.24	
6	ID-6	148.15	s			ID-4	148.36	
7	ID-5	147.16	s			ID-7; β-Glc-1	146.97	
8	ID-4′	146.76	s			ID-2′; ID-5′; ID-6′	146.76	
9	OA-7a′	143.60	s			OA-3′; OA-4′; OA-6′	143.70	
10	ID-3a	137.43	s			ID-1; ID-7; ID-3	137.91	
11	ID-1′	135.70	s			ID-5′; ID-6′; ID-3; ID-2	135.50	
12	ID-7a	134.42	s			ID-1; ID-4	134.51	
13	OA-3a′	130.26	s			OA-2′; OA-5′; OA-7′; OA-2	130.20	
14	OA-6′	129.25	d	7.11 “t”t	OA-7′ (7.7) OA-5′ (7.7) OA-4′ (0.7)	OA-4′	129.20	7.15 td
15	OA-4′	124.98	d	7.05 “t”d	OA-5′ (7.3) OA-6′ (0.7)	OA-6′	125.14	7.07 ddd
16	OA-5′	123.38	d	6.84 “d”t	OA-4′ (7.3) OA-6′ (7.7) OA-7′ (0.8)	OA-7′	123.38	6.84 td
17	ID-6′	121.60	d	6.49 dd	ID-5′ (8.2) ID-2′ (2.0)	ID-3; ID-2′	121.55	6.49 dd
18	ID-5′	116.49	d	6.72 d	ID-6′ (8.2)	ID-6′	116.49	6.67 d
19	ID-4	115.34	d	6.77 br s	ID-3 (0.8)		115.86	6.77 br s
20	ID-7	114.44	d	6.96 br s	ID-1 (0.8)	ID-1	114.50	6.97 br s
21	ID-2′	112.20	d	6.62 d	ID-6′ (2.0)	ID-6′; ID-3	112.20	6.64 d
22	OA-7′	110.86	d	6.86 d	OA-6′ (7.7) OA-5′ (0.8)	OA-5′	110.80	6.87 d
23	Frc-2	109.74	s			Frc-3; Frc-4; Frc-5; Frc-6a; Frc-6b; α-Glc-1	109.74	
24	β-Glc-1	104.42	d	4.69 d	β–Glc-2 (7.6)	β-Glc-2 and/or β-Glc-5	104.21	4.63 d
25	α-Glc-1	94.55	d	5.43 d	α-Glc-2 (3.7)		94.55	5.43 d
26	Frc-5	88.65	d	4.13 br s	Frc-6a (2.3) Frc-6b (1.5) Frc-3, Frc-4 (<1.0)	Frc-4; Frc-3; Frc-6a; Frc-6b	88.59	4.13 br s
27	Frc-3	80.03	d	4.84 br s	Frc-4 (<0.5)	Frc-1a; Frc-1b; Frc-4; Frc-5	80.06	4.84 br s
29	β-Glc-3	77.25	d	3.41 dd	β–Glc-4 (8.6) β-Glc-2 (9.1)	β-Glc-2; β-Glc-4	77.25	3.37 dd
30	β-Glc-5	75.44	d	3.46 dd	β-Glc-4 (9.9) β-Glc-6b (6.5) β-Glc-6a (2.0)	β-Glc-4	75.60	3.29 dd ?
31	α-Glc-3	75.27	d	3.70 “t”	α-Glc-2 (9.1) α-Glc-4 (10.0)	α-Glc-2; α-Glc-4	75.27	3.70 dd
32	β-Glc-2	74.75	d	3.44 dd	β-Glc-1 (7.6) β-Glc-3 (9.1)	β-Glc-3	74.84	3.43 dd
33	α-Glc-5	74.33	d	3.98 m	α-Glc-4 (10.0) α-Glc-6b (4.0) α-Glc-6a (2.2)	α-Glc-6a; α-Glc-6b	74.33	3.98 m
34	Frc-4	73.95	d	4.47 “s”	Frc-5 (<1) Frc-3 (<1)	Frc-3; Frc-5; Frc-6b	73.83	4.48 “s”
35	α-Glc-2	73.66	d	3.45 dd	α-Glc-1 (3.7) α-Glc-3 (9.6)	α-Glc-1; α-Glc-3	73.66	3.45 dd
37	β-Glc-4	71.43	d	3.27 dd	β-Glc-3 (8.6) β-Glc-5 (9.9)	β-Glc-2 and/or β-Glc-5; β-Glc-6a	71.35	3.27 dd
38	α-Glc-4	71.10	d	3.39 dd	α-Glc-3 (10.0) α-Glc-5 (10.0)	β-Glc-3; β-Glc-5; β-Glc-6a	71.13	3.39 dd
39	Frc-6	65.40	t	a) 4.58 dd	Frc-6b (12.7) Frc-5 (1.5)	Frc-4; Frc-5	65.44	4.56 dd
				b) 4.50 dd	Frc-6a (12.7) Frc-5 (2.3)			4.52 dd
40	β-Glc-6	64.78	t	a) 4.36 dd	β-Glc-6b (11.8) β-Glc-5 (2.0)	β-Glc-4	64.67	4.34 dd
				b) 3.99 dd	β-Glc-6a (11.8) β-Glc-5 (6.5)			4.07 dd
41	Frc-1	63.44	t	a) 3.88 d b) 3.79 d	Frc-1b (12.3) Frc-1a (12.3)	Frc-3	63.44	3.88 d 3.79 d
42	α-Glc-6	62.45	t	a) 3.82 dd	α-Glc-6b (12.0) α-Glc-5 (2.2)	α-Glc-4	62.45	3.83 dd
				b) 3.64 dd	α-Glc-6a (12.0) α-Glc-5 (4.0)			3.66 dd
43	ID-2	59.98	d	3.56 dd	ID-1 (9.7) ID-3 (6.8)	ID-1; ID-3	59.98	3.57 dd
44	CH_3-O-(ID-3′)	56.43	q	3.71 s			56.44	3.71 s
45	ID-3	53.86	d	4.47 d	ID-2 (6.8) ID-4 (<1)	ID-2′; ID-6′; ID-1; ID-4	53.98	4.60 d br
46	ID-1	52.71	d	4.76 d	ID-2 (9.7) ID-7 (<1)	ID-7; ID-2	52.73	4.77 d br

Table 1 (Continued)

	secaloside A						secaloside B[d]	
no.	^{13}C assignment	δ_C	CH_n DEPT	δ_H of directly attached protons HSQC	^{1}H−^{1}H homonuclear scalar correlations COSY, TOCSY (J (Hz))	^{13}C−^{1}H scalar ($^{2,3}J_{CH}$) long-range correlations to protons HMBC	δ_C	δ_H
47	OA-3′	43.70	d	3.61 dd	OA-2a (4.5) OA-2b (7.5)	OA-2; OA-4′	(43.30) α-D-shift	exchanged by ^{2}H
48	OA-2	34.75	t	a) 2.69 dd	OA-2b (16.7) OA-3′ (4.5)	OA-3′	34.92	2.73 dd
				b) 2.52 dd	OA-2a (16.7) OA-3′ (7.5)			2.67 dd

[a] Nonequivalent methylene protons are labeled with (a) for the proton resonating at lower field and (b) for the proton resonating at higher field. [b] Carbon signals 28, 36, 49, 50, and 51 belong to an impurity and are not listed. [c] "s", "d", "t": signals with singlet-like, doublet-like, and triplet-like appearance, respectively. [d] ^{13}C assignments for secaloside B are based on analogy to secaloside B and, in part, on HMBC; ^{1}H assignments are based on similarity of coupling patterns and TOCSY, which showed both isomers.

Chart 2. Constitution of the Epimeric Secalosides A and B with the Five Building Blocks and Their Abridged Notation for Atom Labeling



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5) prompted us to verify the presence of a saccharose unit in secalosides A and B by chemical analysis (see below).

Long-range correlation from H(α-Glc-1) to C(*β*-Frc-2) led to the final carbohydrate subunit, which corresponds to spin system **B**. $^{2,3}J_{CH}$− correlations, together with the observed $^3J_{HH}$ coupling constants, identified this unit as α-glucose, bound to C(*β*-Frc-2) via a glycosidic bond.

Formula **1** (Chart 2) summarizes the constitution of secaloside A with the five building blocks (abridgments in brackets): aglycon I (OA), *β*-glucose (*β*-Glc), aglycon II (ID), *β*-fructose (*β*-Frc), and α-glucose (α-Glc).

Subsequent to the NMR experiments, three small aliquots of the NMR sample (equilibrium mixture of secalosides A and B and ca. 12% w/w of the impurity mentioned above) were subjected to mild basic hydrolysis in 0.5 M NaOH. Determination of D-glucose and D-fructose in the neutralized hydrolysates gave negligable quantities (<0.5 mol %) of these sugars. Enzymatic hydrolysis with *β*-fructosidase (invertase) followed by redetermination of D-fructose and D-glucose gave reproduceable contents of 78.8 and 79.9%, respectively, of the amount expected for structure **1**. Considering the impurity found in the NMR spectra and the formation of several minor byproducts in the hydrolysis (HPLC), these values are close to the theoretical yields expected for the presence of one saccharose unit per molecule.

NOESY spectra measured in order to determine the relative configuration of stereogenic centers in aglycon II did not show any interpretable cross-peaks because of unfavorable correlation times. ROESY was used as the best alternative. Only correlations between protons that are not part of the same spin system will be discussed here because they are less prone to the

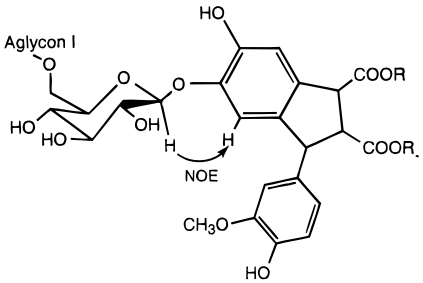
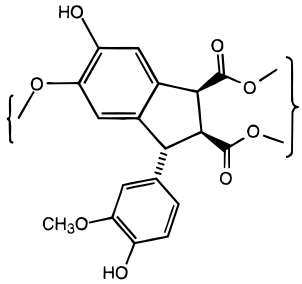


Figure 2. Conformational detail of secaloside A.

Chart 3. Relative Configuration of the Stereogenic Centers in Aglycon II (One of the Two Possible Absolute Configurations Shown)



common ROESY artifacts due to scalar coupling. NOE's between H(OA-4′) and both protons of the methylene group CH_2(OA-2) confirmed that the nitrogen-containing subunit (aglycon I) has indeed the structure suggested by Jaton *et al.*,[1] i.e., 2-oxindole-3-acetic acid. The six-membered ring of the alternative structure, which was isolated after acidic hydrolysis, i.e., 2-oxo-1,2,3,4-tetrahydroquinoline-4-carboxylic acid, is inconsistent with this effect.

A NOE between H(*β*-Glc-1) and H(ID-4) proved that the *β*-glucose unit is attached to C(ID-5), not C(ID-6), of aglycon II. It also showed that the conformation depicted schematically in Figure 2 is populated.

ROE's observed between the protons H(ID-2′) and H(ID-6′) of the phenyl ring and H(ID-1) and H(ID-2) of the cyclopentane unit allowed the determination of the relative configuration at the three stereogenic centers in this five-membered ring. They are consistent only with a configuration where H(ID-1) and H(ID-2) are *cis* oriented and both *trans* to H(ID-3) as shown in Chart 3.

The NMR parameters of secaloside B are provided in Table 1. Because secaloside B was the minor isomer, the set of observed scalar shift correlations was not as complete for this isomer as for secaloside A. However, all observed correlations were completely analogous to

those for isomer A, which indicated that both isomers have the same constitution. To the best of our knowledge, secalosides A and B are epimers at C(OA-3′), the stereogenic center of aglycon I. This is consistent with the observation that H(OA-3′) is the only proton that exchanged with deuterium of the solvent CD_3OD.

The largest differences in chemical shift between the two isomers were observed in the acetic acid residue of aglycon I, in the β-glucose unit, and surprisingly, for H(ID-3) of aglycon II. Within the 10-membered bislactone ring, the CH_2(β-Fruc-6) resonances of the fructose moiety also showed significant chemical shift differences. These observations suggest that aglycon I is stacked onto aglycon II from the side of H(ID-3) (*exo*) in one of the isomers and that epimerization at C(OA-3′) prevents or changes this stacking in the other isomer. However, no NOE's were observed between the aromatic protons of aglycon I or H(OA-3′) on one hand and H(ID-3) or the protons of the phenyl ring on the other hand. Therefore, our interpretation remains hypothetical.

The NMR study presented here allowed to determine the constitution of secalosides A and B unambiguously and confirmed that the two isomers are epimers at C(3′) of aglycon I. However, the configurational relationship between aglycon I, aglycon II, and the sugar moieties could not be deduced from the available NMR data. Therefore, the assignment of the two epimers to the *(R)*-C3′ and *(S)*-C3′ configuration in aglycon I, respectively, as well as the absolute configuration of aglycon II remain open.

Experimental Section

Sample Preparation. The sample for the NMR study was isolated and purified from commercially available Cernilton as described in the preceding paper.[1] An aliquot (16.8 mg) of the isolate was dissolved in 0.6 mL of CD_3OD. In the initial ^{1}H-NMR spectrum, taken less than 30 min after dissolution, the ratio of secalosides A and B was *ca*. 10:1. Signals at 1.33, 1.34, 1.42, 4.74, and 4.93 ppm in the ^{1}H-NMR spectrum and at 24.2, 24.33, 24.51, 73.2, and 77.29 ppm in the ^{13}C-NMR spectrum belonged to an unidentified impurity that integrated to ca. 60% of secaloside A on a per H basis. These signals did not change with time and did not show any correlations with those of the secalosides in the 2D spectra.

Nuclear Magnetic Resonance. 1D- and 2D-NMR spectra of secalosides A and B were recorded on a Bruker AMXII-500 spectrometer (11.7 T) equipped with a pulsed-field gradient unit and a ^{1}H-observe, X-decouple 5 mm probehead with z-gradient coil. 2D-NMR: all spectra were acquired with spectral widths of 8.47 ppm in the ^{1}H- and of 248 ppm in the ^{13}C-dimensions. DQF.COSY with pulsed-field gradient (PFG) coherence selection.[2] Acquisition: 4K(t_2) × 1K-(t_1) data points; two scans per increment. Processing: zero filling and FT to 2K × 1K real/real datapoints after multiplication with $\sin^2$ filter shifted by $\pi/3$ in ω_2 and $\cos^2$ filter in ω_1. HSQC with PFG.[3] Acquisition: 2K-(t_2) × 512(t_1) datapoints; two scans per increment. Processing: zero filling and FT to 1K × 1K real/real datapoints after multiplication with $\cos^2$ filter in ω_2 and cos filter in ω_1. HMBC with PFG.[4] Acquisition: 4K-(t_2) × 611(t_1) datapoints; eight scans per increment. Processing: zero filling and FT to 2K × 1K real/real datapoints after multiplication with $\cos^2$ filter in ω_2 and Gaussian filter in ω_1. Power spectrum in both dimensions. TOCSY. Acquisition: 4K(t_2) × 512(t_1) datapoints; 16 scans per increment. MLEV-17 spin lock of 10 kHz field strength and 200 ms duration. Processing: zero filling and FT to 2K × 1K real/real datapoints after multiplication with $\sin^2$ filter shifted by $\pi/3$ in ω_2 and $\cos^2$ filter in ω_1. ROESY.[5] Acquisition: 2K(t_2) × 416-(t_1) datapoints; 32 scans per increment. CW spin lock of 3.3 kHz field strength and 200 ms duration, flanked by 2.5 ms trim pulses. Processing: zero filling and FT to 1K × 1K real/real datapoints after multiplication with $\sin^2$ filter shifted by $\pi/3$ in ω_2 and $\cos^2$ filter in ω_1.

Basic Hydrolysis of the Equilibrium Mixture of Secalosides A and B. The NMR sample was evaporated to dryness and dried under vacuum at room temperature. Of the residue, 0.434 mg was dissolved in 0.5 mL of 0.5 N NaOH and stirred at room temperature for 5 h. The hydrolysate was brought to pH 6−7 with 0.21 mL of 0.1 N HCl and diluted to 0.7 mL. This procedure was repeated twice with similar amounts of material to give three samples for the enzymatic analysis.

Enzymatic Determination of Saccharose. Analysis for saccharose was performed using a commercial enzyme test (Boehringer-Mannheim Cat. No. 716 260),[6] which consisted of tests for D-glucose, D-fructose, and a reagent for the enzymatic hydrolysis of saccharose with β-fructsidose (isomerase). For calibration, pure saccharose was subjected to identical basic hydrolysis conditions and determined through the enzyme test. Each of the three samples from hydrosis was subjected to the enzymatic analysis five times.

Control analysis of the hydrolysate from the secaloside sample for D-glucose and D-fructose before enzymatic cleavage of saccharose gave less than 0.002 μmol of each (<0.5 mol % based on **1**) of these sugars. After enzymatic hydrolysis with β-fructosidase (isomerase), 0.346 μmol of D-glucose (79.9%) and 0.341 μmol of D-fructose (78.8%) were found.

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References and Notes

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