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Two glucosylated abscisic acid derivates from avocado seeds (*Persea americana* Mill. Lauraceae cv. Hass)

María del Refugio Ramos[a,b,c], Gerold Jerz[a], Socorro Villanueva[c], Fernando López-Dellamary[b], Reiner Waibel[d], Peter Winterhalter[a,*]

[a]*Institute of Food Chemistry, Technical University of Braunschweig, Schleinitz-Strasse 20, D-38106 Braunschweig, Germany*
[b]*University of Guadalajara, CUCEI, Department of Wood, Cellulose and Paper. Las Agujas S/N Zapopan, Jal., Mexico*
[c]*Research Center of Investigation, Technical and Design Assistance of Jalisco State, Av. Normalistas 800 C.P., 44270 Guadalajara, Jal., Mexico*
[d]*Institute of Medicinal Chemistry, University of Erlangen-Nürnberg, Schuhstrasse 19, D-91052 Erlangen, Germany*

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Abstract

Phytochemical investigation of avocado seed material (*Persea americana* Mill., Lauraceae) resulted in the isolation of two glucosylated abscisic acid derivates. One of these was not known as a natural product and can be regarded as a potential 'missing link' in abscisic acid metabolism in plants. After fractionation by high-speed countercurrent chromatography, and multiple steps of column chromatography, structures were elucidated by 1D-, 2D-NMR, electrospray-MS to be the novel β-D-glucoside of (1′*S*,6′*R*)-8′-hydroxyabscisic acid, and (1′*R*,3′*R*,5′*R*,8′*S*)-*epi*-dihydrophaseic acid β-D-glucoside. Absolute configuration was determined by circulardichroism, optical rotation, and by NOE experiments.

Keywords: *Persea americana* Mill. (cv. Hass) Lauraceae; Avocado seeds; Abscisic acid β-D-glucoside; (1′*S*,6′*R*)-8′-Hydroxyabscisic acid β-D-glucoside; (1′*R*,3′*R*,5′*R*,8′*S*)-*epi*-Dihydrophaseic acid β-D-glucoside; High-speed countercurrent chromatography; Stereochemistry; Circulardichroism; Plant metabolism

1. Introduction

Avocado fruits (*Persea americana* Mill.) of the variety Hass are preferably cultivated in Mexico due to a higher content of fruit flesh and superior sensory attributes. After industrial fruit processing, avocado seed material is generally disposed, although it could be a potential source for food supplements and medicinal products. It is noteworthy that ethnopharmacy of the aztec culture used decocts of avocado seeds as a potent agent to treat mycotic and parasitic infections. Also local anesthetic effects of avocado seeds preparations are known to decrease muscle pain (Argueta et al., 1994; Cabrera, 1996).

Previous phytochemical studies on avocado seeds identified various classes of natural products, such as phytosterols, triterpenes (Werman et al., 1990; Lozano et al., 1993), fatty acids with olefinic, and acetylenic bonds (Kashman et al., 1969), furanoic acids (Farines et al., 1995), dimers of flavanols (Geissman and Dittmar, 1965), and oligomeric proanthocyanidins (Thompson et al., 1972; Valeri and Gimeno, 1953), but no abscisic acid (ABA) components were detected before. Here, we report the first isolation and complete structure elucidation of two glucosylated abscisic acid derivates from avocado seed material. ABA and some of its derivates are important phytohormones in regulation of seed development controlling desiccation tolerance, storage product deposition, and dormancy (Krochko et al., 1998).

2. Results and discussion

2.1. Isolation of two glucosylated abscisic acid derivates from avocado seeds

Dried and milled avocado seeds were defatted with *n*-hexane and repeatedly extracted with methanol. For fractionation on preparative scale, we applied high-speed

* Corresponding author. Tel.: +49-0531-391-7200; fax: +49-0531-391-7230.
E-mail address: p.winterhalter@tu-bs.de (P. Winterhalter).

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countercurrent chromatography (HSCCC), a technique that is based on liquid–liquid partitioning effects. Since no solid support is used adsorbing effects on stationary phase material and artifact formation are eliminated (Ito and Conway, 1996). The preparative separation of the concentrated methanolic crude extract by HSCCC yielded nine major fractions (Fig. 1). For further preparation, fractions V, VIII, and IX were subjected to column chromatography on Sephadex LH20. The fractions were finally chromatographed on PVA 500 and silica gel to result in two glucosylated abscisic acid derivates, **1** and **2**, respectively.

2.2. Structure elucidation of (1′*S*,6′*R*)-8′-hydroxyabscisic acid β-D-glucoside (**1**)

Based on spectroscopic data, i.e. ^{1}H-, ^{13}C-, DEPT-NMR, including heteronuclear correlation experiments such as HMQC, and HMBC, compound **1** was identified as (1′*S*,6′*R*)-8′-hydroxyabscisic acid β-D-glucoside (**1**). Glucoside **1** was obtained as an ambar gum, $[\alpha]_D$ +196.3 (*c* 0.13, MeOH), and showed a quasimolecular ion $[M+Na]^+$ at *m*/*z* 465 (positive mode). The corresponding ion $[M-H]^-$ at *m*/*z* 441 (in negative ESI) verified the molecular weight, and further ESI-MS/MS fragmentation of this ion signal resulted in three abundant diagnostic ions: *m*/*z* 397 suggested a decarboxylation reaction (Δ*m*/*z* 44), ion *m*/*z* 330 was attributed to an α-fragmentation with cleavage of the olefinic side chain of the abscisic acid backbone, and loss of a hexose unit is indicated by the fragment ion *m*/*z* 161.

The ^{1}H NMR spectrum of **1** in MeOH-d_4 contained abundant singlet signals of three methyl groups [δ 1.08 (3H, *s*; C<u>H</u>$_3$-9′), 1.93 (3H, *s*; C<u>H</u>$_3$-7′), 2.01 (3H, *s*; C<u>H</u>$_3$-6)], four methylene signals [δ 2.41 (1H, *d*, *J* = 17 Hz; H-5′a), 2.66 (1H, *d*, *J* = 17 Hz; H-5′b), 3.62 (1H, *d*, *J* = 10 Hz, H-8′a), 3.97 (1H, *d*, *J* = 10 Hz; H-8′b)], and four signals of double bond protons [δ 5.78 (1H, s_{br}; H-2), 5.94 (1H, s_{br}; H-3′), 6.15 (1H, *d*, *J* = 16.5 Hz; H-5) and 7.75 (1H, *d*, *J* = 16.5 Hz; H-4)]. In the aliphatic region, coupling constants of *J* = 17 Hz elucidated two geminal protons, two olefinic resonances with a *J* = 16 Hz coupling confirmed a *trans*-configured double bond, whereas two remaining double bond singlet signals led no direct coupling partners. The complex oxymethine region integrated for six protons which belong to a glucose unit [δ 3.15 (1H, *dd*, J_1 = 8.0 Hz, J_2 = 8.5 Hz; H-2″), 3.24 (1H, m_{br}, H-4″), 3.27 (1H, *m*, H-3″), 3.66 (1H, *dd*, J_1 = 5.0 Hz, J_2 = 10.0 Hz; H-6″b), 3.85 (1H, *dd*, J_1 = 2.0 Hz, J_2 = 10.0 Hz; H-6″a)], and resonance δ 4.16 (1H, *d*, *J* = 8.0 Hz; H-1″) was assigned to the anomeric glucose proton, with a diaxially coupling constant of a β-glucosidic linkage.

The ^{13}C NMR spectrum revealed 20 carbon resonances, consisting of three methyl, three methylene, nine methine-groups, and five quaternary C-atoms, overall data suggested a glucosylated abscisic acid derivate. In comparison to abscisic acid (Millborrow, 1975a), substance **1** revealed a hydroxymethylene instead of four methyl groups, which was proposed to be the linkage to the glucose moiety. The quarternary nature of hydroxyl group C-1′ (δ 80.0) was verified by a DEPT135 experiment.

The constitution of structure **1** was unambiguously confirmed by heteronuclear correlation experiments such as ^{1}J-^{1}H{^{13}C}- and $^{2,3}J$-^{1}H{^{13}C}-COSY. All long-range correlations are summarised in Table 1, and structurally relevant correlations of the HMBC spectrum are presented in Fig. 2.

The $^{2,3}J$-CH cross peak from the anomeric sugar proton H-1″ (δ_H 4.16) elucidated the glucosidic linkage to the hydroxymethylene position C-8′ (δ_C 74.4). Several abundant cross signals corroborated the location of C-8′ (H-9′, H-5′a, H-5′b, H-1″) (Fig. 2).

Absolute stereochemistry of two chiral carbons at C-1′*S*, and C-6′*R* in the aglycone of **1** were derived by circulardichroism spectroscopy (CD), and multiple *diff*-NOe experiments (in MeOH-d_4).

The CD-curve of **1** showed two intense cotton effects at λ 233 nm and λ 266 nm (Fig. 3), and elucidated a 1′*S*-configuration for the tertiary hydroxyl function. This well known chiroptical effect for abscisic acid derivates was the key information for determining stereochemistry at the second chiral position C-6 (Hirai et al., 1986; Ohloff et al., 1973). Since steric flexibility of cyclohexenone rings is limited, such as for other abscisic acid derivates (Todoroki and Hirai, 2000), only two main conformers—envelope or inverted envelope type—are possible. Our results of multiple *diff*-NOE experiments on substance **1** are in agreement to NOE experiments reported for abscisic acid derivates (Millborrow, 1975b), and corroborate a predominant envelope-type conformer with the axially oriented side chain instead of this bulky substituent in equatorial orientation.

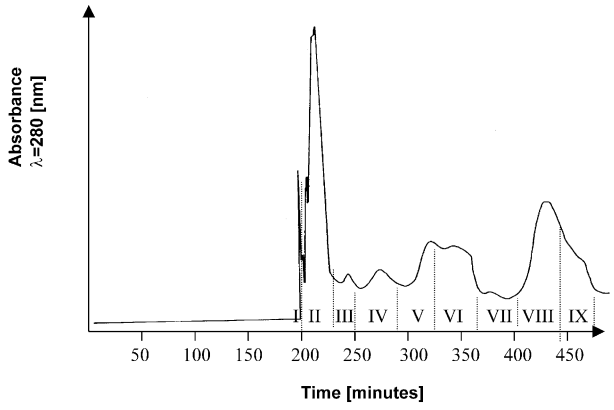

Fig. 1. HSCCC-separation of the methanolic avocado seed extract. Solvent system: *tert*.-butylmethylether–*n*-BuOH–MeCN–H_2O (1:3:1:5, v:v:v:v), flow rate: 2.5 ml min^{-1}. UV-trace: λ = 280 nm, velocity: 1000 rpm; mobile phase: lower aqueous phase; elution mode: head to tail.

Table 1
^{1}H-, *diff*-NOE, ^{13}C-, DEPT-NMR, and $^{2,3}J$-HC long-range correlations for compound **1** (δ [ppm]; J [Hz]) in MeOH-d_4

Position	^{1}H	*diff*-NOE	^{13}C	DEPT	$^{2,3}J$-HC correlation
1			n.d.	n.d.	
2	5.78 *s* $_{br}$	C$\underline{H}_3$-6	121.2	CH	C-4, C-6
3			150.0	C	
4	7.75 *d* (16.5)	n.d.	129.6	CH	C-1′, C-3, C-6
5	6.15 *d* (16.5)	C$\underline{H}_3$-6, H-5′$_a$, C$\underline{H}_3$-9′	136.3	CH	C-1′, C-2′, C-3, C-4
6	2.01 *s*	H-2, H-5	20.8	CH_3	C-2, C-3, C-4
1′			80.0	C	
2′			166.3	C	
3′	5.94 *s* $_{br}$	C$\underline{H}_3$-7′	127.9	CH	C-1′, C-4′, C-5′, C-7′
4′			200.5	C	
5′$_a$	2.41 *d* (17.0)	CH_3-9′, H-5, H-5′$_b$	45.2	CH_2	C-1′, C-4′, C-6′,C-8′, C-9′
5′$_b$	2.66 *d* (17.0)	H-5′$_a$			C-1′, C-3′, C-4′, C-6′, C-8′, C-9′
6′			46.6	C	
7′	1.93 *s*	H-3′	19.3	CH_3	C-1′, C-2′, C-3′
8′$_a$	3.62 *d* (10.0)	H-1″, H-8′$_b$, C$\underline{H}_3$-9′	74.4	CH_2	C-1″, C-5′, C-6′, C-9′
8′$_b$	3.97 *d* (10.0)	H-8′$_a$, C$\underline{H}_3$-9′			C-1′, C-1″, C-5′, C-6′, C-9′
9′	1.08 *s*	H-5, H-8′$_b$, H-5′$_a$	20.0	CH_3	C-1′, C-5′, C-6′, C-8′
1″	4.16 *d* (8.0)	H-8′$_a$	104.4	CH	C-8′
2″	3.15 *dd* (8.5, 8.0)		74.9	CH	C-1″, C-4″
3″	3.27 *		71.3	CH	
4″	3.24 *m* $_{br}$		77.8	CH	C-3″
5″	3.30 *		77.7	CH	
6″$_b$	3.66 *dd* (10.0, 5.0)		62.5	CH_2	
6″$_a$	3.85 *dd* (10.0, 2.0)				

* Signal overlapped by MeOH-d_4 signal, n.d. not detected.

Observation of strong NOE resonances (Fig. 4) between the side chain double bond proton H-5 (δ 6.15), protons of methyl group CH_3-9′ (δ 1.08), and also H-5′a (δ 2.41), clearly indicated that all interfering protons are located on the same side of the cyclohexene ring plane. This implies an equatorial orientation for CH_3-9′ (Fig. 4), and the olefinic side chain in axial orientation, hence the hydroxymethylene β-D-glucose moiety is located on the opposite side of the ring system, and is also axially oriented.

Relative stereochemical data gained by NOE-irradiation in combination with the absolute configuration at carbon 1′*S*, unequivocally determined a *R*-configuration for the second chiral position C-6′.

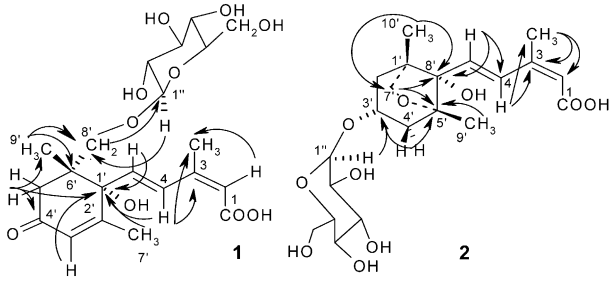

Fig. 2. Structure relevant long-range correlations in the HMBC of structures **1** and **2**.

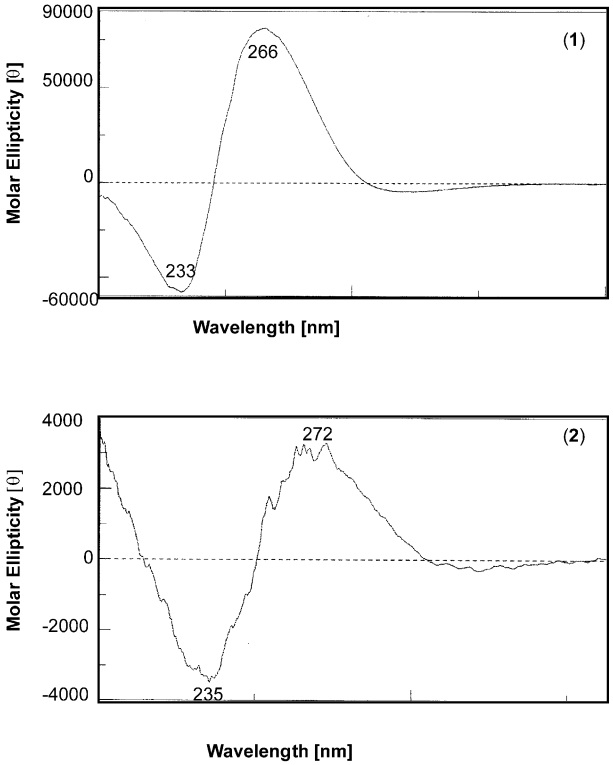

Fig. 3. CD-curves and cotton effects for *S*-configures chromophores of (1′*S*,6′*R*)-8′-hydroxyabscisic acid β-D-glucoside (**1**) and (1′*R*,3′*R*,5′*R*, 8′*S*)-*epi*-dihydrophaseic acid β-D-glucoside (**2**). For details see text.

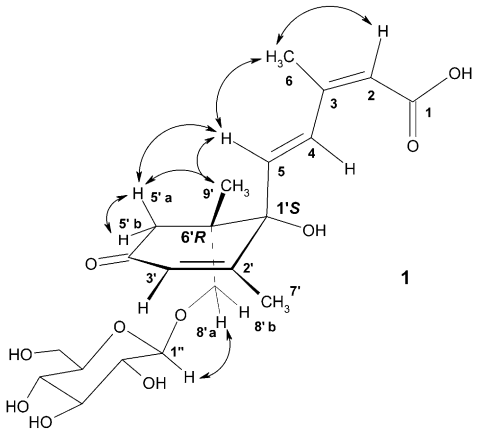

Fig. 4. Important NO-effects determining the stereochemistry of carbon 6′*R* in respect to the tertiary hydroxyl function at C-1′*S* of structure **1**.

Confirmation for the stereochemistry at C-6′ was obtained by selective irradiation of H-5′b (δ 2.66) which resulted in a NOE resonance to H-5′a (δ 2.41) but not in a through-space interaction to CH_3-9′.

The double bond configurations in the olefinic side chain were visible by selective NOE enhancements: *trans* configuration of Δ4-bond was observed by a significant NO effect between H-5 (δ 6.15) and CH_3-6 (δ 2.01), also irradiation of CH_3-6 (δ 2.01) resulted in a NOE for H-2 (δ 5.78) showing a *trans*-configuration of double bond Δ2. Also glucosidation at C-8′ was recognized by abundant NOE resonances between H-8a′ (δ 3.62), and the anomeric proton (δ 4.16) (Fig. 4) in vice-versa direction.

To the best of our knowledge the 8′-*O*-β-D-glucoside of (1′*S*,6′*R*)-8′-hydroxyabscisic acid isolated from avocado seed material is a novel natural product.

2.3. Structure elucidation of (1′*R*,3′*R*,5′*R*,8′*S*)-epi-dihydrophaseic acid β-D-glucoside (**2**)

^{1}H-, ^{13}C-, DEPT NMR, and heteronuclear correlation experiments (HMQC, HMBC) (Fig. 2) led to the identification of compound **2**, a β-D-glucoside of *epi*-dihydrophaseic acid, which was not isolated before from avocado seeds.

This compound was obtained as a brown transparent gum, $[\alpha]_D$ −5.0 (*c* 0.16, MeOH). ESI-MS in positive mode revealed a quasimolecular ion $[M+Na]^+$ at *m*/*z* 467, and negative ESI resulted in a $[M-H]^-$ signal at *m*/*z* 443, respectively. Therefore the M_r of compound **2** was 2 amu higher than for substance **1**. ESI-MS/MS of ion *m*/*z* 443 yielded two abundant ions: the formal loss of 18 mass units to fragment *m*/*z* 425 was correlated to a dehydration process, and due to the structure of **2**, ion *m*/*z* 237 is related to the cleavage of a glucose unit, and simultaneous decarboxylation.

In comparison to the ^{1}H NMR of substance **1**, the aliphatic region of **2** appeared highly complex, and signals in the carbinol region overlapped.

In detail, the ^{1}H spectrum in MeOH-d_4 indicated three singlet resonances [δ 0.90 (3H, *s*; C$\underline{H}_3$-10′), 1.15 (3H, *s*; C$\underline{H}_3$-9′), and 2.10 (3H, *s*; C$\underline{H}_3$-6)], five methylene protons [δ 1.78 (1H, *dd*, J_1 = 12 Hz, J_2 = 10 Hz; H-2′eq), 1.90 (1H, *dd*, *J* = 12 Hz; H-2′ax), 2.18 (2H, m_{br}; H-4′), 3.63 (1H, m_{br}; H-6″)], and 3.78 (2H, *d*, *J* = 11 Hz; H-7′a/H-3″), six carbinol protons, 3.10 (1H, *dd*, J_1 = 7 Hz, J_2 = 8 Hz; H-2″), 3.25 (1H, m_{br}; H-4″), 3.27 (1H, m_{br}; H-5″), 3.85 (1H, *d*, *J* = 10 Hz; H-6″), 4.23 (1H, m_{br}; H-3′)], and the anomeric signal δ 4.30 (1H, *d*, *J* = 8 Hz; H-1″) indicative for a β-glucosidic linkage.

Only three olefinic resonances [δ 5.70 (1H, s_{br}; H-2), 6.45 (1H, *d*, *J* = 16 Hz; H-5), and 7.90 (1H, *d*, *J* = 16 Hz; H-4)] appeared, proposing a similar substitution pattern, and double bond configuration as elucidated for the side chain moiety of **1**, Interestingly, the olefinic proton δ 5.94 of the cyclohexene ring system **1** was missing in **2**, hence a ring closure to a phaseic acid derivate was assumed.

Formal numbering of the carbon ring skeleton of phaseic acid derivate **2** is different to abscisic acids (Addicott, 1983), defining the chiral tertiary hydroxyl group to be C-8′ instead of carbon 1′ in structure **1**.

^{13}C NMR data of compound **2** revealed 20 carbon signals: three methyl, four methylene, nine methine, and four quarternary signals, but like in the case of previous studies (Champavier et al., 1999) the carboxylic function was not detected. Beside the tertiary hydroxyl group (δ_C 83.2) similar to structure **1**, a ^{13}C-DEPT135 experiment revealed the quarternary nature of a second tertiary oxymethine function (δ_C 87.6), and also an additional hydroxymethylene carbon (δ_C 77.2). Overall data—including 2D-HMQC and HMBC spectral results—elucidated *epi*-dihydrophaseic acid with a β-D-glucose moiety at position C-3′ (Fig. 2, Table 2).

The glucosidic linkage was recognized via a strong ^{2,3}J-CH cross signal from the anomeric sugar proton (δ_H 4.30) to the C-3′ (δ_C 74.0). Intense ^{2,3}J-CH-long-range cross signals proved the intramolecular ether ring formation in the dihydrophaseic acid structure: protons of CH_2-7′ (δ_H 3.78) correlated to C-1′ (δ_C 49.3), C-8′ (δ_C 83.2), and most important to C-5′ (87.6) (Fig. 2).

In good accordance to chiroptical reference data of abscisic acid derivates (Hirai et al., 1986), the CD-curve of *epi*-dihydrophaseic acid glucoside (**2**) (Fig. 3) showed two characteristic cotton effects (λ 235 and λ 272 nm), which were similar to the CD-effects of **1**, and clearly assigned an 8′*S*-configuration.

From a biosynthetic viewpoint, 8′-hydroxyabscisic acid β-D-glucoside (**1**) is a potential precursor of *epi*-dihydrophaseic acid and phaseic acid (Balsevich et al., 1994; Hirai and Koshimizu, 1983). Hence stereochemistry of the formed ether bridge in **2** should directly

Table 2
^{1}H-, *diff*-NOE, ^{13}C-, DEPT-NMR, and $^{2,3}J$-HC long-range correlations for compound **2** (δ [ppm]; J [Hz]) in MeOH-d_4

Position	^{1}H	*diff*-NOE	^{13}C	DEPT	$^{2,3}J$-HC correlation
1			n.d.	n.d.	
2	5.70 *s*	C<u>H</u>$_3$-6	120.5	CH	C-4, C-6
3			149.9	C	
4	7.90 *d* (16.0)	C<u>H</u>$_3$-9′, C<u>H</u>$_3$-10′	132.1	CH	C-2, C-3,C-6, C-8′
5	6.45 *d* (16.0)	C<u>H</u>$_3$-6, C<u>H</u>$_3$-9′,C<u>H</u>$_3$-10′, H-2′ax	134.4	CH	C-3, C-4,C-8′
6	2.10 *s*	H-2, H-5	21.1	CH_3	C-2, C-3,C-4
1′			49.3	C	
2′$_{ax}$	1.78 *m*	H-2‘eq, H-4‘ax, H-5	42.90	CH_2	
2′$_{eq}$	1.90 *m*	H-2′ax			C-3′
3′	4.23 *m* $_{br}$	H-1″	74.0	CH	
4′$_{ax, eq}$	2.18 *m* $_{br}$	C<u>H</u>$_3$-9′, H-2′ax	42.95	CH_2	C-2′, C-3′,C-5′, C-8′
5′			87.6	C	
7′	3.78 *m* $_{br}$	H-4″, C<u>H</u>$_3$-10′	77.2	CH_2	C-2′, C-5′,C-8′
8′			83.2	C	
9′	1.15 *s*		19.7	CH_3	C-4′, C-5′
10′	0.90 *s*	C<u>H</u>$_2$-7	16.3	CH_3	C-2′, C-7′,C-8′
1″	4.30 *d* (8.0)		103.1	CH	C-3′
2″	3.10 *dd* (7, 8)		75.1	CH	C-1″
3″	3.78 *d* (11)		78.0	CH	
4″	3.25 *		71.7	CH	
5″	3.27 *		78.1	CH	
6″$_a$	3.63 br	C<u>H</u>$_2$-7	62.8	CH_2	
6″$_b$	3.85 *d* (10.0)				

* Signal overlapped by MeOH-d_4 signal, n.d. not detected.

correlate to the likely precursor substance **1**. As a steric assumption for a successful ether ring formation in system **2**, hydroxymethylene carbon C-7′ has to be in axial orientation of the cyclohexane plane (Fig. 5). Ring closure is causing a partly rigid cyclohexane system, were H-2$_{ax}$, and H-4$_{ax}$, are preferably in axial orientation.

Selective NOE irradiation (in MeOH-d_4, 25 °C) on axial proton H-2′ax (δ 1.78) resulted in strong NO effects to H-4′ax (δ 2.18) and the olefinic proton H-5 (δ 6.45), clearly indicating that these interfering protons are located on the same side of the cyclohexane plane (Fig. 5). Equatorial orientation of CH_3-9′ (δ 1.15) was shown by NO enhancement of the olefinic proton H-5. There are no other stereochemical configurations which are eligible for the observed NO effects. Therefore the olefinic side chain has to be in axial orientation as well, and ether bridge CH_2-7′ to C-5′ is in *trans*-location, on the opposite side of the ring plane.

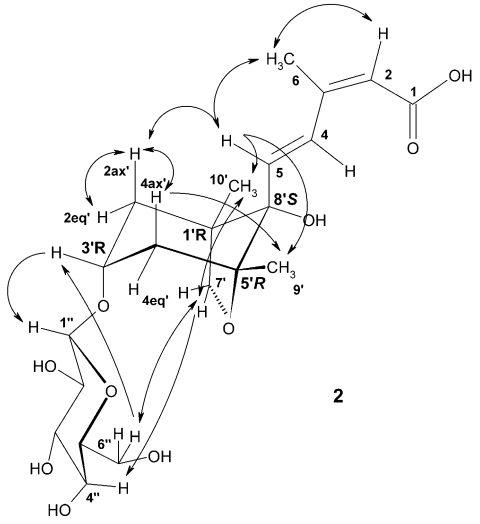

Fig. 5. Structure relevant NOE-resonances in compund **2** determining the absolute configuration of carbon 1′*R*,3′*R*, and 5′*R* in respect to the tertiary (8′*S*)-hydroxyl function, and a $^{4}C_1$-conformation of the glucose moiety.

A significant NO effect from the glucose proton H-6a″ (δ_H 3.63) to protons of the ether bridge CH_2-7′ (δ_H 3.78)—and in vice-versa direction also to H-4″ (δ_H 3.25)- elucidates that β-D-glucose unit and location of the ether ring are on the same side of cyclohexane plane. Interestingly, the observed NO effects are only possible if the glucose cyclohexane ring is in a $^{4}C_1$-conformation.

All NO effects observed for the olefinic side chain elucidated that both double bonds are *trans*-configured, similar to substance **1**.

Combining all results of the *diff*-NOE experiments with the known absolute configuration of carbon 8′*S*, elucidate the stereogenic C-atoms to be 1′*R*, 3′*R*, and 5′*R*.

The determined configurations of **1** and **2** are able to support the theory of a biosynthetical linkage between these components (Fig. 6). Enzymatic hydroxylation of abscisic acid at C-8′ (Krochko et al., 1998) leads to the highly instable intermediate 8-OH-ABA, which immediately

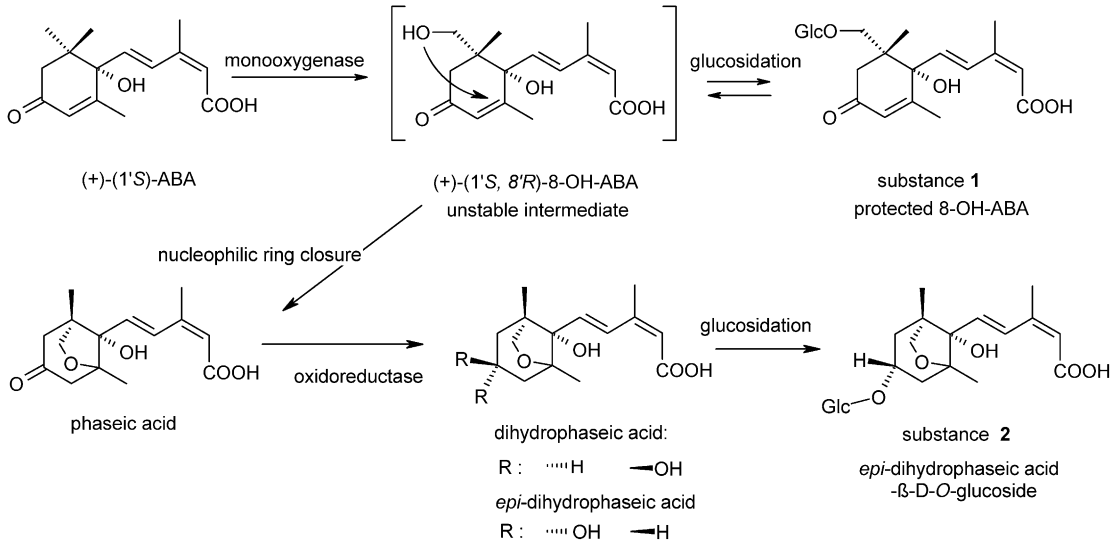

Fig. 6. Proposed pathway of substances **1**, and **2**.

reacts to the bicyclic phaseic acid. This metabolic pathway was assured by feeding experiments done with abscisic acid marked with [^{14}C], and [^{18}O] (Addicott, 1983).

Hence, we postulate that the newly isolated substance **1**, which is protected by glucosidation at C-8′—is a missing link—in the biosynthetical conversion of hydroxyabscisic acid structures to phaseic acid derivates.

Beside substance **2**, we screened for the glucoside of dihydrophaseic acid, which is the C-3′-epimer. But ESI-LC/MS analysis of the methanolic crude avocado seed extract, co-elution experiments, and selective ion extraction of *m*/*z* 443 detected solely substance **2**. This result implies that in avocado seeds the conversion of phaseic acid to the *epi*-dihydrophaseic acid might be a stereoselective oxidoreductase reaction.

3. Experimental

3.1. General experimental procedures

^{1}H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded on a Bruker AMX 300 spectrometer in MeOH-d_4 at 25 °C, and chemical shifts are given in δ values (ppm), based on those of the solvent signals (δ_H 3.31 and δ_C 49.0 ppm). The two-dimensional NMR experiments (HMQC, HMBC), and one-dimensional *diff*-NOE spectra were measured on a Bruker AM 360 (360 MHz for ^{1}H, and 90.6 for ^{13}C) in MeOH-d_4.

ESI-LC-MS, and MS^n experiments were performed on a BRUKER Esquire LC-MS-MS ion trap mass spectrometry system. For ESI MS-MS fragmentation studies, samples were introduced via a syringe pump at a flow rate of 240 μl min^{-1}, drying gas nitrogen (7.0 l min^{-1}, 330 °C), and nebulizer pressure (5 psi). ESI-MS (neg. mode): capillary +4500 V, end plate +4000 V, cap exit-90 V, cap exit offset −60 V, skim 1 −30 V, skim 2 −10 V; ESI-MS (pos. mode): capillary −4500 V, end plate −4000 V, cap exit +90 V, cap exit offset +60 V, skim 1 +30 V, skim 2 +10 V.

For HPLC-ESI-MS-MS analysis, a Hewlett Packard binary gradient pump G1312A was coupled to the ESI-LC-MS system. ESI-MS (neg. mode): dry gas nitrogen 9.0 l min^{-1}, nebulizer pressure 40 psi, capillary +3500 V, end plate +3000 V, capillary exit −95 V, skim 1 −25 V, skim 2 −10 V. Column for HPLC-MS was a Prontosil C18 Aqua, 5 μm, 250 × 2.0 mm (Bischoff), flow rate was 0.25 ml min^{-1}, and eluents were nanopure® water (solvent A), and acetonitrile (solvent B). Initial conditions of the gradient were 97% A, and 3% B, hold over 10 min, starting a linear gradient in 30 min to 40% A, and 60% B, in 15 min to 0% A, and 100% B for 10 min.

Specific optical rotations $[\alpha]_D$ were measured on a Perkin-Elmer polarimeter type-24 at 21 °C ($\lambda = 589$ nm, cell length: 10 cm). Curves of CD and ORD were recorded on a Jasco spectropolarimeter type J-715 at 21 °C (scan range: $\lambda = 200$–450 nm, cell length: 0.1 cm). Fractionation of the methanolic extract was done on a high-speed countercurrent chromatograph (HSCCC) model CCC-1000 (Pharma-Tech Research Corp.) equipped with three preparative coils made of a polytetrafluoroethylene tubing (2.6 mm i.d. × 165 m), and connected in series (850 ml total volume). Further sample purification was done by multiple column chromatography steps on silica gel (Merck, 0.040–0.063 mm), on Sephadex LH-20 (Pharmacia), and on Fractogel PVA 500 (Merck). Thin-layer-chromatography was done on normal phase plates silica gel 60 F_{254} (Merck) with the solvent system (S1) CH_2Cl_2–MeOH–H_2O (74:25:5), and on reversed phase plates RP-18W (Macherey-Nagel) with system (S2) MeOH–H_2O (1:1). Visualization was

done with anisaldehyde–concentrated sulfuric acid–glacial acid (1:2:97), and flash heating (110 °C) on a hot plate.

3.2. Plant material

Seeds of freshly picked avocados (*Persea americana* Mill. cv. Hass) from Tingüindín Michoacán, México, were gently dried in an oven (40 °C), vacuum packaged and kept frozen until extraction.

3.3. Extraction and isolation of **1** and **2**.

Dried avocado seeds *P. americana* (1600 g) of the variety Hass were ground to powder in a laboratory mill, defatted with petrolether (F_p 55–75 °C) and macerated with freshly distilled methanol until exhaustion. After filtration all extracts were concentrated under vacuum at 35 °C. The methanolic extract was fractionated by preparative high-speed countercurrent chromatography (HSCCC). *Tert*.-butylmethylether (TBME)–*n*-BuOH–MeCN–H_2O (1:3:1:5, v:v:v:v) was used as solvent system. The separation was run at a revolution speed of 1000 rpm. The upper more lipophilic phase was used as the stationary phase, the lower aqueous phase was pumped as mobile phase at a flow rate of 2.5 ml min^{-1}, elution mode of the system was head to tail. Peak monitoring was done at $\lambda = 280$ nm with a UV-detector (Knauer, variable wavelength monitor) using a preparative cell. The high tannin content of the fraction—having a strong emulsifying effect—limited the maximum sample load to 3.2 g applicable to the HSCCC-system. The chromatography of the four separations was highly reproducible, and yielded fractions I to IX (Fig. 1). Fractions VIII and IX (51 mg) were combined due to results of TLC-visualization, and purified by CC on Sephadex LH-20 (1.5 cm i.d. × 70 cm) eluting with MeOH resulting in 2 mg of (1′*S*, 6′*R*)-*8*′-hydroxyabscisic acid β-D-glucoside (**1**).

For isolation of **2**, HSCCC fraction V (20 mg) was chromatographed on Sephadex LH-20 (1.5 cm i.d. × 70 cm) using MeOH, then subjected to a silica gel 60 column (1 cm i.d. × 15 cm) CH_2Cl_2- MeOH- H_2O (75:20:1), and finally purified on PVA 500 (1.5 cm i.d. × 40 cm) with MeOH to yield 2.5 mg of *epi*-dihydrophaseic acid-β-D-glucoside (**2**).

3.4. Characterization of (1′S,6′R,2Z,4Z)-5-[(1′-hydroxy-2′,6′-dimethyl-6′-hydroxymethyl-4′-oxo-8′-β-D-glucosyl)-cyclohex-2′-en-1′-yl]-3-methyl-penta-2,4-dienoic acid, (1′S,6′R)-8′-hydroxyabscisic acid β-D-glucoside (**1**)

Ambar gum compound **1** (2 mg). TLC: $R_f = 0.34$ (S1) and $R_f = 0.49$ (S2), anisaldehyde: grey-green, $[\alpha]_D$ +196.5 ($c = 0.13$, MeOH); ESI MS (neg) *m*/*z*: 883 $[2M-H]^-$, 441 $[M-H]^-$; MS^2 (883): 441, 331; MS^3 (441): 330, 179; ESI MS (pos) *m*/*z*: 907 $[2M+Na]^+$, 465 $[M+Na]^+$; MS^2 (907): 465; MS^3 (465): 447, 285; LC-MS: $R_t = 27.4$ min; CD (MeOH): $[\theta]_{266}$ +81 263, $[\theta]_{233}$ −57 162. ORD (MeOH): $[\Phi]_{245}$ −96501, $[\Phi]_{291}$ +41064. ^{1}H- and ^{13}C NMR (see Table 1).

3.5. Characterization of (1′R,3′R,5′R,8′S,2Z,4Z)-5-[(8′-hydroxy-1′,5′-dimethyl-3′-β-D-glucosyl-6′-oxabicyclo[3.2.1]oct-8′-yl)]-3-methyl-penta-2,4-dienoic acid, (1′R,3′R,5′R,8′S)-epi-dihydrophaseic acid β-D-glucoside (**2**)

A brown transparent gum (2.5 mg). TLC : $R_f = 0.16$ (S1), $R_f = 0.79$ (S2), anisaldehyde: grey-green, $[\alpha]_D$ −5.0 ($c = 0.16$, MeOH); ESI MS (neg) *m*/*z*: 443$[M-H]^-$; MS^2 (443): 425, 237; ESI MS (pos) *m*/*z*: 467 $[M+Na]^+$; MS^2 (467): 449, 287; LC-MS: $R_t = 22.4$ min; CD (MeOH): $[\theta]_{272}$ +3310, $[\theta]_{235}$ −3481. ORD (MeOH): $[\Phi]_{252}$ −4312, $[\Phi]_{294}$ +1575. ^{1}H and ^{13}C data (see Table 2).

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