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METABOLISM OF THE PLANT GROWTH REGULATOR DIHYDROJASMONIC ACID IN BARLEY SHOOTS

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Key Word Index—*Hordeum vulgare*, Gramineae, barley, (±)-9,10-dihydrojasmonic acid metabolism, (−)-9,10-dihydro-11ξ-hydroxyjasmonic acid, (−)-9,10-dihydro-12-hydroxyjasmonic acid, (−)-9,10-dihydro-11ξ-hydroxyjasmonic acid *O*(11)-β-D-glucopyranoside

Abstract—The biotransformation of (±)-9,10-dihydrojasmonic acid (DJA) was studied in six-day-old barley seedlings, Both [2-^{14}C] and [U-^{3}H]DJA were fed to excised shoots and the formed metabolites analysed after 72 hr DJA was converted into two major and some minor metabolites, purified by chromatographic methods The major metabolites were identified mainly by spectroscopic investigations as (−)-9,10-dihydro-11ξ-hydroxyjasmonic acid and its *O*(11)-β-D-glucopyranoside To a lesser extent (−)-9,10-dihydro-12-hydroxyjasmonic acid was also found

INTRODUCTION

(−)-Jasmonic acid [(−)-JA, **1**] and its methyl ester (**2**) were found to be widespread in plants [1] and are considered to be members of a new type of endogenously occurring plant growth regulators with hormone-like properties [cf. 2, 3].

A few studies dealing with the biosynthesis of jasmonic acid have been published [4, 5]. However, knowledge of its metabolism in plants is very limited [6–8]. Reports on the plant growth regulating activities of 9,10-dihydrojasmonic acid (DJA, rac-**3**) [9–11] and recent results demonstrating its natural occurrence in broad bean fruits [12] prompted us to start tracer experiments on the metabolism of radiolabelled rac-**3**

In this paper we report studies on the isolation and structural elucidation of the major metabolities formed in barley shoots from exogenously applied [2-^{14}C](±)-DJA. Some preliminary results obtained in experiments with [2-^{14}C](±)-JA are also presented.

RESULTS AND DISCUSSION

After a feeding period of 72 hr exogenously applied radiolabelled (±)-DJA or (±)-JA was taken up by excised barley shoots in the range of *ca* 90% The radioactivity was almost completely extracted by 80% methanol from the plant material After evaporation of methanol the remaining aqueous phase was subsequently partitioned with *n*-hexane and ethyl acetate (pH 2, 5). The radiolabelled compounds were found to be distributed to *ca* 60% in the ethyl acetate and to *ca* 40% in the aqueous phase.

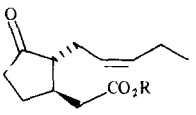
In studies with [2-^{14}C](±)-DJA (rac-**3**), in addition to the starting compound DJA six radioactive zones (E1–E6) were found in the ethyl acetate extract by TLC, system I, as detected by radioscanning. With respect to the total radioactivity of the methanol extract (100%) their relative amounts were: non-metabolized DJA (23.9%), E1 (8 6%), E2 (5.3%), E3 (4 2%), E4 (12 3%), E5 (4.0%), E6 (2 9%). Thus, component E4 represents the major metabolite in the ethyl acetate extract. The aqueous phase contained two metabolites (W1, W2) in relatively high amounts (20 5 and 18 3%, respectively) which were separated by TLC in system IV.

Feeding [2-^{14}C](±)-JA (rac-**1**) instead of radiolabelled rac-**3** a similar pattern of metabolites both in the ethyl acetate extract and the aqueous phase was observed suggesting the metabolic fates of both compounds being closely related to each other in barley shoots However, JA was metabolized faster than DJA After 72 hr the applied [^{14}C]JA had disappeared totally, whereas in the corresponding experiment using [^{14}C]DJA *ca* 24% remained still unmetabolized and disappeared only after *ca* 96 hr. At that time the level of E3 and E4 was decreased, and simultaneously more polar products were formed.

Additional feeding experiments using either [U-^{3}H]DJA or [2-^{14}C]DJA or a mixture of both gave about the same pattern of radiolabelled metabolities. The [^{3}H]/[^{14}C] ratio of the isolated metabolites was nearly the same as that of the labelled DJA applied to the plants Thus, the basic structure of the major metabolites should be closely related to DJA The isolation of the metabolites contained in the EtOAc-extract and the remaining aqueous phase was achieved by DEAE-Sephadex A-25 chromatography, preparative TLC, CC on LiChroprep RP 18, followed by HPLC (see Experimental).

As shown by the following data, the structures of the major metabolites E4 and W1 of DJA (rac-**3**) in barley shoots were elucidated (for E4) as a 9 : 1 mixture of (−)-(3*R*, 7*R*)-9,10-dihydro-11ξ-hydroxyjasmonic acid (**5**) and (−)-(3*R*, 7*R*)-9,10-dihydro-12-hydroxyjasmonic acid (**8**), and (for W1) as (−)-(3*R*, 7*R*)-9,10-dihydro-11ξ-hydroxyjasmonic acid *O*(11)-β-D-glucopyranoside (**11**)

Thus, the mass spectrum of E4-Me obtained by esterification of E4 with diazomethane shows a molecular ion at *m/z* 242 and key fragments of type *a* and *b* characterizing a hydroxylated dihydrojasmonic acid methyl ester



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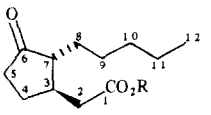
2 R = Me



3 R = H
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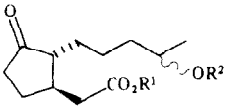
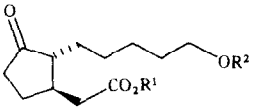
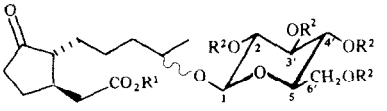


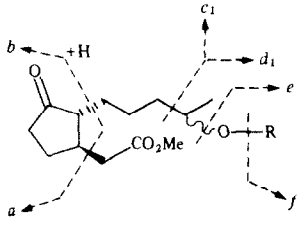
5 R^1 = H, R^2 = H
6 R^1 = Me, R^2 = H
7 R^1 = Me, R^2 = TMSi



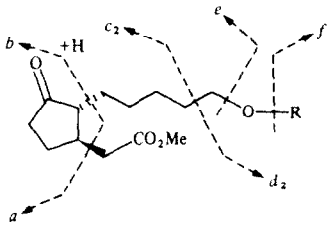
8 R^1 = H, R^2 = H
9 R^1 = Me, R^2 = H
10 R^1 = Me, R^2 = TMSi



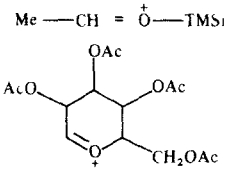
11 R^1 = H, R^2 = H
12 R^1 = H, R^2 = Ac
13 R^1 = Me, R^2 = Ac



6 R = H
7 R = TMSi
13 R = β-D-(2,3,4,6-tetra-O-acetyl)-glucopyranosyl



9 R = H
10 R = TMSi



7 *m/z* 117 (d_1)

13 *m/z* 331 (*f*)

Scheme 1 Mass spectral fragmentation of the DJA-derivatives **6**, **7**, and **13** and of the isomers **9** and **10**, respectively

(Scheme 1) The hydroxy group is located in the side chain because ion *b* (*m/z* 156) is not shifted compared with methyl jasmonate or methyl dihydrojasmonate [13, 14] The exact position of the hydroxy group was determined by GC/MS of the trimethylsilyl ether of E4-Me demonstrating the existence of the two isomers **7** and **10** which are hydroxylated in position C-11 and C-12, respectively The ratio of **7** to **10** is in the range of *ca* 9 1 In component **7** the α-cleavage at C-11 gives the complementary key ions $\mathbf{c}_1$ (*m/z* 197) and $\mathbf{d}_1$ (*m/z* 117, base peak) (Scheme 1) This is in accordance to the mass spectral behaviour of branched aliphatic trimethylsilyl ethers [15] The C-12 hydroxylated isomer **10** is characterized by ions at *m/z* 103 ($\mathbf{d}_2$, $CH_2=\overset{+}{O}TMSi$) and *m/z* 211 ($\mathbf{c}_2$), which are formed by an α-fission at C-12

In accordance, the ^1H NMR spectrum of E4-Me showed the occurrence of an isomeric mixture of **6** and **9** in the ratio of *ca* 17 3 The position of the hydroxy group in the side chain of each isomer was determined by analysing the signal pattern of the proton(s) attached to the hydroxy substituted carbon atom and of the methyl group, respectively The major component was identified as the 11-hydroxy isomer **6** characterized by the doublet of the methyl group at δ1 17 and the multiplet (*tq*) of the methine proton H-11 at 3 79 This was proved by spin decoupling. The minor component, which showed no C-methyl signal, but a triplet (2H) at δ3 62, was recognized as the 12-hydroxy isomer **9** In addition this was proved by comparing the chemical shift values of the 12-protons and of the adjacent protons (identified by a spin decoupling difference spectrum) with those of simple aliphatic alcohols [16]

Derivatives of C-12 hydroxylated jasmonic acid, like the jasmine ketolactone [17] and *N*-(12-acetoxy) jasmonoyl-phenylalanine methyl ester [18], have been isolated as endogeneous compounds from *Jasminum grandiflorum* and *Praxelis clematidea*, respectively On the contrary, DJA exogenously applied to barley shoots, was mainly hydroxylated in position C-11

The $[\alpha]_D$ of E4-Me is slightly negative (−10 7°) and in the same order of magnitude as for (−)-DJA-Me [19] The ORD curve showed a negative Cotton effect with extrema at 312 nm and 274 nm and zero rotation at 296 nm as described for (−)-JA-Me [20] These results indicate that from racemic **3** the (−)-isomers (−)-9,10-dihydro-11ξ-hydroxyjasmonic acid (**5**) and (−)-9,10-dihydro-12-hydroxyjasmonic acid (**8**) in a ratio of *ca* 9 1 are formed preferentially However, the presence of the (+)-isomers is low amounts can not be excluded The polar metabolite W1 (**11**) could be esterified with diazomethane and acetylated, indicating a free COOH-group and OH-group(s), respectively By enzymatic hydrolysis with β-glucosidase it was cleaved to an aglycone cochromatographing with metabolite E4 (**5**, **8**) in TLC and HPLC Additionally, it gave the same reduction product with sodium borohydride as E4 By GC/MS of the trimethylsilyl ether, the aglycone was identified as component **5** of metabolite E4 The sugar moiety was characterized as glucose by glucose-oxidase-peroxidase reaction[21] Only treatment with β-glucosidase was successful in hydrolysing W1 (**11**), α-glucosidase was not effective, demonstrating a β-glucoside linkage

The positive and negative ion mass spectra of peracetylated W1-Me (**13**) reveal a glucoside of a hydroxylated DJA, as indicated by ions of type **a**, **b**, **e** and **f** The mass spectral behaviour of compound **13** is mainly

characterized by bond cleavages at the glucosidic linkage leading to the key ions **e** and **f** (Scheme 1). The presence of a glucosyl moiety is evidenced by the appearance of the oxonium ion **f** (*m/z* 331) and ions deriving from this fragment (*m/z* 289, 271, 229, 169, 127, 115, 109, 98 [22]). While the positive ion mass spectrum of **13** does not show a molecular ion, the negative one displays a $[M-1]^-$ ion at *m/z* 571 and intense ions originating by losses of acetyl and ketene units (see Experimental).

Glucosylation apparently took place only with the C-11 hydroxylated DJA (**5**). The 11-*O*-glucosyl position is proved by GC-MS of the methylated and trimethylsilylated aglycone (**7**) and confirmed in ^{1}H NMR studies of the peracetylated W1 (**12**) by the doublet structure of the methyl signals of C-12. The ^{1}H NMR spectrum additionally shows the existence of a mixture of (11*R*)- and (11*S*)-isomers in the ratio 2·1 or *vice versa* in the glucoside by the occurrence of two signals for the methyl group of the side chain (δ1 07 and 1 19) as well as for the anomeric sugar proton (δ4.53 and 4.56) A similar but less pronounced effect was also observed in the ^{1}H NMR spectrum of the methyl ester of the 9,10-dihydro-11-hydroxyjasmonic acid (**6**)

The $[\alpha]_D$ of W1 (**11**) was negative (−35.8°). The ORD curve exhibited, like that of the aglycone E4, a negative Cotton effect, supporting the favoured formation of the (−)-isomers of both metabolites. The extrema of the ORD curve agreed with those reported for E4.

Thus, structural elucidation proved (−)-3*R*, 7*R*)-9,10-dihydro-11ξ-hydroxyjasmonic acid *O*(11)-β-D-glucopyranoside (**11**) to be the major component of the metabolite W1 The minor metabolites have only partly been characterized up to now. E5 consists of three amino acid conjugates of compound **3** The metabolite fraction E3 contains also conjugates of this type derived from metabolite E4. Amino acid conjugates of jasmonic acid and related compounds have already been shown to occur naturally [13, 18, 23, 24]. Additionally, fraction E3 contains a compound having a cyclopentanol moiety, and thus, being structurally related to cucurbic acid, another plant growth regulator of the jasmonic acid type originally isolated from *Cucurbita pepo* [20, 25] Furthermore, an *O*-glucoside of this metabolite E3 is formed (W2)

Esters of E3, E4 and DJA were found as in metabolic studies using (−)-JA in tissue cultures of tomato, where a glucosyl ester of JA represents the major metabolite [8]. The identification of the minor metabolites formed from DJA and of the JA metabolites in barley shoots and other plant systems is in progress

EXPERIMENTAL

Radiochemicals [2-^{14}C](±)-Dihydrojasmonic acid (2 1 mCi/mM) and [2-^{14}C](±)-jasmonic acid (2 0 mCi/mM) were obtained by synthetic procedures already reported [26, 27]. [U-^{3}H]Dihydrojasmonic acid was prepared by tritium exchange [28] Radioactivity was measured by liquid scintillation counting, TLC plates were monitored with a radioscanner

Plant material and feeding experiments. Caryopses of barley (*Hordeum vulgare* L cv 'Certina') were germinated at 25–28° under greenhouse conditions in soil for 5 to 6 days The roots were cut off and 10 g (fr wt) shoots of *ca* 8 cm length were placed into a beaker containing 5 mg [^{14}C]-labelled DJA or JA (total radioactivity about 10^7 dpm) in 10 ml H_2O Feeding experiments with 5 mg [U-^{3}H]DJA were performed with a total radioactivity of *ca* 10^8 dpm The incubation was performed at 20° for 72 hr, in some cases for 24, 48 and 96 hr Large scale feeding experiments were carried out with 440 mg (±)-DJA supplied to 880 g barley shoots for 72 hr.

Isolation of radiolabelled metabolites After feeding the barley shoots were rinsed with H_2O, and the washings combined with the remaining feeding soln Aliquots were analysed for radioactivity which was used for calculation of the rate of uptake After homogenization of the shoots in MeOH the plant material was extracted with 80% MeOH The methanolic phase was concd *in vacuo* and the remaining aq phase frozen overnight, thawed and the ppt filtered off The filtrate was extracted x5 with *n*-hexane, which was discarded

The aq phase was acidified to pH 2 5 and extracted with EtOAc The dried EtOAc layer (Na_2SO_4) and the remaining aq phase were evapd All extracts were analysed for radioactivity The EtOAc-extract (E) and the aq phase (W) were separated by TLC on analytical and prep scale and yielded non-metabolized DJA (R_f = 0 92) as well as its radioactive metabolites E1 (R_f = 0 02), E2 (R_f = 0 12), E3 (R_f = 0 33), E4 (R_f = 0 55), E5 (R_f = 0 73) and E6 (R_f = 0 82) using system I and W1 (R_f = 0 63) and W2 (R_f = 0 78) using system IV

The radioactive extracts also served as internal marker added to the corresponding extract of the non-radioactive large scale feeding experiments, in which 880 g barley shoots were extracted as described above The EtOAc-extract (residue 1 65 g) was purified by CC on DEAE-Sephadex A-25 (50 × 2 0 cm) using a gradient of HOAc in 80% aq MeOH [29] The greatest proportion of radioactivity was eluted from the column with 0 5 M HOAc in MeOH This fraction (77 mg) was subjected to prep TLC, system II, yielding mainly 2 radioactive zones, corresponding to E4 (R_f = 0 50) and non-metabolized DJA (R_f = 0 86), which gave by MeOH elution 24 mg E4 and 28 mg DJA, respectively

Further purification of E4 was achieved by CC (20 × 1 1 cm) on LiChroprep RP 18 using a discontinous gradient of MeOH in 0 2% HOAc, giving 10 4 mg E4 in the 50–60% MeOH containing fraction It was methylated with CH_2N_2 and finally separated by prep HPLC, system I At R_t = 14 1 min, 4 7 mg of E4-Me were received for identification About 1/10 equivalent of E4-Me was trimethylsilylated for GC/MS analysis.

The fraction corresponding to DJA was methylated, too, and further purified by prep TLC, system V, yielding 11 2 mg DJA-Me at R_f = 0 54, identified by GC/MS R_t value (3.4 min) and fragmentation pattern were identical with those of authentic rac-**4** and lit data [13]

The aq residues, obtained after EtOAc-extraction of several feeding studies were combined and subjected to prep TLC, system IV, resulting in 2 radioactive bands W1 and W2 W1 was eluted with MeOH and rechromatographed on cellulose plates, system VI (R_f = 0 80) After extraction with MeOH, W1 was separated on a DEAE-Sephadex A-25 column (50 × 1 1 cm) by elution with 0 5 M HOAc in 80% aq MeOH and subsequently purified by prep HPLC, system II (R_t = 7 4 min) HPLC gave 12 2 mg W1, which was characterized by $[\alpha]_D$, ORD and enzymatic hydrolysis as well as MS after methylation and peracetylation and ^{1}H NMR after peracetylation Prior to ^{1}H NMR purification of the acetylated W1 by prep TLC had been performed in system III (R_f = 0 53)

TLC Silica gel GF_{254} for analytical and PF_{254} = 1 mm for prep TLC Solvent systems (I) $CHCl_3$–EtOAc–HOAc (5 4 1), × 3, (II) $CHCl_3$–EtOAc–HOAc (5 4 1), twice, (III) $CHCl_3$–EtOAc–HOAc (5·4 1), once, (IV) PrOH–H_2O (3 1),

× 3, (V) *n*-hexane–EtOAc–HOAc (60 40 1), once Cellulose MN 300, Macherey Nagel, 1 mm, prewashed with *t*-BuOH HOAc H_2O (3 1 1), system VI, *t*-BuOH HOAc H_2O (3 1 1) Detection by radioscanning or spraying with anisaldehyde reagent and heating for 5–10 min at 120 [30]

HPLC For both analytical and prep HPLC a LiChrosorb RP 18 column (4.6 × 250 mm), was used Solvent systems I, MeOH–H_2O (9 11) II, MeOH–H_2O–HOAc (200 300 1), III, MeOH–H_2O–HOAc (250 250 1) Flow rate 0.6 ml/min UV detector, 228 nm, radiodetector

Mass spectroscopy The positive (10–16 eV) and negative (2–4 eV) ion mass spectra were obtained with an electron attachment mass spectrograph 'M v Ardenne'

GC-MS This was performed with MAT 111 GC/MS system (electron energy 80 eV), GC steel column (1 5 m × 2 mm) containing 3% OV-225 on Gaschrom Q (100 120 μm), He at 15 ml/min, column temp 180 °, EID

^1H *NMR* 200 MHz, $CDCl_3$, TMS as int standard

Hydrolysis of the glucoside **11** Aliquots of W1 were enzymatically hydrolysed with cellulase (24 hr, 37°, McIlvain buffer, pH = 3 0), the resulting aglycone was extracted with EtOAc, the sugar component in the aq layer determined by glucose-oxidase-peroxidase reaction [21]

The aglycone was compared by TLC, system II and HPLC, system III with the metabolites occuring in the EtOAc-extract Finally it was methylated and trimethylsilylated (Sil-Prep) for GC/MS analysis In addition, a part of W1 was treated in McIlvain buffer with α-glucosidase from yeast at pH = 6 0, another one with β-glucosidase from almonds at pH = 5 0 followed by glucose determination according to the described method

Identification of metabolites

E4-Me, mixture of isomers of methyl(−)-(3R, 7R)-9,10-dihydro-11ζ-hydroxyjasmonate (**6**), *and methyl (−)-(3R, 7R)-9,10-dihydro-12-hydroxyjasmonate* (**9**). $[\alpha]_D^{25}$ − 10 7° (MeOH, *c* 0 243), ORD (MeOH, *c* 0 115) $[\phi]_{274}$ + 631 $[\phi]_{296}$ 0 , $[\phi]_{312}$ − 716 , IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3610 (OH), 1730 (CO_2Me, *c* 0). ^1H NMR δ 1 17 (3H, *d*, *J* = 6 3 Hz, H-12), 1 2–2 7 (15H), 3 79 (1H, *t*, *q*, *J* = *J'* = 6 3 Hz, H-11), 3 69 (3H, s, OMe), characterizing **6**, ^1H NMR δ 1 2–2 7 (16H), 3 62 (2H, *t*, *J* = 6 4 Hz, H-12), 3 69 (3H, s, OMe), characterizing **9** MS *m/z* (rel int), 242 [M]$^+$ (5), 224 [M − H_2O]$^+$ (1), 211 [M − OMe]$^+$ (4), 209 (5), 198 (3), 182 (3), 169 (**a**, 20), 156 (**b**, 81), 151 [**a** − H_2O] (35), 137 (10), 125 (22), 109 (29), 96 (27), 83 [**a** − $C_5H_{10}O$] (100), see Scheme 1

Silylation of E4-Me with Sil-Prep for GC/MS analysis gave a mixture of **7** and **10** which were separated by GC R_t = 4 2 min for **7**, R_t = 6 8 min for **10**, MS (80 eV), **7** (TMSi-derivative of **6**) *m/z* (rel int), 314 [M]$^+$ (1), 299 [M − Me]$^+$ (5), 255 (3), 243 (6), 241 (**a** and [M − TMSi]$^+$) (6), 225 (**e**, 10), 197 (**c**$_1$, 24), 169 (6), 156 (**b**, 7), 151 (20), 133 (14), 117 (**d**$_1$, 100), 83 (12), 79 (16), 75 (52), 73 (**f**, 87), MS (80 eV), **10** (TMSi-derivative of **9**) *m/z* (rel int) 299 [M − Me]$^+$ (3), 241 (**a** and [M − TMSi]$^+$) (5), 228 (9), 225 [M − OTMSi]$^+$ (19), 211 (**c**$_2$ 5), 169 (5), 156 (**b**, 17), 151 [**a** − TMSiOH] (22), 133 (23), 117 (12), 103 (**d**$_2$, 14), 83 [**a** − TMSiOH]-C_5H_8] (40), 79 (37), 75 (100) 73 (**f**, 50) see Scheme 1

W1 [(−)-(3R,7R)-9,10-*dihydro*-11ζ-*hydroxyjasmonic acid* 0(11)-β-D-*glucopyranoside* (**11**)] $[\alpha]_D^{25}$ − 35 8 (MeOH, *c* 0 555), ORD (MeOH, *c* 0 124) $[\phi]_{274}$ + 566 , $[\phi]_{296}$ 0 , $[\phi]_{312}$ − 629 , W1, **11** (3 7 mg) was acetylated and methylated to give **13** See Scheme 1

MS (10 − 16 eV, positive ions) *m/z* (rel int) 499 ([M − CH_2OAc]$^+$ and **a**) (1), 456 (2), 439 (2), 397 (2), 370 (2), 331 (**f**, 25), 289 (12), 271 (5), 247 (6), 242 (5), 239 (8), 229 (8), 225 (**e**, 100), 207 (17), 193 (16), 169 (39), 156 (**b**, 17), 151 (48), 142, (20), 133 (15), 127 (11), 115 (12), 109 (20), 98 (17), 83 (15), MS (2 − 4 eV, negative ions) *m/z* (rel int) 571 [M − 1]$^-$ (6), 529 [M − Ac]$^-$ (61), 487 [M − Ac − CH_2CO] (29), 455 [M − Ac − CH_2CO − MeOH]$^-$ (82), 413 [M − Ac − 2CH_2CO − MeOH]$^-$ (100), 371 [M − Ac − 3CH_2CO − MeOH]$^-$ (61), 353 (13), 311 (21), 227 (24), 201 (26), 185 (17), 167 (30), 155 (20), 143 (23), 125 (56), 119 (39), 113 (37), 101 (40), 97 (68) Another part of W1 **11** (2 mg) was hydrolysed, the aglycone was methylated and trimethylsilylated and analysed by GC/MS Its GC/MS data were identical with those of compound **7**

^1H NMR was done with 5 2 mg of acetylated W1, **12** δ 1 07 (3H, *d*, *J* = 6 3 Hz, H-12, minor component), 1 19 (3H, *d*, *J* = 6 3 Hz, H-12, major component), 1 99, 2 01, 2 03 and 2 07 (4 × 3H, 4 × s, OAc), 3 78 (2H, *m*, unresolved, H-5' and H-11), 4 11 (1H, *dd*, *J* = 2 8 Hz *J'* = − 12 4 Hz H-6'A), 4 22 (1H, *dd*, *J* = 4 5 Hz, *J'* = − 12 4 Hz, H-6'B), 4 53 (1H, *d*, *J* = 7 9 Hz, H-1', major component), 4 56 (1H, *d*, *J* = 7 9 Hz H-1', minor component)

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