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METABOLISM OF 24-*EPI*-CASTASTERONE IN CELL SUSPENSION CULTURES OF *LYCOPERSICON ESCULENTUM*

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; brassinosteroids; cell suspension cultures; 24-*epi*-castasterone; glucosides; metabolism.

Abstract—The metabolism of ^{3}H-labelled 24-*epi*-castasterone in cell suspension cultures of *Lycopersicon esculentum* involved epimerization, hydroxylation and glucosidation, and yielded several new brassinosteroids and brassinosteroid glucosides. Their structures were elucidated by MS and NMR analysis.

INTRODUCTION

Brassinosteroids are endogenous plant growth regulators which exhibit a multitude of physiological activities and are of ubiquitous distribution in the plant kingdom [1, 2]. They are considered as a new class of phytohormones [3]. Recently, increasing attention has been directed towards the biosynthesis and metabolism of this interesting group of compounds. The biosynthetic work has been concentrated on several steps of the biogenetic sequence between teasterone and brassinolide [4, 5]. We have focused on interconversions and metabolic reactions. Recently, we have demonstrated that brassinosteroids in cell suspension cultures of *Omithopus sativus* are conjugated with fatty acids [6]. In the same cell culture, 24-*epi*-castasterone and 24-*epi*-brassinolide, respectively, are metabolized by side-chain cleavage to give pregnane-like compounds [7]. In cell suspension cultures of *Lycopersicon esculentum*, 24-*epi*-brassinolide is converted to a novel type of pentahydroxylated brassinosteroid glucosides and the reactions involved in these conversions have been characterized [8, 9]. In this paper, we report on the isolation and structural elucidation of several new metabolites formed from exogenously supplied 24-*epi*-castasterone in cell suspension cultures of *L. esculentum*.

RESULTS AND DISCUSSION

24-*epi*-Castasterone (**1**), first found in the green alga *Hydrodictyon reticulatum* [10], is widely distributed in the plant kingdom [11–13]. It is assumed that **1** is the immediate precursor of 24-*epi*-brassinolide, in an analogous manner to the related pair castasterone/brassinolide from the 24-*S* series [14]. ^{3}H-labelled **1** [15] (final concentration $1.3\ 10^{-5}$ M, $218\ 10^3$ Bq) was administered to cell suspension cultures of *L. esculentum* on day 3 of the growth cycle. Radioactivity measurements indicated a very rapid uptake during the initial incubation period (45.9% after 2 hr) which later became slower (91.7% after 4 days). After 4 days, the cells were harvested and extracted with MeOH followed by partition of the aqueous residue between *n*-hexane and water. The aqueous phase (183×10^3 Bq) was subjected to XAD-2 chromatography. The aqueous efflux (12×10^3 Bq) and 40% methanol eluate from the XAD-2 column (13×10^3 Bq) were not investigated further. Radio TLC of the methanol eluate (32×10^3 Bq) exhibited only one major peak which was due to the parent compound **1**. The 70% MeOH eluate (124×10^3 Bq) was further purified by ion exchange chromatography. The chromatographic behaviour on silica gel TLC of the combined radioactive fractions of the DEAE A-25 column indicated several hydrophilic compounds, probably glycosides. This fraction was divided into two halves. One half was enzymatically hydrolysed (aglycone fraction) and the other one was examined as follows.

Radio TLC of the non-hydrolysed glucosidic fraction gave four major radioactively labelled zones. Two major components (1.1 mg each) were recovered as pure compounds by RP-HPLC (R_t 8 and 11 min, respectively; gradient 1). The FAB-MS and NMR data of these glucosides indicated that they were 25-β-D-glucopyranosyloxy-24-*epi*-castasterone (**3**) and 26-β-D-glucopyranosyloxy-24-*epi*-castasterone (**5**) (Fig. 1). Thus, both FAB mass spectra exhibited identical fragmentation patterns but the relative intensities were significantly different, e.g. *m/z* 643 $[M + H]^+$ (rel. int. 9 for **3** and 71 for **5**, respectively), 481 $[\text{aglycone} + H]^+$ (4, 100), 463 $[\text{aglycone} + H - H_2O]^+$ (16, 44), 445 $[\text{aglycone} + H - 2H_2O]^+$

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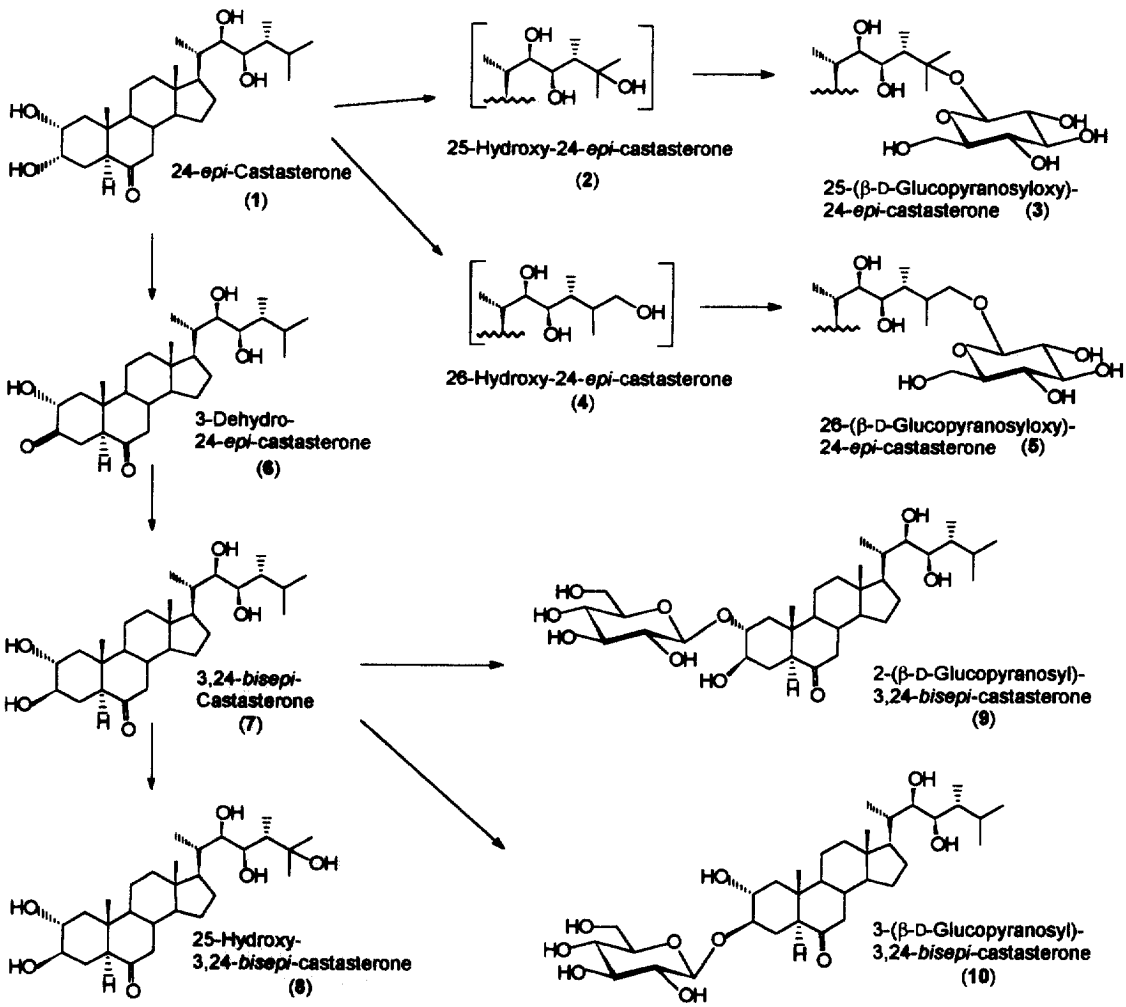

Fig. 1. Metabolism of 24-*epi*-castasterone (**1**) in cultured cells of *L. esculentum*.

(12, 34), 427 [aglycone + H − $3H_2O$]$^+$ (9, 23), 393 (100, 16). The latter fragments, due to bond fission between C-23 and C-24, confirmed the position of the glucosyloxy moiety at the terminal part of the side-chain beyond C-23. The ^{1}H NMR spectrum of **3** contained two methyl group doublets (δ1.06, J = 6.2 Hz and δ0.89, J = 7.0 Hz) and four methyl singlets (δ1.35, 1.29, 0.76 and 0.73) suggesting hydroxylation at C-25 (Table 1). This was confirmed by a ^{1}H, ^{13}C long-range shift correlation experiment (HMBC). Both ^{1}H singlets at δ1.35 and 1.29 showed correlations with C-25 (δ84.1) and C-24 (δ45.5). Furthermore, the anomeric proton of the glucose (δ4.56, *d*, J = 7.8 Hz) showed a HMBC correlation with C-25, indicating glucosylation at this position. In the case of **5**, side-chain hydroxylation resulted in the disappearance of one of the four methyl ^{1}H NMR doublets. The three methyl doublets exhibited ^{1}H–^{1}H COSY cross-peaks with different proton signals, thus hydroxylation had to have occurred at C-26 (or C-27). Unfortunately, superimposition of C-26 and C-2′ (Table 2) prevented estimation of the glycosylation site by means of the HMBC spectrum. However, after peracetylation of **5** the ^{1}H NMR signals of H-26A and H-6B were shifted slightly in comparison with the underivatized compound, thus confirming the proposed structure.

Obviously, the metabolic pathway recently established for 24-*epi*-brassinolide [9] is also operating for **1**, namely regiospecific hydroxylation of **1** followed by glucosidation of the newly formed hydroxyl groups in intermediates **2** and **4**, respectively.

The FAB-MS of another labelled fraction obtained by TLC and HPLC (R_t 12 min; gradient 1) from the 70% MeOH eluate of the XAD-2 column indicated a glucoside structure without preceeding hydroxylation: *m/z* 627 [M + H]$^+$ (15), 465 [aglycone + H]$^+$ (100), 447 [aglycone + H − H_2O]$^+$ (58), 429 [aglycone + H − $2H_2O$]$^+$ (21), 411 [aglycone + H − $3H_2O$]$^+$ (11). The ^{1}H NMR spectrum revealed this fraction to be a mixture of two compounds which could not be separated by HPLC (40% aq. MeCN). Thus, the structures of these glucosides were elucidated by NMR analysis of the mixture (1.0 mg) and shown to be 2-*O*-β-D-glucopyran-

Table 1. ^{1}H NMR spectral data of the metabolites of 24-*epi*-castasterone (**1**) produced in cell suspension cultures of *L. esculentum*. Chemical shifts were obtained from the ^{1}H NMR, ^{1}H–^{1}H COSY or 2D HMQC spectra (500 MHz)

H	1*	3†	5†	6*	7*	8†	10†
1α/1β	1.74/1.55	1.68/1.57	1.68/1.57	2.54/1.44	2.06/1.24	1.99/1.22	2.05/1.23
2β	3.77	3.64	3.65	4.25	3.60	3.47	3.63
3α‡/3β§	4.05‡	3.94‡	3.96‡		3.39§	3.60§	2.48§
4α/4β	1.92/1.72	1.76/1.66	1.78/1.67	2.72/2.52	1.96/1.60	1.83/1.50	2.01/1.52
5	2.69	2.72	2.73	2.64	2.32	2.46	2.45
7α/7β	2.30/2.00	2.20/2.11	2.21/2.11	2.39/2.00	n.d.	n.d.	n.d.
8	1.76	1.80	1.79	1.85	n.d.	n.d.	n.d.
9	1.40	1.43	1.42	1.37	n.d.	n.d.	1.42
11α/11β	1.65/1.34	1.68/1.34	1.68/1.41	1.68/1.42	n.d.	n.d.	n.d.
12α/12β	2.02/1.28	2.09/1.32	2.07/1.31	2.06/1.30	n.d.	n.d.	n.d.
14	1.31	1.37	1.37	1.33	n.d.	n.d.	1.34
15α/15β	1.58/1.11	1.59/1.14	1.59/1.15	1.60/1.15	n.d.	n.d.	n.d.
16α/16β	1.98/1.30	1.99/1.35	2.00/1.37	2.02/1.34	n.d.	n.d.	n.d.
17	1.56	1.57	1.60	1.59	n.d.	n.d.	1.54
H_3-18	0.68	0.73	0.74	0.70	0.68	0.73	0.72
H_3-19	0.76	0.76	0.76	1.04	0.81	0.78	0.79
20	1.47	1.54	1.59	1.48	1.46	1.49	1.49
H_3-21	0.98	1.06	0.98	0.98	0.98	1.02	0.98
22	3.70	3.67	3.66	3.69	3.69	3.66	3.65
23	3.41	3.54	3.35	3.41	3.41	3.48	3.33
24	1.50	1.99	1.93	1.50	1.51	1.72	1.47
25	1.90		2.20	1.89	1.90		1.95
H_3-26‡	0.92	1.35	3.71/3.41§	0.92	0.92	1.22	0.91
H_3-27‡	0.87	1.29	0.87	0.87	0.87	1.20	0.86
H_3-28	0.85	0.89	0.85	0.85	0.85	0.83	0.83
1′		4.56	4.23				4.36
2′		3.12	3.17				n.d.
3′		n.d.	3.36				n.d.
4′		n.d.	3.24				n.d.
5′		n.d.	3.24				n.d.
6′		3.80/3.61	3.86/3.64				3.84/3.65

Typical multiplicities and coupling constants if not otherwise mentioned in the text: H-2β: *br m* ($\Delta_{1/2}$ = 23 Hz); H-3β: *br s*; H-3α: *ddd* (*J* = 14.4, 9.5, 4.9 Hz); H-5α: *dd* (*J* = 11.9, 4.9 Hz); H-7β: *br d* (*J* = 12.2 Hz); H-7α: *dd* (*J* = 12.2, 9.1 Hz); H_3-18: *s*; H_3-19: *s*; H_3-21; *d* (*J* = 6.7 Hz); H-2: *br dd* (*J* = 4.5, 4.5 Hz); H-23: *ddd* (*J* = 5.0, 4.5, 4.5 Hz); H_3-26: *d* (*J* = 7.0 Hz); H_3-27: *d*(*J* = 7.0 Hz); H_3-28: *d* (*J* = 7.0 Hz). n.d.: not detected because of poor signal to noise ratio and/or overlapping with other signals.

*$CDCl_3$.

†CD_3OD.

‡May be reversed.

§H-26A/H-26B.

osyl-3,24-*bisepi*-castasterone (**9**) and 3-*O*-β-D-glucopyranosyl-3,24-*bisepi*-castasterone (**10**). The ^{1}H–^{1}H COSY spectrum showed cross-peaks belonging to the methyl doublets typical of an unchanged side-chain. The proton chemical shifts of the ring A protons, derived from the COSY spectrum, indicated epimerization at C-3. However, the low-field signals of **9** and **10** were superimposed. Only the two anomeric protons showed well-separated signals (δ4.36 and 4.32, intensity ratio about 3:1). After acetylation, the COSY spectrum exhibited two sets of H-2β/H-3α signals at δ4.92/3.61 (major component) and δ3.76/4.74 (minor component), respectively. Consequently, **10** was the major and **9** the minor glucosylated metabolite. The ^{1}H NMR and ^{13}C NMR data of compound **10** are given in Tables 1 and 2, respectively.

Compounds **9** and **10** are the first brassinosteroid glucosides bearing the sugar moiety on ring A hydroxyl groups.

The most hydrophilic compound from the 70% MeOH eluate of the XAD-2 column (R_t 7 min, gradient 2) appeared to be a disaccharide conjugate of a pentahydroxy 6-keto brassinosteroid. This was indicated by the FAB-MS data, e.g. *m/z* 805 $[M + H]^+$ (5) and 643 $[M - \text{glucose} + H]^+$ (4). The fragments *m/z* 333 (56) and 393 (13) due to bond fission between C-20/C-22 and C-23/C-24, respectively, demonstrated the unchanged constitution of the ring system. The latter fragment was also indicative of the position of the newly formed hydroxyl group which must be located beyond C-23 and must bear the disaccharide moiety. Complete structure

Table 2. ^{13}C NMR spectral data of metabolites of 24-*epi*-castasterone (1) produced in cell suspension cultures of *L. esculentum*. Chemical shifts of compound 1 were obtained from the $^{13}C\{^1H\}$ NMR spectrum (125 MHz) and compounds 3, 5, 6, and 10 from the ^1H detected HMQC and HMBC spectra (500 MHz)

C	1*	3†	5†	6*	10†
1	39.9	40.9	40.9	47.8	45.8
2	68.0	69.1	69.1	72.0	n.d.
3	68.1	69.4	69.4	211.4	n.d.
4	26.2	27.8	27.7	35.0	28.9
5	50.7	52.0	52.0	58.6	57.0
6	212.9	214.5	n.d.	208.2	n.d.§
7	46.6	47.4	47.4	46.3	n.d.
8	37.7	39.0	39.1	37.8	n.d.
9	53.6	55.0	55.0	53.4	54.8
10	42.5	43.6	43.6	42.1	39.8
11	21.1	22.3	22.4	21.8	n.d.
12	39.3	40.8	40.8	39.2	40.7
13	42.7	44.0	44.0	42.8	43.9
14	56.4	57.8	57.8	56.3	57.8
15	23.8	24.9	24.8	23.8	n.d.
16	27.6	29.0	28.6	27.6	n.d.
17	52.5	54.7	54.1	52.6	54.1
18	11.7	12.2	12.3	11.8	12.2
19	13.4	13.7	13.8	13.8	15.6
20	40.1	43.2	41.0	40.2	41.6
21	12.3	13.7	13.0	12.4	13.0
22	72.4	73.2	73.8	72.6	73.4
23	75.9	79.0	77.8	76.4	77.3
24	41.4	45.5	36.2	41.4	42.7
25	26.9	84.1	33.4	27.0	27.9
26‡	22.0	20.9	75.1	22.0	22.5
27‡	17.1	26.9	12.0	17.2	17.4
28	10.7	13.8	11.5	10.8	11.1
1′		98.1	104.8		102.8
2′		75.1	75.1		74.9
3′		n.d.	67.3		n.d.
4′		n.d.	71.7		n.d.
5′		n.d.	77.7		n.d.
6′		62.7	62.8		62.5

*$CDCl_3$.
†CD_3OD.
‡May be reversed.
§n.d.: not detected because of poor signal to noise ratio and/or overlapping with other signals.

elucidation was not possible because of the very small amounts produced and the impurity of the sample. This finding presents the first indication of a brassinosteroid diglucoside.

From the hydrolysed part of the extract, 24-*epi*-castasterone (1) and three major metabolites (0.3–0.5 mg) were isolated by silica gel TLC and RP-HPLC. These metabolites belong to a metabolic sequence starting with the reduction of the 3α-OH group. The first compound of this sequence is 3-dehydro-24-*epi*-castasterone (6, R_t 6.5 min, HPLC 75% MeOH), a new 3,6-diketobrassinosteroid. The EI-MS showed only a weak molecular ion peaks at *m/z* 462 M^+ (1) and peaks at *m/z* 444 $[M - H_2O]^+$ (0.5), 361 (79), 343 (100) and 317 (71). The ^1H chemical shifts of side chain protons of 6 were identical with those of 1, whereas the protons of rings A and B showed quite different shifts and coupling patterns (Table 1). Only three of the four low-field ^1H signals showed correlation peaks in a one-bond ^1H–^{13}C shift correlation experiment (HMQC) and belonged, therefore, to methine protons. Combined use of homo- and heteronuclear shift correlation experiments proved that these signals were attributable to H-2β (δ4.25), H-22 (δ3.69) and H-23 (δ3.41), respectively. Moreover, an additional C=O signal at δ211.4 was assigned to C-3, thus confirming the proposed structure (Table 2).

Compound 6 can be regarded as an intermediate in the epimerization reaction to give 3,24-*bisepi*-castasterone (7, R_t 14 min, HPLC 40% MeOH). EI-MS: *m/z* 465 $[M + H]^+$ (2), 446 $[M - H_2O]^+$ (3), 364 (100), 345 (44) and 319 (31). The ^1H chemical shifts and linewidth of H-2 (δ3.60, $\Delta_{1/2}$ = 24 Hz) and H-3 (δ3.39, $\Delta_{1/2}$ = 21 Hz) as well as the high field shift of H-5α of about 0.4 ppm in comparison with 1 indicated the axial position of both protons H-2 and H-3 and therefore epimerization at C-3, whereas the side-chain ^1H signals show no difference to those of 1 in terms of chemical shifts and coupling patterns (Table 1).

3,24-*bisepi*-Castasterone (7) is known both as a naturally occurring compound [16] and as an intermediate in the metabolism of 1 in cell cultures of *Onithopus sativus* [16]. As a branching point in this metabolic sequence, it is either glucosylated at 3β-OH or at 2α-OH yielding compounds 9 and 10, respectively, or it can be hydroxylated at C-25 to give 25-hydroxy-3,24-*bisepi*-castasterone (8, R_t 7 min, HPLC 40% MeOH). The major fragment of the EI mass spectrum, *m/z* 363 (79), was due to bond fission between C-22/C-23. The fragments *m/z* 393 (bond fission between C-23/C-24, 9), and 345 $[363 - H_2O]^+$ (100) also confirmed the suggested structures. As in the case of 7, the ^1H chemical shifts and coupling patterns of the ring A protons of 8 indicated epimerization at C-3. The methyl group chemical shifts and signal multiplicities of 8 (δ1.22, *s*; δ1.02, *d*; δ0.83, *d*; δ0.78, *s*; δ0.73, *s*) confirmed hydroxylation at C-25.

The structures of the metabolites shown in Fig. 1 indicate the involvement of pairs of aglycones and glucosides, for instance, for compound 7 and its glucosides 9 and 10, respectively. This may be true also for 2/3, 4/5 as well as for 6 and 8, although in these cases only the glucoside or the aglycone could be detected in our experiments. This may be due to very small pool sizes of the compounds or to the fact that it was not possible to obtain all the metabolites in pure form. Similarly the intermediate 25- and 26-hydroxy-24-*epi*-brassinolides, respectively, were not found in *L. esculentum* cell cultures [9]. Compounds 6 and 8 were isolated after hydrolysis. Thus, originally they must have been present in the cultured cells as glucosides.

EXPERIMENTAL

Radiochemicals and measurement of radioactivity. The synthesis of 24-*epi*-[5,7,7-^3H] castasterone (1) was re-

cently described [15]. Compound **1** with a sp. act. of 22.2 MBq mmol^{-1} was used in this study. Radioactivity was measured by liquid scintillation counting (LSC). TLC plates were analysed for radioactive zones with an automatic TLC linear analyser.

Cell culture and administration of **1**. Plant cell cultures of *L. esculentum* were grown in Linsmaier–Skoog medium [18] and maintained in 300 ml Erlenmeyer flasks containing 150 ml cell suspension as previously described [8, 9]. Filter-sterilized ethanolic solns (< 1 ml) of 24-*epi*-[5,7,7-^{3}H]castasterone (14.4 mg) were administered to the cell suspension cultures at day 3 of growth (final concn of cell suspension 1.3×10^{-5} M). The cell suspensions were maintained under identical conditions for another 7 days.

Isolation and purification of **3**. The cells (285 g) were harvested by suction filtration through a nylon mesh and then homogenized in aq. 80% EtOH with an ultra-turrax grinder at room temp., filtered and washed with MeOH. The aq. soln remaining after concn of the combined filtrates *in vacuo* at less than 40° was partitioned between *n*-hexane and H_2O. The aq. layer was purified CC on XAD-2 eluted successively with MeOH–H_2O in ratios 0:100, 2:3, 7:3, 100:0. The 70% MeOH was subjected to ion exchange chromatography (DEAE A-25, MeOH). The combined radioactive fractions were purified by TLC (Merck silica gel 60; 0.5 mm layer for prep. mode; silica gel sheets for analytical mode; successively developed in $CHCl_3$–MeOH, 9:1 and 4:1) and RP-HPLC (Nucleosil C18; 10 μm; 250 × 4 mm; flow rate 4 ml min^{-1}; detection UV 204 nm and LSC of aliquots from 2 ml HPLC fractions; gradient 1: MeCN–H_2O from 3:7 to 1:1 in 15 min; gradient 2: MeCN–H_2O from 1:4 to 9:11 in 15 min; isocratic mode: MeCN–H_2O, for ratios see text).

Enzymatic hydrolysis and acetylation. The sample was dissolved in McIlvain buffer, pH 4.0, and incubated with 1 mg cellulase per ml soln for 20 hr at 37°. The EtOAc extract of this soln was subjected to radio-TLC. Acetylation was performed at room temp. with pyridine–Ac_2O (1:1) containing 1% 4-(dimethylamino)-pyridine.

Spectrometric methods. El-MS: 70 eV; FAB-MS: 9 kV; NMR: 499.84 MHz (^{1}H), 125.7 MHz (^{13}C), $CDCl_3$ or CD_3OD, NALORAC 3 mm microsample inverse detection probe.

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