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ISOLATION AND CHARACTERIZATION OF CONJUGATED GIBBERELLINS IN MATURING SEEDS OF *SECHIUM EDULE*

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Key Word Index—*Sechium edule*; Cucurbitaceae; conjugated gibberellins; plant hormones.

Abstract—Endosperm and cotyledons of *Sechium edule* at different stages of seed development were found to contain three novel GA conjugates. 3-propyl- or 3-acetyl- $GA_{4/7}$ were characterized by mass spectral analysis and were found to be biologically active in contrast to synthetic $GA_{4/7}$ propyl esters. An unusual GA glucoside showing biological activity was also isolated from both tissues and characterized by mass spectrometry and NMR analysis as 16,17-dihydro-16-hydroxy-GA_{15} alcohol glucoside. The functional significance of this novel GA conjugate is discussed.

INTRODUCTION

Maturing seeds of *Sechium edule* contain very high levels of gibberellins. We have previously reported [1] on the identity and localization of various biologically active GAs in endosperm and cotyledons of seeds at different stages of development. GA_4 and GA_7 were the major gibberellins present in both tissues at all stages of seed growth while GA_9 was present at a much lower level and GA_1 and GA_3 were identified only in cotyledons. Moreover, we have demonstrated that endosperm and cotyledon cell-free systems prepared from *Sechium edule* maturing seeds synthesize [^{14}C]GA_9 and [^{14}C]$GA_{4/7}$ from [^{14}C]MVA [2].

In a recent paper Albone *et al.* [3] have reported a detailed analysis on the localization of these and other gibberellins in the seed components of *Sechium edule*.

The presence of bound gibberellins in *Sechium edule* was first suggested by Ogawa [4] who reported on the presence of 'water-soluble' gibberellin-like substances in developing seeds. Albone *et al.* [3] have reported the presence and localization of gibberellin catabolites, particularly GA_8 and a GA_8-catabolite mainly localized in the testa together with large amounts of polyhydroxylated kaurenoids widely distributed in the seed components.

During our previous work [1] we noticed the presence of GA-like substances showing a peculiar chromatographic behaviour. As a part of a project investigating the relation between hormones and seed development in *Sechium edule* the purification and identification of these substances has been pursued. We now report on the characterization of three novel GA conjugates.

RESULTS

Time-course studies on the levels and identity of GAs throughout seed development [1] showed the presence of unknown compounds showing gibberellin-like activity. These substances were extracted and purified from endosperm and cotyledons as described [1]. On reverse-phase HPLC analysis using system 1 two biologically active fractions eluting after a GA_9 standard were isolated from both tissues and are referred to as fraction A (elution volume 64–70 ml) and fraction B (elution volume 128–136 ml).

Fraction A was further purified on HPLC system 2. The biological activity was associated with a well defined peak which was separately collected. An aliquot was trimethylsilylated and the TMS derivative was subjected to analytical and preparative GC analysis. Only one peak was present in the chromatogram and after preparative GC it was shown to be biologically active.

EIMS of the TMS derivative showed a spectrum with the following major ions: 446 (7.8%), 444 (10.6), 386 (13.2), 384 (11.2), 356 (17.3), 354 (5.8), 311 (32.5), 309 (19.3), 224 (31.5), 222 (48.4) and 73 (100). Nevertheless in this mass spectrum neither molecular or $[M-15]^+$ ions were clearly identifiable.

To establish the molecular ions a non-derivatized aliquot of the HPLC fraction was subjected to FAB-MS. The sample was analysed by direct introduction after mixing with glycerol. This ionization technique produces mass spectra with intense peaks corresponding to the molecular ion $+n92$ ($n = 1, 2 \ldots$; 92 = glycerol molecule) and molecular ion $\times 2$ (dimer) + or -1 under positive or negative ionization. In the negative ion spectrum shown in Fig. 1 it is possible to assign ions as follows: 371 $[M_x-H]^-$ and 373 $[M_y-H]^-$; 463 $[M_x-H+gly]^-$ and 465 $[M_y-H+gly]^-$; the dimers 743 $[2\times M_x-H]^-$ and 747 $[2\times M_y-H]^-$, 745 $[M_x+M_y-H]^-$. Similar conclusions can be drawn from the positive ion spectrum shown in Fig. 1. The above evidence together with that obtained from EIMS analysis were consistent with the presence of propyl derivatives of GA_4 and GA_7.

To distinguish between C-3 or C-7 derivatives the 7-*n*-propyl esters of $GA_{4/7}$ were synthesized following the procedure described by Sell *et al.* [5]. The purified natural products and the synthesized mixture of 7-*n*-propyl esters of $GA_{4/7}$ were subjected either to methylation and subsequent silylation or directly to silylation. GC-MS analysis with EI ionization of the differently prepared samples showed as expected the same mono-TMS spectrum for the synthesized 7-*n*-propyl esters of $GA_{4/7}$. Instead the natural product showed alternatively a methyl

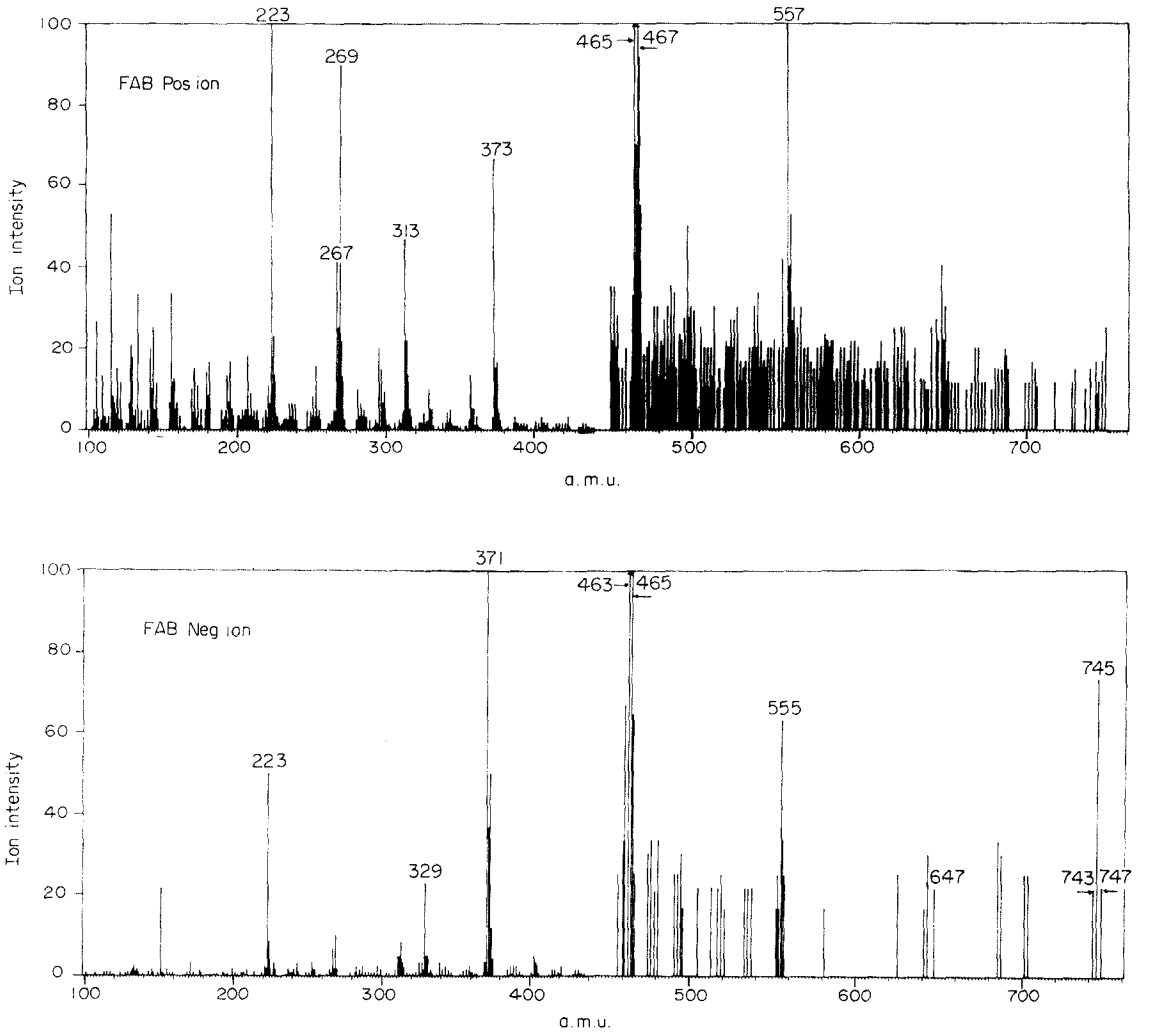


Fig 1. Positive and negative FAB-MS of fraction A.

ester or a TMS spectrum according to the derivatization procedure, demonstrating the presence of a free carboxyl on the molecule. Therefore, we conclude that the GA derivatives contained in fraction A are conjugates of $GA_{4/7}$ via the hydroxyl at C-3. However, we cannot distinguish between 3-propyl and 3-acetyl derivatives of $GA_{4/7}$ owing to the very similar mass spectra of these compounds [Professor J. MacMillan, personal communication]. A mixture of 3-acetyl GA_4 and 3-acetyl GA_7 (supplied by J. MacMillan) was analysed by EIMS as the TMS or methyl derivatives and the spectra were indistinguishable from those obtained with compounds of fraction A run under the same conditions. Moreover, we were unable to separate the substances of fraction A from 3-acetyl GA_4 and 3-acetyl GA_7 by HPLC or GC procedures.

The content of these $GA_{4/7}$ derivatives relative to the content of free $GA_{4/7}$ in endosperm (calculated by GLC analysis) was observed to change during seed development showing a much higher ratio (8 : 1) in correspondence of B and C stages (see ref. [1]). The biological activity of natural $GA_{4/7}$ derivatives contained in fraction A, 3-acetyl $GA_{4/7}$, $GA_{4/7}$ propyl esters, GA_4 and GA_7 was compared in lettuce, dwarf rice and α-amylase bioassays. While the $GA_{4/7}$ propyl esters did not show appreciable biological activity in any of these bioassays, confirming the previous findings from Hemphill *et al.* [6] and Sell *et al.* [5] with GA_3 propyl esters, the natural $GA_{4/7}$ conjugates and the 3-acetyl $GA_{4/7}$ were biologically active. The biological activity was comparable to that of free GA_4 and GA_7 in the rice and lettuce bioassays while in the α-amylase test it was only 30%.

Fraction B from HPLC system 1 was further purified on HPLC systems 3 and 4. After this procedure a fraction was obtained containing a single well-defined peak associated with high biological activity. The same procedure was used starting either from endosperm or cotyledons and the presence of the same active substance was demonstrated in both tissues. The isolated compound was silylated and analysed by analytical and preparative GLC. However the biological activity was not recovered after preparative GLC and the active compound must undergo degradation during the GLC procedure.

The substance was subjected to mass spectral analysis

using positive and negative FAB ionization techniques. With both positive or negative ionization intense spectra were obtained as shown in Fig. 2 but in the positive ion spectrum more ions were produced. In the negative ion spectrum major ions were clearly recognizable and were attributed as follows: 495 $[M-H]^-$; 587 $[M-H+gly]^-$; 679 $[M-H+2gly]^-$. In the positive ion spectrum major ions were attributed as follows: 497 $[M+H]^+$; 589 $[M+H+gly]^+$; 681 $[M+H+2gly]^+$; 773 $[M+H+3gly]^+$; 865 $[M+H+4gly]^+$; 993 $[2M+H]^+$. Therefore, we concluded that the M_r of the active substance was 496. Moreover in the positive ion spectrum some low molecular weight fragments were shown among which were 335 $[M+H-162]^+$, 317 $[M+H-180]^+$, 289 $[M+H-208]^+$ and 271 $[M+H-226]^+$. These results suggested the presence of a gibberellin conjugate, possibly a glucoside. The compound was therefore subjected to enzymatic hydrolysis with β-glucosidase. The resulting biologically active aglycone showed on TLC a different chromatographic behaviour (R_f 0.8) from that of the active compound (R_f 0.1).

A sample of the substance after hydrolysis was subjected either to methylation with diazomethane and subsequent silylation or directly to silylation. GC-MS analysis produced in both cases the same spectrum, attributable to a compound carrying two TMS groups, demonstrating the absence on the hydrolysed molecule of free carboxylic groups. Major ions in the spectrum were at m/z 463 (56%), 435 (100), 388 (12), 345 (47), 298/299 (15/17), 271 (13). This fragmentation pattern suggested a molecular weight of 478 but a molecular ion was not seen in the spectrum. The fragments observed were therefore tentatively assigned as: $[M-15]^+$; $[M-43]^+$; $[M-90]^+$; $[M-(90+43)]^+$; $[M-179/180]^+$; $[M-207]^+$ respectively. These assignments are consistent with an aglycone having a molecular weight of 334.

The presence of glucose in the molecule was confirmed by EIMS analysis of the fragments deriving from thermal degradation of the substance introduced via probe in the mass spectrometer. During a probe thermal gradient from 20 to 300° a spectrum corresponding to that of α-D-glucopyranose was observed at a probe temperature of 100°. Successively, when the probe temperature reached 230–250° a spectrum with the following major ions was

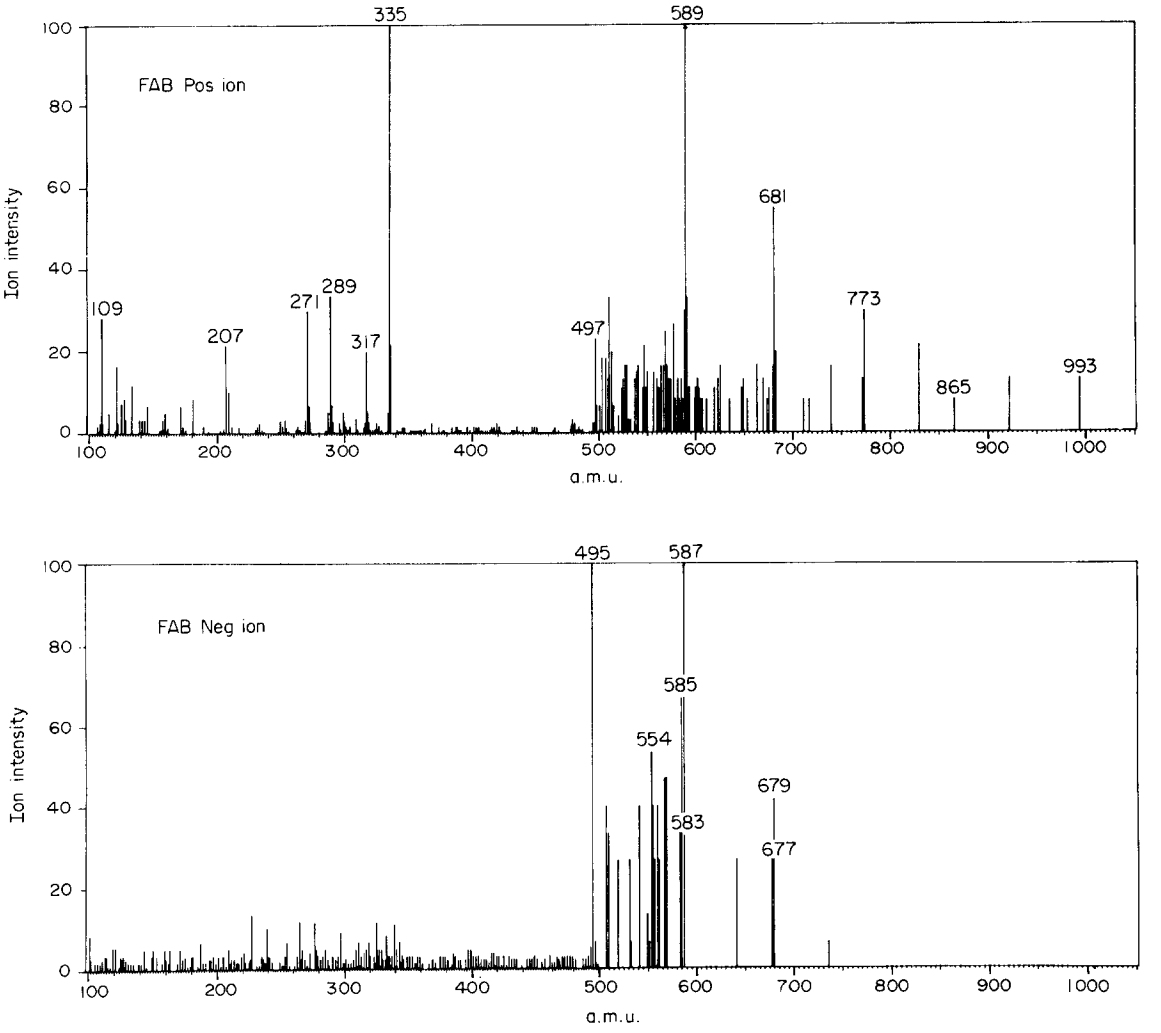
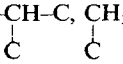


Fig. 2. Positive and negative FAB-MS of fraction B.

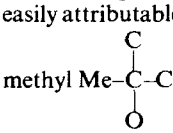
obtained: *m/z* 317 (34%), 299 (22), 289 (34), 271 (84). The ion at *m/z* 317 is the result of the loss of 179 (glucose-H) from the intact molecule having a molecular weight of 496. To elucidate the elemental composition of the fragment with *m/z* 317 the intact compound was analysed by high resolution mass spectrometry. The spectrum obtained exhibited prominent ions at *m/z*: 335.2206, 317.2101, 289.2160, 271.2054 with the composition: $C_{20}H_{31}O_4$ (calc. 335.2219), $C_{20}H_{29}O_3$ (calc. 317.2115), $C_{19}H_{29}O_2$ (calc. 289.2165), $C_{19}H_{27}O_1$ (calc. 271.2061) respectively. These data are all consistent with a molecular formula of $C_{20}H_{29}O_3$ for the aglycone fragment resulting after the glucose cleavage of the intact GA glucoside.

In order to obtain structural information on the gibberellin glucoside ^{1}H NMR spectra were run in $CDCl_3$ and D_2O solutions. Solubility in $CDCl_3$ is very poor (*ca* 10^{-6} molar) but a spectrum in this solvent was necessary in order to observe the δ4.5–5.5 range. No signal attributable to vinylidenic protons was found thus demonstrating the absence of this functional group.

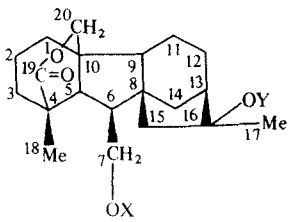
In D_2O the ^{1}H NMR spectrum (Fig. 3) lies in two well defined spectral ranges, i.e. between δ3 and 4.5 due to O–CH and O–CH_2 groups and between 0.7 and 2.2, due to C–CH–C, CH_2–C and Me–C groups. Integration of



signals between δ3 and 4.5 shows 11 protons; while in the δ0.7–2.2 region two singlets of intensity 3 were found easily attributable to methyl in position 4 at δ0.82 and to a



methyl Me–C–C at 1.33. Only two structures can account for the observed NMR and mass spectra differing only for the position of the sugar residue, as shown in the structure below.



Y = H, X = Glucose
Y = Glucose, X = H

The GA glucoside is present both in endosperm and cotyledons at all developmental stages of seed growth. The endosperm has a much higher level (16 μg/g fr. wt) than the cotyledons (2 μg/g fr. wt) and both organs contain the glucoside from the earlier stage without considerable variations during seed development.

The glucoside, biologically active in the lettuce hypocotyl bioassay as above reported, was shown to be active also in the rice bioassay (root application).

DISCUSSION

Previous observations [1] on the presence of biologically active GAs with peculiar chromatographic properties has led to the isolation and characterization of three novel GA conjugates in endosperm and cotyledons of maturing *Sechium edule* seeds namely a glucoside of 16,17-dihydro-16-hydroxy-GA_{15}, the 3-propyl- or 3-acetyl-GA_4 and the 3-propyl- or 3-acetyl-GA_7.

We cannot distinguish between 3-propyl- or 3-acetyl-derivatives of $GA_{4/7}$ as explained in the results but in any

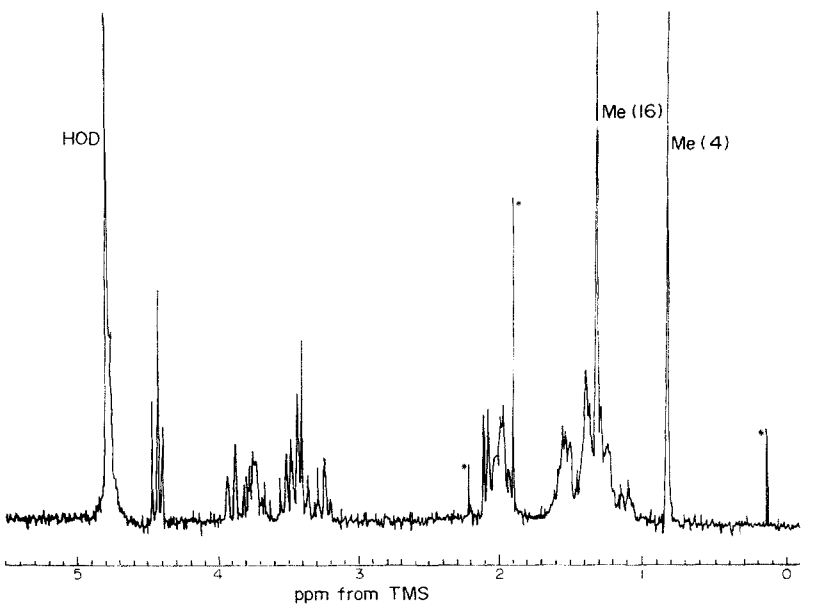


Fig. 3. 200 MHz ^{1}H NMR spectrum of fraction B in a 0.5 mg/0.5 g solution in D_2O. Ppm were from TMS but actually measured from the residual HOD peak assumed at 4.8 ppm. *Signals due to sample impurities.

case the conjugation is through the hydroxyl at C-3. The only previous report of GA propyl derivatives in plants is the identification of 7-*n*-propyl GA_1 and 7-*n*-propyl GA_3 in *Cucumis sativus* [5]. The presence of 3-acetyl GAs has never been reported in higher plants but only in *Gibberella fujikuroi* culture medium. The GA_3 and GA_1 propyl esters found in *Cucumis sativus* by Hemphill *et al.* [6] were reported to be almost inactive in several bioassays. Our results with 7-*n*-propyl GA_4 and 7-*n*-propyl GA_7 esters confirm that the esterification of the COOH at C-6 with a propyl group is associated with the loss of biological activity. On the contrary the $GA_{4/7}$ derivatives isolated from *S. edule* retain a biological activity almost equivalent to free $GA_{4/7}$. The 3-acetyl GA_4 and the 3-acetyl GA_7 show a very similar biological activity.

The function of these conjugates remains unknown but the retention of the biological activity associated with a lower polarity excludes inactivation or storage hypotheses and would instead suggest a role in the compartmentation of these substances.

The GA glucoside identified in *S. edule* bears an unusual structural feature. The GA aglycone is a C_{20}-GA with two alcoholic functions at C-7 and C-16, the latter associated to the saturation of the 16,17-double bond. Whether the ether linkage between the GA moiety and the glucose is through the hydroxyl group at C-7 or at C-16 has not been ascertained. GAs with a saturated 16,17-double bond were not known in advance in plants as they were isolated only from *Gibberella fujikuroi* cultures and we are aware of only one report of a GA alcohol in biological systems. This is the GA_{12}-alcohol produced from GA_{12}-aldehyde in cell-free systems prepared from seeds of *Pisum sativum* [7]. Recently the occurrence of GA_6-16,17-diol in a bound form in *Sechium* has been reported [3].

Interestingly, the natural GA glucoside identified in *S. edule* bears a 19,10-lactone as all the natural C_{20}-GA glucosides whether ether or ester with the exception of a GA_{12}-ald-glucosyl ester formed in a *Pisum sativum* cell-free system after feeding with GA_{12}-aldehyde [8].

Another unique feature of the GA glucoside identified in *S. edule* is the oxidation at C-20 to alcohol before the complete oxidation at C-7.

The presence of neutral GA-like substances in *S. edule* was reported by Ogawa [4]. This glucoside, lacking the carboxyl group at C-6 can at least partly account for the neutral fraction of GA-like activity.

The *S. edule* GA glucoside is biologically active in the lettuce and rice bioassay. The activity of GA glucosides is commonly attributed to the free GAs resulting from enzymatic hydrolysis rather than to the glucoside per se [9]. In the case of the *S. edule* GA glucoside the aglycone resulting eventually from enzymatic hydrolysis would not bear structural characteristics assuring biological activity thus it seems reasonable to hypothesize that the observed biological activity would result from its further metabolism. These considerations suggest that this GA glucoside might serve as a transport or storage form of a GA precursor in *S. edule*.

The presence of large amounts of a GA glucoside from the early stage of seed development is unusual as GA glucosides have been reported to accumulate in seeds during maturation while the level of free GAs is concomitantly reduced [10, 11], Moreover, GA glycosylating enzymes are usually absent in the early immature seed stages as they appear only in the late stages of the maturation process [12].

EXPERIMENTAL

Plant material. Cotyledons and endosperm of *S. edule* seeds at different developmental stages were separately collected and classified as in ref. [1]. The material was stored at −24° prior to extraction. Extraction, solvent partitioning, analytical and prep. GLC, GC-MS and probe MS by EI were also performed as previously described [1].

HPLC. This was performed on a LDC instrument equipped with a UV absorbance detector operating at 214 nm. Solvent systems were obtained by mixing different percentages of MeCN in H_2O at pH 8 (500 ml H_2O + 150 μl 32% NH_4OH). A 15 cm × 1 cm i.d. column packed with Licrosorb RP18 10 μ, eluted at a flow rate of 4 ml/min, was used in system 1. GA standard elution vols in this system were: $GA_{4/7}$ 30–33 ml; GA_9 50–54 ml while the elution vol. of fraction A and fraction B were 64–70 ml and 128–136 ml respectively. A 15 cm × 6 mm o.d. column packed with Licrosorb RP18, 5 μ eluted at a flow rate of 1 ml/min was used in systems 2, 3 and 4.

System 1: 5% MeCN in H_2O followed by two successive linear gradients of MeCN in H_2O from 5 to 20% for 20 min and from 20 to 100% for 20 min; System 2: 10% MeCN in water for 8 min then a linear gradient of MeCN in H_2O from 10 to 20% for 20 min; System 3: 20% MeCN in water for 6 min then a linear gradient of MeCN in water from 20 to 60% for 20 min; System 4: 36% MeCN in H_2O. Elution vol. of fraction A in system 2 was 12–13.5 ml while the elution volume of GA_9 standard was 5.8–6.5 ml; elution volume of fraction B was 15–17 ml in System 3 and 2.8–3.4 ml in System 4.

Enzymatic hydrolysis was carried out with β-glucosidase from sweet almonds (Boehringer) at 37° in 0.1 M acetate buffer at pH 5.4 for 24 hr.

FAB and high resolution MS. Analyses were performed on a VG 7070 EQ instrument. For FAB positive and negative MS analysis the above instrument was fitted with its own standard FAB ion source employing argon atoms at 7 keV, kinetic charge 1 mA; M/ΔM = 1000, dynamic resolution; scan rate: 10 sec × decade^{-1}. The sample was dissolved in glycerol, transferred onto the FAB target and inserted into the ion source. The sample was dissolved in glycerol to obtain long lasting and intense ion signals. The computer subtracted the characteristic glycerol peaks from all the spectra. High resolution mass spectra were obtained by EI after direct introduction of the sample. The probe temperature was raised manually to achieve intense signals at m/z 317. Dynamic resolution was M/ΔM = 10000. The reference compound was PKF introduced by system inlet; scan rate was 1 sec × decade^{-1}. All the data were recorded with a PDP 8/A computer system.

NMR. ^{1}H NMR spectra were taken on a Bruker WP 200 instrument. Spectral conditions were: NS = 2000 for D_2O soln, 27000 for $CDCl_3$ soln; PW = 6 μsec (75° flip angle); relaxation delay: 1 sec; acquisition time: 1.5 sec; sweep width: 6000 Hz; data points: 16 K; solvents: $CDCl_3$, isotopic purity 99.96% and D_2O, isotopic purity 99.996% from Aldrich.

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