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Mass spectral characterization of C-glycosidic flavonoids isolated from a medicinal plant (*Passiflora incarnata*)

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ABSTRACT

The four major C-glycosidic flavonoids isolated from *Passiflora incarnata* were identified as schaftoside, isoschaftoside, isovetexin-2″-O-glucopyranoside and isoorientin-2″-O-glucopyranoside on the basis of mass spectral and ^{13}C NMR data. The daughter ion spectra of $[M + H]^+$ ions of schaftoside and isoschaftoside showed differences for the $[M + H - 104]^+$ ions, which could be rationalized by hydrogen bonding effects. In the negative-ion mode, pronounced differences were found for the $[M - H - 90]^-$ and $[M - H - 120]^-$ ions, formed by prevalent fragmentation in the C-6-linked sugar moiety. With respect to isovitexin-2″-O-β-glucopyranoside and isoorientin-2″-O-β-glucopyranoside, the daughter ion spectra of both the $[M + H]^-$ and $[M - H]^-$ ions provided evidence for a 1→2 linkage in the diglucosidic moiety. Support for C-6 glucosylation was obtained by recording the daughter ion spectra of $[M - H - 162]^-$ ions, which were in good agreement with that obtained for $[M - H]^-$ ions of isovitexin.

INTRODUCTION

Phytochemical preparations of the herbage of *Passiflora incarnata* are commercially available and widely used medically for sedative and tranquillizing purposes. Physico-chemical methods for quality control of these pharmaceuticals are still available to only a limited extent. High-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) methods are generally used for analysis and identification based on chromatographic retention data [1,2]. However, identification problems arise when standards are not available, which, in the case of phytochemical preparations, often occurs. In order to support an HPLC quality control programme on the analysis of *Passiflora incarnata* extracts, we have characterized the four major C-glycosidic flavonoids. C-Glycosidic flavonoids are polyphenolic pigments which are abundant in plants, have biological activity, are of interest for chemotaxonomy and are used as tracers in medicinal plant preparations. With regard to the C-glycosidic flavonoid composition of *Passiflora incarnata*, literature data are contradictory. Vitexin (**I**), isovitexin (**II**), orientin (**III**), isoorientin (**IV**) and saponarin (**V**) have been reported to be the major C-glycosidic flavonoids [3—5] (structures are shown in Table I). As a

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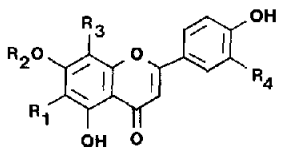
TABLE I

STRUCTURES OF FLAVONOIDS



Compound	No.	Substituents[a]			
		R_1	R_2	R_3	R_4
Vitexin	**I**	H	H	glc	H
Isovitexin	**II**	glc	H	H	H
Orientin	**III**	H	H	glc	OH
Isoorientin	**IV**	glc	H	H	OH
Saponarin	**V**	glc	glc	H	H
Schaftoside	**VI**	glc	H	ara	H
Isoschaftoside	**VII**	ara	H	glc	H
Vicenin-2	**VIII**	glc	H	glc	H
Isovitexin-2″-O-β-glucopyranoside	**IX**	soph	H	H	H
Isoorientin-2″-O-β-glucopyranoside	X	soph	H	H	OH

[a] glc = β-D-glucopyranosyl; ara = α-L-arabinopyranosyl; soph = sophorose.

consequence, this information can be found in reviews on the pharmacognosy of *Passiflora* [6,7]. We found only one study, by Geiger and Markham [8], in which firm mass spectral and NMR spectroscopic evidence was presented. They could not detect saponarin (**V**) and found isovitexin (**II**), isoorientin (**IV**), schaftoside (**VI**), isoschaftoside (**VII**), vicenin-2 (**VIII**), isovitexin-2″-O-β-glucopyranoside (**IX**) and isoorientin-2″-O-β-glucopyranoside (**X**) as the major components.

The advantages of using fast atom bombardment (FAB) in combination with collisionally activated decomposition (CAD) and tandem mass spectrometry for the direct analysis of O-glycosidic flavonoids were first documented by De Koster *et al.* [9] and Crow *et al.* [10]. Recently, this methodology was also explored for the characterization of C-glycosidic flavonoids by Becchi and Fraisse [11]. They demonstrated that characteristic fragment ions of $[M - H]^-$ ions allow the differentiation between C-glycosylation at the 6- and 8-positions. In this study, we applied liquid secondary ion mass spectrometry (SIMS), which is a variant of FAB, in combination with CAD and *B/E* linked scanning to obtain daugther ion spectra of $[M + H]^+$ and $[M - H]^-$ ions. In addition, we also carried out ^{13}C NMR in order to confirm the identity of the isolated compounds.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC apparatus consisted of two Model 303 pumps, a Model 811 dynamic mixer, a Model 802C manometric module, a Model 621 data module (all from Gilson, Middleton, WI, U.S.A.), a variable-wavelength UV detector (Hewlett-Packard, Avondale, PA, U.S.A.), a Rheodyne injector equipped with a 20-μl loop and an IBM PC for control of the gradient, data acquisition and processing. The column (250 × 4 mm I.D.) was LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.) with 7-μm particles. A gradient of methanol and 5% formic acid in water was applied; for the first 2 min the column was run isocratically with 12% methanol and thereafter the gradient was started, reaching 25% methanol after 8 min and 40% methanol after 25 min. The flow-rate was 1.8 ml min^{-1} and detection was carried out at 350 nm.

Mass spectrometry

The analyses were performed on a VG70SEQ instrument (VG Analytical, Manchester, U.K.), equipped with a caesium ion source. Caesium ions with energies of *ca.* 22 keV and a beam flux of 1 μA were used as the ionizing beam. Daughter ion spectra were obtained by CAD in the first field-free region and *B/E* linked scanning. The helium pressure in the gas cell was adjusted until the main ion beam decreased to 30%. Daughter ion spectra were acquired in the multichannel analyser mode at a scan rate of 8 s per decade under control of the VG11-250J data system and accumulation of ten scans. The analyses were carried out using glycerol as a matrix. Samples were dissolved in dimethyl sulphoxide (5 $\mu g\ \mu l^{-1}$) and 1 μl of the solution was added to the matrix. Accurate mass data were obtained by carrying out a linear voltage scan at resolution 10 000 and by using poly(ethylene glycol) 600 as a matrix and reference. Two reference peaks were used to establish the mass scale.

NMR spectra

^{13}C NMR spectra were recorded on a JEOL JNM-FX-200 instrument at 50.10 MHz and room temperature. For the assignment of the ^{13}C NMR signals the SEFT (spin echo Fourier transform) multiple sequence was used, with an interval time of 7 ms ($1/J$), yielding positive signals for CH and CH_3 groups and negative signals for quaternary C atoms and CH_2 groups, since for both groups of resonances a phase difference of 180° exists. The samples were dissolved in $[^2H_4]CH_3OH$ (99.5 atom-% ^2H). Chemical shifts are reported in ppm (δ scale) relative to $[^2H_4]CH_3OH$ (49.00 ppm).

Samples

Vitexin was purchased from Roth (Karlsruhe, F.R.G.). Isovitexin was prepared by isomerization of vitexin according to Markham [12] and purified by

reversed-phase HPLC. The source of the four unknown products was a commercial dry extract of *Passiflora incarnata* (Pharma Beta, Basle, Switzerland). The products were isolated by common chromatographic techniques, including column chromatography and droplet counter-current chromatography, and their purity was checked by reversed-phase HPLC. The conditions for reversed-phase HPLC were those used for quality control of extracts. The structures of the C-glycosidic flavonoids found in *Passiflora incarnata* in this study and in others mentioned in the Introduction are given in Table I.

RESULTS AND DISCUSSION

Fig. 1 shows the reversed-phase HPLC of a crude extract of *Passiflora incarnata*. The products investigated correspond to peaks A–D. As preliminary tests using vitexin and orientin as reference standards indicated that the four major peaks did not correspond with these standards, a more detailed chemical characterization was initiated.

In a first series of experiments, liquid SIMS spectra were obtained in the positive- and negative-ion modes, which gave $[M + H]^+$ and $[M - H]^-$ ions, respectively, and as such gave complementary information about the molecular mass. The accurate mass data obtained for the $[M + H]^+$ ions are listed in Table II. These data indicate that compounds A and C are isomeric and correspond to di-C-glycosyl derivatives of apigenin, containing a hexose and a pentose substituent. Compounds A and C were identified as isoschaftoside and schaftoside, respectively, on the basis of mass spectral and ^{13}C NMR data (see below). As mass spectrometry does not allow characterization of the linkage and configuration of the sugar moieties without derivatization, ^{13}C NMR was necessary to support the proposed structures [13]. Compounds B and D were identified as isovitexin-2″-O-β-glucopyranoside and isoorientin-2″-O-β-glucopyranoside, respectively. Again, ^{13}C NMR was required to confirm the presence of the sophorose moiety (see below).

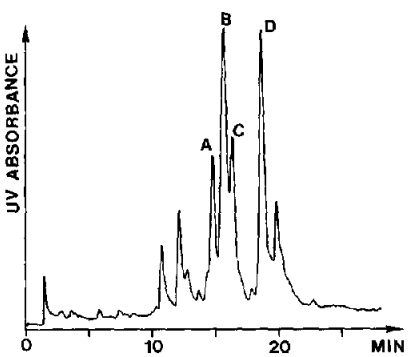


Fig. 1. Reversed-phase HPLC of a crude extract of *Passiflora incarnata*.

TABLE II

ACCURATE MASS DATA FOR THE $[M + H]^+$ IONS OF C-GLYCOSIDIC FLAVONOIDS ISOLATED FROM *PASSIFLORA INCARNATA*

Compound	Identification	Elemental composition	Calculated mass	Observed mass	Error (mu)
A	VII	$C_{26}H_{29}O_{14}$	565.1557	565.1598	−4.1
B	IX	$C_{27}H_{31}O_{15}$	595.1663	595.1678	−1.5
C	VI	$C_{26}H_{29}O_{14}$	565.1557	565.1581	−2.4
D	X	$C_{27}H_{31}O_{16}$	611.1612	611.1620	−0.8

Mass spectral and NMR characterization of compounds A and C

The daughter ion spectra obtained for $[M + H]^+$ and $[M - H]^-$ ions of compounds A and C are listed in Table III and IV. The spectra obtained for the $[M - H]^-$ ions are illustrated in Fig. 2. The results obtained in the negative-ion mode are consistent with those reported for schaftoside and isoschaftoside by Becchi and Fraisse [11]. The presence of both a C-hexosyl and C-pentosyl group is evident from the formation of the sets of ions typical for each group as shown in Fig. 3. By comparison of the ^{13}C NMR spectra obtained for the isolated compounds with published data, the structures of compounds A and C were confirmed as isoschaftoside and schaftoside, respectively [15–18]. The ^{13}C NMR spectral data of the sugar moieties are listed in Table V. Differentiation between the two isomers was made on the basis of mass spectral correlations. Becchi and

TABLE III

DAUGHTER ION MASS SPECTRA OF $[M - H]^-$ IONS OF COMPOUNDS A AND C

m/z	A: isochaftoside (VII)	C: schaftoside (VI)	Fragment ion assignments
545	14	16	$[M - H - 18]^-$
303	12	13	$[M - H - 60]^-$
473	100	64	$[M - H - 90]^-$
459	16	20	$[M - H - 104]^-$
433	68	100	$[M - H - 120]^-$
429	16	16	$[M - H - 134]^-$
413	6	12	$[M - H - 150]^-$ or $[M - H - 60 - 90]^-$[a]
383	23	24	$[M - H - 90 - 90]^-$
353	31	27	$[M - H - 120 - 90]^-$

[a] For rationalization of this ion structure, see ref. 14.

TABLE IV

DAUGHTER ION MASS SPECTRA OF $[M + H]^+$ IONS OF COMPOUNDS A AND C

m/z	A: isoschaftoside (**VII**)	C: schaftoside (**VI**)	Fragment ion assignments
547	100	100	$[M + H - 18]^+$
529	62	33	$[M + H - (2 \times 18)]^+$
511	26	10	$[M - H - (3 \times 18)]^+$
499	38	10	–[a]
475	74	66	$[M + H - 90]^+$
461	62	17	$[M + H - 104]]^+$
457	27	40	$[M + H - 90 - 18]^+$
445	48	67	$[M + H - 120]^+$
431	39	40	$[M + H - 134]^+$
427	82	15	$[M + H - 120 - 18]^+$
409	58	11	$[M + H - 120 - (2 \times 18)]^+$
403	14	16	$[M + H - 162]^+$
379	50	35	$[M + H - 150 - (2 \times 18)]^+$
365	24	13	–[a]
355	33	19	$[M + H - 120 - 90]^+$
349	21	14	$[M + H - 180 - (2 \times 18)]^+$
341	40	22	$[M + H - 134 - 90]^+$
337	33	21	–[a]
325	56	33	$[M + H - 120 - 120]^+$
311	26	13	$[M + H - 120 - 134]^+$
295	25	14	$[M + H - 120 - 150]^+$
289	18	23	–[a]
283	14	9	A_1^{+b}
121	8	5	B_2^{+b}

[a] No assignment made.

[b] For a rationalization of these ion structures, see ref. 14.

Fraisse [11] demonstrated that linkage information can be derived from the relative abundances of characteristic ions because the C-6 sugar substituent gives rise to the most abundant fragments. They found that schaftoside (**VI**), which has a C-6 glucose substituent, yields the most abundant $[M - H - 120]^-$ ion, formed by a B-type cleavage, whereas its isomer, isoschaftoside (**VII**), which has a C-6 arabinose group, results in the most abundant $[M - H - 90]^-$ ion, due to a similar B-type cleavage. Our spectra (Fig. A and Table III) show that the most abundant $[M - H - 120]^-$ and $[M - H - 90]^-$ ions are found for compounds C and A, respectively, indicating that C can be characterized as schaftoside and A as isoschaftoside.

The daugher ion spectra of $[M + H]^+$ ions were also recorded in order to evaluate whether schaftoside and isoschaftoside can be distinguished in the positive-ion mode. The data in Table IV show that there are clear differences in

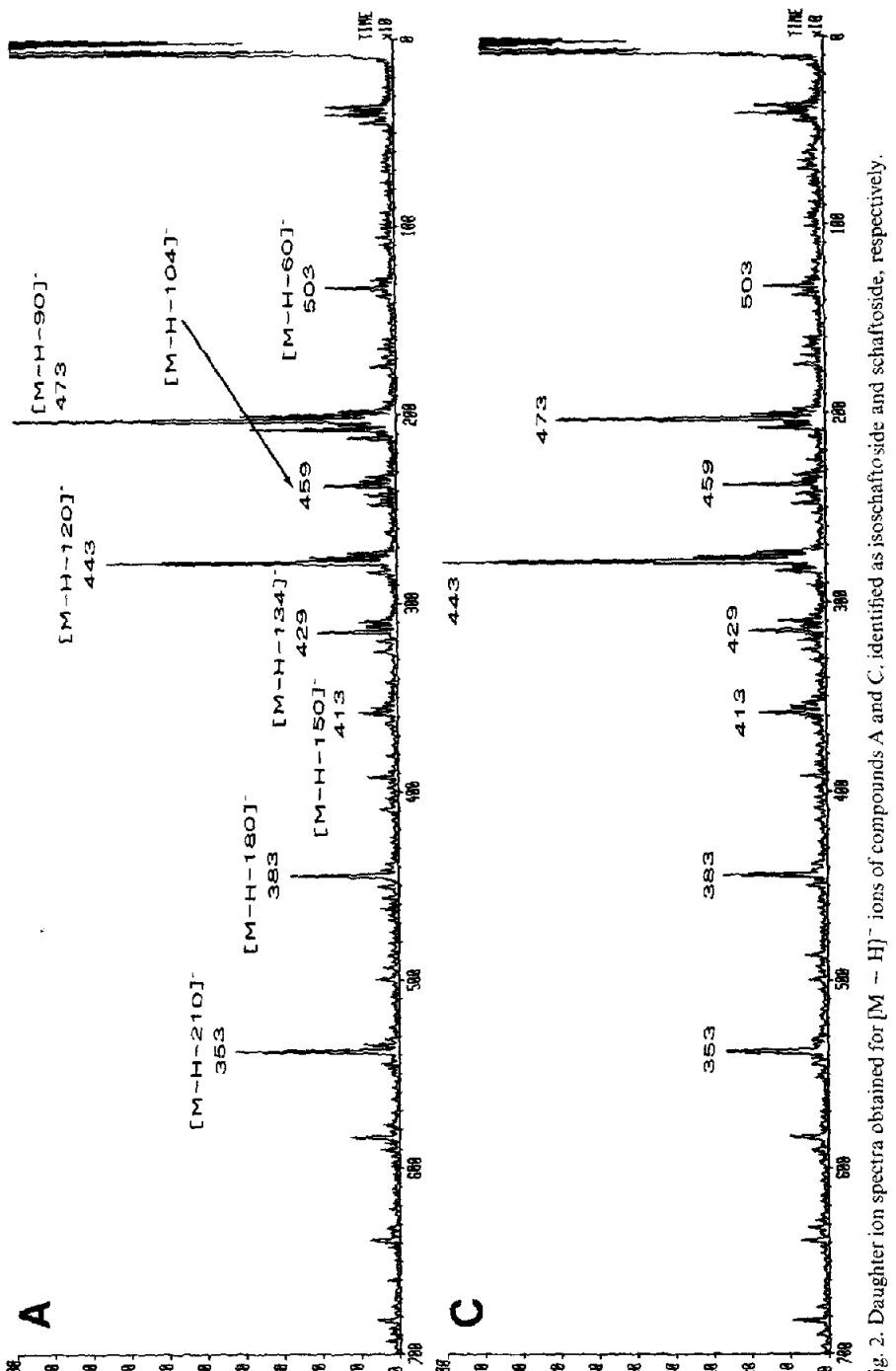
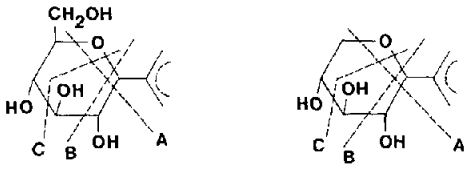


Fig. 2. Daughter ion spectra obtained for $[M - H]^-$ ions of compounds A and C, identified as isoschaftoside and schaftoside, respectively.



C-hexosyl	C-pentosyl
A: [M-H-134]⁻	[M-H-104]⁻
B: [M-H-120]⁻	[M-H-90]⁻
C: [M-H-90]⁻	[M-H-60]⁻

Fig. 3. Characteristic ions for C-hexosyl and C-pentosyl moieties.

relative abundances. It is worth noting that the loss of two and three molecules of H_2O is much more prominent with isoschaftoside. Another striking difference concerns the $[M + H - 104]^+$ ion, which is formed by an A-type cleavage and is most abundant in the daughter ion spectrum of $[M + H]^+$ ions of isoschaftoside. This phenomenon can be rationalized by hydrogen bonding, which is more pro-

TABLE V

^{13}C NMR CHEMICAL SHIFTS OF THE SUGAR MOIETIES OF ISOSCHAFTOSIDE (A), SCHAFTOSIDE (C), ISOVITEXIN-2″-O-β-GLUCOPYRANOSIDE (B) AND ISOORIENTIN-2″-O-β-GLUCOPYRANOSIDE (D)

Carbon No.	Compound		Carbon No.	Compound	
	A (VII)	C (VI)		B (IX)	D (X)
Glucose			*Glucose*		
G-1	75.2	75.4	G-1	73.4	73.5
G-2	73.2	72.8	G-2	81.6	81.7
G-3	80.3	79.9	G-3	79.8	79.9
G-4	72.5	71.6	G-4	71.6[a]	71.6[a]
G-5	83.0	82.6	G-5	82.4	82.5
G-6	63.1	62.6	G-6	62.8[a]	62.8[a]
Arabinose			*(Glucose)'*		
A-1	75.2	76.0	G-1′	106.0	106.1
A-2	70.5[a]	70.8[a]	G-2′	75.9	76.0
A-3	76.6	77.5	G-3′	77.4	77.4
A-4	71.2[a]	70.7[a]	G-4′	71.1[a]	71.1[a]
A-5	71.9	72.3	G-5′	77.8	77.8
			G-6′	62.4[a]	62.4[a]

[a] Assignments may be reversed.

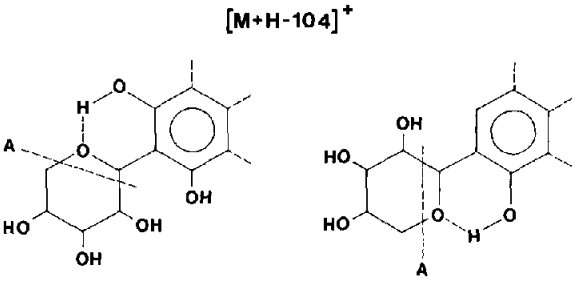


Fig. 4. Mechanism for the formation of $[M + M - 104]^+$ ions.

nounced in the case of C-6 substitution and can promote an A-type cleavage (Fig. 4).

Mass spectral and NMR characterization of compounds B and D

The daughter ion spectra obtained for $[M - H]^-$ and $[M + H]^+$ ions of compounds B and D are listed in Tables VI and VII, respectively. The data for the $[M - H]^-$ ions (Table VI) are in good agreement with the results reported by Becchi and Fraisse [11] for isovitexin-2″-O-β-glucopyranoside (**IX**) and isoorientin-2″-O-β-glucopyranoside (**X**), respectively. The most abundant fragment ion noted for both B and D corresponds to the $[M - H - 180]^-$ ion. Evidence for a 1→2 linkage in the diglucosidic moiety is given by the presence of a $[M - H - 180 - 120]^-$ ion, which is rationalized by the loss of a terminal glucose unit and a B-type cleavage in the remaining C-6-linked glucose moiety (Fig. 5). Further

TABLE VI

DAUGHTER ION MASS SPECTRA OF $[M - H]^-$ IONS OF COMPOUNDS B AND D

B: isovitexin-2″-O-β-glucopyranoside (IX)		D: isoorientin-2″-O-β-glucopyranoside (X)		Fragment ion assignments
m/z	Relative abundance (%)	*m/z*	Relative abundance (%)	
577	7	593	10	$[M + H - 18]^-$
473	53	489	95	$[M + H - 120]^-$
431	36	447	32	$[M + H - 162]^-$
413	100	429	100	$[M + H - 180]^-$
353	5	369	6	$[M + H - 120 - 120]^-$
341	12	357	16	$[M + H - 162 - 90]^-$
323	84	339	87	$[M + H - 150 - 120]^-$
311	27	327	27	$[M + H - 162 - 120]^-$
293	78	309	73	$[M + H - 180 - 120]^-$
282	25	298	48	$[M + H - 162 - 150]^-$
269	14	285	13	$[M + H - (2 \times 162)]$

TABLE VII

DAUGHTER ION MASS SPECTRA OF $[M + H]^+$ IONS OF COMPOUNDS B AND D

B: isovitexin-2″-O-β-glucopyranoside (IX)		D: isoorientin-2″-O-β-glucopyranoside (X)		Fragment ion assignments
m/z	Relative abundance (%)	*m/z*	Relative abundance (%)	
577	26	593	49	$[M + H - 18]^+$
505	75	521	60	$[M + H - 90]^+$
475	14	491	24	$[M + H - 120]^+$
461	31	477	49	$[M + H - 134]^+$
433	100	449	100	$[M + H - 162]^+$
415	73	431	89	$[M + H - 180]^+$
397	28	413	68	$[M + H - 180 - 18]^+$
379	19	395	42	$[M + H - 180 - (2 \times 18)]^+$
367	14	383	29	$[M + H - 120 - 90 - 18]^+$
341	22	357	41	$[M + H - 120 - 134]^+$
337	53	353	78	$[M + H - 150 - 90 - 18]^+$ or $[M + H - (2 \times 120) - 18]^+$
325	63	341	74	$[M + H - 150 - 120]^+$
313	86	329	98	$[M + H - 162 - 120]^+$
299	75	315	84	$[M + H - 162 - 134]^+$
283	68	299	86	$[M + H - 162 - 150]^+$
271	30	287	55	$[M + H - 162 - 162]^+$
121	4	137	12	B_2^+ [a]

[a] For a rationalization of this ion structure, see ref. 14.

support for a sophorose moiety, in which two glucose units are 1→2 linked, is provided by the ^{13}C NMR data (Table V), which show the typical signals of sophorose and are in good agreement with published spectra [8].

Daughter ion spectra were also taken for the $[M + H]^+$ ions (Table VII) in

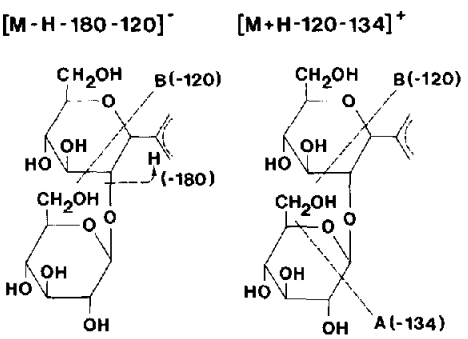


Fig. 5. Mechanism for the formation of $[M - H - 180 - 120]^-$ and $[M + H - 120 - 134]^+$ ions.

TABLE VIII

RELATIVE ABUNDANCES (%) OF MAJOR FRAGMENTS OBTAINED FOR $[M - H - 162]^-$ IONS OF COMPOUNDS B AND D AND FOR $[M - H]^-$ IONS OF VITEXIN AND ISOVITEXIN

Fragment ion[a]	B (IX)	D (X)	Vitexin (I)	Isovitexin (II)
$[M - H - 18]^-$	14	10	–	13
$[M - H - 90]$	57	75	15	46
$[M - H - 120]^-$	100	100	100	100
$[M - H - 134]^-$	25	26	23	24
$[M - H - 150]^-$	20	22	12	14
$[M - H - 162]^-$	14	9	10	8

[a] For compounds B and D: M = M − 162.

order to evaluate whether structural information comparable to that obtained for the $[M - H]^-$ ions could be achieved. As can be noted in Table VII, an $[M + H - 120 - 134]^+$ ion is found, which results from an A-type cleavage in the terminal glucose unit and a β-cleavage in the C-6-linked glucose moiety, which gives proof of a 1→2 linkage (Fig. 5). Surprisingly, this ion is absent from the daughter ion spectra of the corresponding $[M - H]^-$ ions. In order to find support for C-6 glycosylation of sophorose, which is a diglucose, daughter ion spectra were also recorded for the $[M - H - 162]^-$ ions (Table VII) and compared with those obtained for $[M - H]^-$ ions of vitexin and isovitexin (Table VIII). The data in Table VIII show that, with compounds B and D, the fragmentation pattern of the $[M - H - 162]^-$ ions is similar to that observed for $[M - H]^-$ ions of isovitexin, thus indicating C-6 glycosylation.

CONCLUSIONS

The mass spectral data of the C-glycosidic flavonoids illustrate that daughter ion spectra of both $[M + H]^+$ and $[M - H]^-$ ions show diagnostic ions, which (i) allow differentiation between the isomeric 6,8-C-diglycosides, schaftoside (**VI**) and isoschaftoside (**VII**), and (ii) provide proof for a 1→2 linkage in the diglucosidic sophorose moiety of isovitexin-2″-O-β-glucopyranoside (**IX**) and isoorientin-2″-O-β-glucopyranoside (**X**). With the latter compounds, evidence for a C-6-linked sophorose moiety was also obtained by recording daughter ion spectra of $[M - H - 162]^-$ ions and comparison with those of $[M - H]^-$ ions of vitexin (**I**) and isovitexin (**II**).

The mass spectral and ^{13}C NMR data obtained in this study demonstrate that the four major C-glycosidic flavonoids found on reversed-phase HPLC of crude extracts of *Passiflora incarnata* correspond to isoschaftoside, isovitexin-2″-O-β-glucopyranoside, schaftoside and isoorientin-2″-O-β-glucopyranoside, in order of increasing retention time. These results agree with those obtained by Geiger

and Markham [8] but not with less documented investigations reported in the literature [1,2].

ACKNOWLEDGEMENTS

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