Phytochemistry, 1973, Vol. 12, pp. 2765 to 2772. Pergamon Press. Printed in England.

GC–MS OF PERDEUTERIOMETHYLATED FLAVONOID AGLYCONES

ROLF D. SCHMID*, RÜDIGER MUES†, JAMES H. MCREYNOLDS, GEORGE VANDER VELDE, NOBUJI NAKATANI, ELOY RODRÍGUEZ and TOM J. MABRY‡

The Cell Research Institute and Department of Botany, The University of Texas at Austin, TX 78712, U.S.A.

(*Revised Received* 20 *May* 1973. *Accepted* 19 *June* 1973)

Key Word Index—GC–MS; gas chromatography; MS fragmentation; flavones; flavonols; flavanones; chalcones; flavonoid glycosides; perdeuteriomethylation; ethylation.

Abstract—GC–MS of perdeuteriomethylated flavonoid aglycones, singly and in mixtures, yields information about both the aglycone types and their substitution patterns. Fragmentation patterns of flavonoid aglycones are discussed. Acid hydrolysis of perdeuteriomethylated flavonoid glycosides, singly and in mixtures, followed by ethylation with diazoethane provides derivatives suitable for GC–MS; the introduced ethyl groups permit identification of the position of attachment of sugars in flavonoid *O*-glycosides.

INTRODUCTION

WE DESCRIBE here GC–MS procedures for analyzing mixtures of flavonoid aglycones which supplement previous UV, NMR[1] and MS[2–6] spectroscopic methods. Recently one of us[4–6] established that flavonoid glycosides can be readily analyzed by MS of their perdeuteriomethylated (PDM) derivatives; this method provides information about the sugar sequence, their interglycosidic linkages and the position of sugar attachment to the aglycone.

In addition to describing GC–MS procedures for the analysis of PDM mixtures of flavonoid aglycones, we include here a GC–MS scheme which may permit determination of the point of attachment of the sugars which were present in natural mixtures of flavonoid glycosides.

RESULTS AND DISCUSSION

GC–MS Analysis of PDM Flavonoid Aglycones

Three flavones, 3 flavanones and 7 flavonols were perdeuteriomethylated with CD_3I and NaH in DMF;[7] under these reaction conditions flavanones are converted to chalcones and not deuteriomethylated at C-3 as previously suggested.[4]

* On temporary leave from Institut de Chimie des Substances Naturelles C.N.R.S., Gif-sur-Yvette, France. Present address: Henkel & Cie Gmbh, Biochem. Abteilung, D-4 Duesseldorf, West Germany.

† On temporary leave from Institut fur Botanik der Universitat des Saarlandes, 66 Saarbrücken, West Germany.

‡ To whom correspondence should be addressed.

[1] MABRY, T. J., MARKHAM, K. R. and THOMAS, M. B. (1970) *The Systematic Identification of Flavonoids*, Springer, New York.

[2] KINGSTON, D. G. I. (1971) *Tetrahedron* **27**, 2691.

[3] (a) VALESI, A. G., RODRIGUEZ, E., VANDER VELDE, G. and MABRY, T. J. (1972) *Phytochemistry* **11**, 2821; (b) RODRIGUEZ, E., CARMAN, N. J., VANDER VELDE, G., MCREYNOLDS, J. H., MABRY, T. J., IRWIN, M. A. and GEISSMAN, T. A. (1972) *Phytochemistry* **11**, 3509.

[4] SCHMID, R. D. (1972) *Tetrahedron* **28**, 3259.

[5] SCHMID, R. D., VARENNE, P. and PARIS, R. R. (1972) *Tetrahedron* **28**, 5037.

[6] SCHMID, R. D. and HARBORNE, J. B. (1973) *Phytochemistry* **12**, 2269.

[7] BRIACOMBE, J. S., JONES, B. D., STACEY, M. and WILLARD, J. J. (1966) *Carbohyd. Res.* **2**, 167.

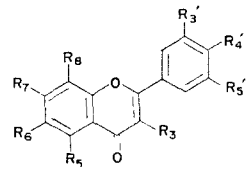


TABLE 1. GC–MS DATA FOR PERDEUTERIOMETHYLATED FLAVONOLS

Parent compounds	Substitution pattern after perdeuteriomethylation							
	R_3	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$	$R_{5'}$
3-Methoxygalangin	Me_3	OCD_3	H	OCD_3	H	H	H	H
Kaempferol	OCD_3	OCD_3	H	OCD_3	H	H	OCD_3	H
Quercetin	OCD_3	OCD_3	H	OCD_3	H	OCD_3	OCD_3	H
Myricetin	OCD_3	OCD_3	H	OCD_3	H	OCD_3	OCD_3	OCD_3
3,3',4'-Trihydroxyflavone	OCD_3	H	H	H	H	OCD_3	OCD_3	H
3,5,6,7,8-Pentamethoxyflavone	Me	Me	Me	Me	Me	H	H	H
3,5,6,7,8,4'-Hexamethoxyflavone	Me	Me	Me	Me	Me	H	Me_3	H

GC*		MS†					
RR_{t260}	RR_{t280}	$M^{+\cdot}$	$[M-1]^+$	$[M-CD_3]^+$	a_1	a_2	b_1
0·72	—	318 (65)	317 (100)	300 (9)	187 (8)	—	105 (12)
1·72	1·63	354 (85)	353 (100)	336 (15)	188 (3)‡	—	138 (7)
2·70	2·41	387 (100)	386 (67)	369 (46)	188 (5)‡	—	171 (9)
3·44	3·03	420 (100)	419 (33)	402 (69)	188 (<1)‡	—	204 (<1)
0·62	—	321 (100)	320 (67)	303 (47)	122 (2)‡	—	171 (3)
0·74	0 79	372 (100)	371 (49)	357 (37)§	241 (2)	240 (3)	105 (4)
1·78	1·68	402 (69)	401 (30)	387 (100)§	241 (<1)	240 (<1)	135 (1)

* RR_{t260} = relative retention time at 260°, relative to 4,2',4',6'-tetradeuteriomethoxychalcone (180 sec = 1; RR_{t280} = relative retention time at 280°, relative to 4,2',4',6'-tetradeuteriomethoxychalcone (90 sec = 1).

† MS = data are given as *m/e* values; relative intensity in parenthesis (base peak RI = 100). Fragments are explained in the text.

‡ Formed by D-transfer.

§ $[M-15]^+$.

GLC

In the few published papers on GC of permethylated[8] or pertrimethylsilylated[9–11] flavonoids, SE30, OV1 or OV101 have been used as liquid phases. In our studies, OV17 gave superior separation relative to OV1. GC data obtained on short analytical columns coated with OV17 are presented in Tables 1–3. Retention times (R_ts) of flavonols (Table 1), flavones (Table 2) and the chalcones obtained from flavanones (Table 3) generally increase with the number of substituents. For flavones and flavonols (e.g. see data for flavonols in Table 1) substitution of the B-ring increases the retention time considerably more than does substitution in the A-ring. An exception to this finding is the behavior of 3,3',4'-deuteriomethoxyflavone which, although bearing two substituents in the B-ring, has a short retention time. The R_ts of all 5,7-substituted compounds are compared in Table 4.

[8] NARASIMHACHARI, N. and VON RUDLOFF, E. (1962) *Can J. Chem.* **40**, 1123.

[9] FURUYA, T. (1965) *J. Chromatog.* **19**, 607.

[10] PIERCE, A R., GRAHAM, H N., GLASSNER, S , MADLIN, H and GONZALES, J. G. (1969) *Anal Chem.* **41**, 301.

[11] COFFIN, D. E. and DUPONT, J. E. (1971) *J. AOAC* **54**, 1211.

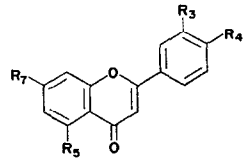


TABLE 2. GC–MS DATA FOR PERDEUTERIOMETHYLATED FLAVONES*

Parent compounds	Substitution patterns after perdeuteriomethylation R_5	R_7	$R_{3'}$	$R_{4'}$	RR_{t260}	GC RR_{t280}
Tectochrysin	OCD_3	Me	H	H	0·72	—
Acacetin	OCD_3	OCD_3	H	Me	1·75	1·75
5,7-Dihydroxy-3',4'-dimethoxyflavone	OCD_3	OCD_3	Me	Me	2·86	2·61

MS $M^{+\cdot}$	$[M-1]^+$	$[M-2]^+$	$[M-CD_3]^+$	$[M-CO-D]^+$ (M-30)	$[M-CD_2O_2]^+$	a_1	b_2
285 (100)	284 (33)	283 (39)	267 (6)	255 (39)	237 (44)	183 (<1)	102 (1)
318 (100)	317 (25)	316 (32)	300 (7)	288 (26)	270 (33)	186 (<1)	132 (20)
348 (100)	347 (24)	346(28)	330 (9)	318 (28)	300 (34)	186 (<1)	162 (13)

* For explanation of symbols see Table 1.

Chalcones, flavonols and flavones with equally substituted B-rings are eluted from the column in this order. Although only 4 compounds without B-ring substitution were available, their R_ts are also in accord with this general pattern. The chalcone derived from the flavanone pinocembrin has the shortest R_t of all the compounds studied eluting well before the flavonol galangin 3-methyl ether and the flavone tectochrysin. These latter two

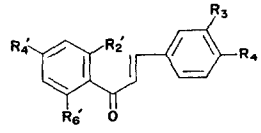


TABLE 3. GC–MS DATA FOR PERDEUTERIOMETHYLATED CHALCONES DERIVED FROM FLAVANONES*

Compounds	Substitution patterns for perdeuteriomethylation $R_{2'},R_{4'},R_{6'}$	R_3	R_4	GC RR_{t260}	RR_{t280}	$M^{+\cdot}$
PDM Chalcone from Pinocembrin	OCD_3	H	H	0·43	—	307 (16)
PDM Chalcone from Naringenin	OCD_3	H	OCD_3	1·00	1·00	340 (46)
PDM Chalcone from Eriodictyol	OCD_3	OCD_3	OCD_3	1·72	1·59	373 (86)

MS $[M-1]^+$	$[M-CD_3]^+$ (M-18)[3]	$(M-28)^{+\cdot}$	a_4	a_5†	b_4
306 (2)	289 (15)	279 (100)	204 (28)	186 (6)	131 (3)
339 (7)	322 (14)	312 (100)	204 (13)	186 (3)	164 (5)
372 (19)	355 (21)	345 (100)	204 (30)	186 (6)	187 (9)

* For explanation of symbols see Table 1.

† The a_5 fragment is equivalent to a_4–CD_3.

compounds have the same R_ts indicating that in the absence of a 4′ substituent they have similar polarities. The flavonol 3,5,6,7,8-pentamethoxyflavone elutes at a slightly longer time in this series in accordance with its additional A-ring substitution (see Table 4).

With the less-polar SE30 liquid phase, retention times of pertrimethylsilylated flavonol aglycones are longer than those observed for similarly substituted flavones.[10]

TABLE 4. R_tS OF 5,7-SUBSTITUTED FLAVONOIDS (DIFFERING IN B-RING SUBSTITUENTS) ON OV17

	B-ring substitution pattern (No. and position of B-ring Me or OCD_3 groups			
	Aglycone type			
Parent compounds	Flavanones†	Flavonols	Flavones	RR_t* (260°)
Pinocembrin†	None			0·43
Galangin 3-*O*-methyl ether		None		0·72
Tectochrysin			None	0·72
Naringenin†	4′			1·00
Eriodictyol†	3′4′			1·72
Kaempferol		4′		1·72
Acacetin			4′	1·75
Quercetin	×	3′4′		2·70
5,7-Hydroxy-3′,4′-dimethoxyflavone			3′4′	2·86
Myricetin		3′4′5′		3·44

* R_t at 260° relative to 2′,4′,6′,4-tetradeuteriomethoxychalcone.
† Converted to chalcones during PDM derivatization.

MS

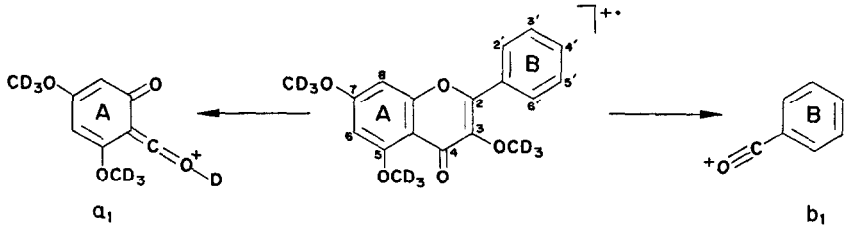
The intense molecular ion ($M^{+\cdot}$) peak shown by all compounds clearly indicates the number and type of substituents present in the aglycone derivative. A closer inspection of the MS allows differentiation between isomeric structures (e.g. a 3,5,7,4′-tetradeuteriomethoxyflavone or a 5,7,3′,4′-tetradeuteriomethoxyflavone). Since MS of flavonoid aglycones and their derivatives have been discussed in detail elsewhere (see Refs. 2, 3, 12, 13) we limit our discussion here to problems pertinent to the differentiation of isomeric compounds and distinguishing the substitution patterns of the A- and B-rings. Fragments derived from the A- and B-rings are named a_1, a_2 . . . and b_1, b_2 . . . , respectively.

Flavonols (Table 1)

In the MS of most permethylated flavonols, $M^{+\cdot}$, $[M\text{-}1]^+$ and $[M\text{-}15]^+$ are prominent peaks. The most abundant a-type and b-type fragments are a_1 (with an H-transfer) and b_1, respectively.[2] In GC–MS analysis of PDM flavonols, strong M^+, $[M\text{-}1]^+$ moderately intense $[M\text{–}CO_3]^+$ and weak a_1 and b_1 fragments were observed (see Scheme 1).

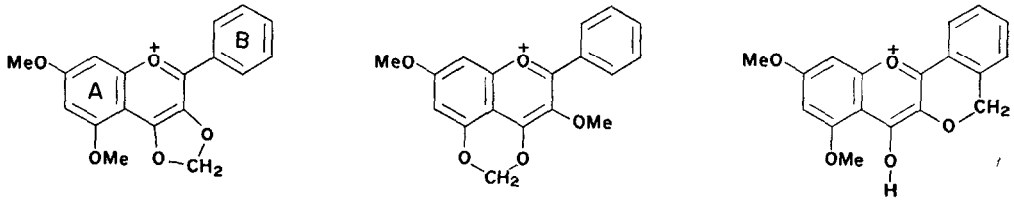
The presence of a strong $[M\text{-}1]^+$ peak in flavonol permethyl ethers has been attributed[2] to structures I or II which could be formed after loss of hydrogen from the 3- or 5-OMe groups, respectively.

However, this interpretation requires reinvestigation since in our studies with flavonols perdeuteriomethylated at C_3 and C_5 a comparable $[M-2]^+$ was not observed while an $[M-1]^+$ peak was still present. Therefore, the formation of this $[M-1]^+$ peak in these flavonols presumably involves an aromatic proton, perhaps as in a structure such as III.



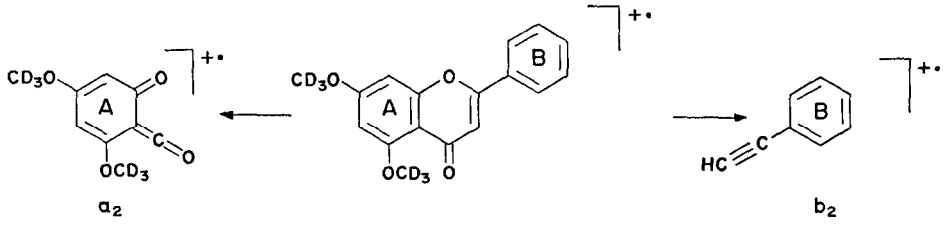
SCHEME 1. MS FRAGMENTS FROM PDM FLAVONOLS.

After perdeuteriomethylation the weak a_1 fragment was observed at 1 m.u. higher than calculated for a simple H-transfer in all compounds except galangin 3-*O*-methyl ether. It is therefore concluded that in these compounds deuterium or hydrogen is transferred from the C_3 substituent. The a_1 and b_1 fragments in PDM flavonols allow identification of the number and type of substituents in the A- and B-rings of the parent compounds; however, the degree of oxygenation appears to determine the intensities of these peaks.



Flavones (Table 2)

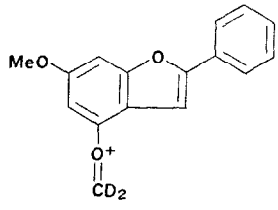
The MS of flavones are distinguished by the presence of a strong M^+, weak $[M-28]^{+\cdot}$, a_2 and b_2 fragments (due to retro-Diels–Alder cleavage) and very weak b_1 peaks (see Scheme 2).



SCHEME 2.MS FRAGMENTS FROM PDM FLAVONES.

In the GC–MS analysis of the three PDM flavones the a_1 and b_1 peaks were either not detected or were very weak. The b_2 peaks were detected; this information coupled with the GC R_ts and other MS information gives a good indication of the substitution patterns of the A and B rings.

The $[M-2]^+$ peak is stronger in the PDM flavones examined than is the $[M-1]^+$ peak; by contrast, PM flavones show only an $[M-1]^+$ peak. Since PDM tectochrysin has only a single deuteriomethoxyl group at C_5 and shows the [M-2] peak, it must result from loss of deuterium from the C_5 group. In addition, the PDM compounds show a strong $[M-30]^+$, as opposed to $[M-29]^+$, [M-CO-H], in the permethylated compounds and an $[M-28]^+$ in the compounds with free hydroxyl groups. Again, since PDM tectochrysin shows the $[M-30]^+$ fragment, it must involve a loss of deuterium from the C_5 group, possibly giving rise to a structure such as IV.

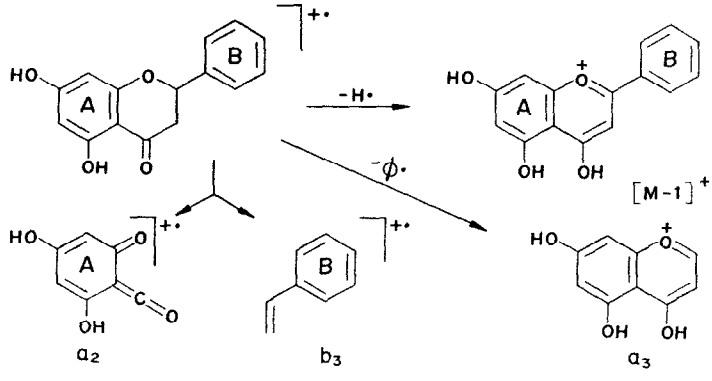


(IV)

An intense fragment at $[M-48]^{+\cdot}$ ($[M-CD_2O_2]$) is observed in the PDM flavones and is shifted to $[M-46]^{+\cdot}$ ($[M-CH_2O_2]^{+\cdot}$) in the permethylated compounds (see Table 2). The structure of this fragment is under investigation.

Flavanones (see Table 3 for data on PDM chalcones derived from flavanones)

MS of flavanones usually are distinguished by the presence of a $[M-1]^+$, an a_2-type fragment, a phenylethylene type fragment b_3, and the chromene type fragment a_3[12–14] (Scheme 3).



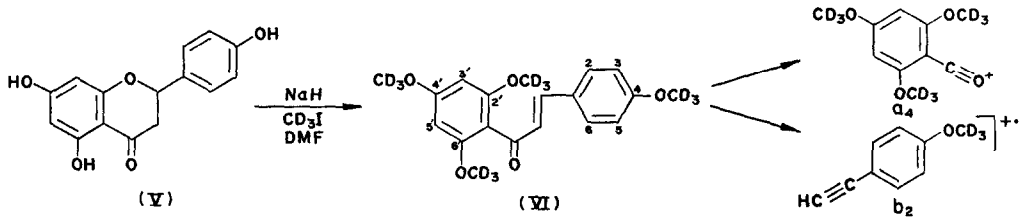
SCHEME 3. MS FRAGMENTS FROM FLAVANONES.

In the presence of the strong base used in the perdeuteriomethylation procedure, flavanones undergo ring opening to chalcones which are subsequently converted to PDM chalcones (Scheme 4). For example, naringenin (V) gave VI (by UV and NMR).

In the GC–MS analysis of the PDM chalcones employed in this study a strong $[M-28]^{+\cdot}$ peak is observed; this appears to be typical for chalcones with 2′ and 2′,6′ methoxyl groups.

[12] AUDIER, H. (1966) *Bull. Soc. Chim. Fr* 2894.
[13] PELTER, A., STAINTON, P. and BARBER, M. (1965) *J. Heterocyclic Chem.* **2**, 262.
[14] VAN DE SANDE, C., SERUM, J. W. and VANDERWALLE, M. (1972) *Org. Mass. Spect.* **6**, 1333.

The proposed structure for the diagnostic fragment a_4 is supported by its shift from *m/e* 204 to *m/e* 195 in the permethylated compounds indicating the presence of 3 deuteriomethyl groups in this fragment. The a_4, a_5 (which is equivalent to a_4–CD_3) and b_4 fragments in the mass spectra of the chalcones usually allow identification of the substitution patterns of the A- and B-rings in the parent flavanones.



SCHEME 4. CONVERSION OF FLAVANONES TO CHALCONES DURING PERDEUTERIOMETHYLATION; MS FRAGMENTS FROM 1 PDM CHALCONE.

GC–MS Analysis of an Aglycone Mixture Obtained by Hydrolysis of PDM Flavone Glycosides

Starting with a mixture of flavonoid glycosides, it is possible to identify the ring (and often the position) to which sugars are attached in the different compounds. The mixture of flavonoid glycosides are first perdeuteriomethylated and then subjected to acid hydrolysis.

TABLE 5. GC–MS DATA FOR A MIXTURE OF DERIVATIZED FLAVONOID AGLYCONES OBTAINED BY HYDROLYSIS OF THEIR DERIVATIZED GLYCOSIDES

Aglycone mixture	Experimental procedure		MS data*				
Mixture A	MS on mixture						
Apigenin 7,4′-dimethyl ether			$M^{+\cdot}$ 298s	$[M-29]^+$ 269m	a_2 166m	b_2 135w	b_2 132v
Apigenin 7-methyl 4′-deuteriomethyl ether			301s	272m	166m	138m	135v
Luteolin 7,4′-dimethyl 3′-deuteriomethyl ether			331s	302s	166m	168w	165w
Mixture B‡	GC–MS on mixture		$M^{+\cdot}$	$[M-15]^+$	$[M-44]^+$	a_2	b_2
	RR_{t260}§	RR_{t280}					
GC-peak 1							
Apigenin 5-ethyl, 7,4′-dimethyl ether	1·56	1·56	326s	311s	282m	197m	132m
Apigenin 5-ethyl, 7-methyl, 4′-deuteriomethyl ether	1·56	1·56	329s	314s	285m	197m	135m
GC-peak 2							
Luteolin 5-ethyl, 7,4′-dimethyl, 3′-deuteriomethyl ether	2·61	2·45	359s	344s	315s	197m	165v

* Intensities are given as s = strong, m = medium, w = weak, v = visible.
† Mixture A was obtained by acid hydrolysis of a mixture of three PDM 5-O-xylosylglucosides.
‡ Mixture B was obtained by ethylation of mixture A.
§ See Table 1 and text for an explanation of symbols.

The deuteriomethylated aglycones containing one or more free hydroxyl groups after acid hydrolysis of the sugar residues are ethylated using diazoethane; this enhances volatility and permits location of the point of attachment of the sugars. The aglycone derivatives obtained in the above manner now contain up to three different substituents: (a) methoxyl

groups which were present in the natural compound; deuteriomethyl groups which were introduced during derivatization of the glycosides; (c) and ethyl groups which were introduced at the positions where the sugars were attached before hydrolysis.

Since most perdeuteriomethylated flavonoids give MS fragments derived from both the A- and B-rings, the ring to which an ethyl residue is attached can be readily identified. In a typical experiment, we applied this method to the analysis of an aglycone mixture obtained after hydrolysis of PDM flavone 5-*O*-xylosylglucosides.[15] The results (see *m/e* values for a_2 fragments in Table 5) give clear evidence for the ethyl groups being attached to the A-ring in all aglycones. The GC of the mixtures gave two peaks, the first representing two apigenin ethers: 5-ethyl, 7-methyl, 4′-deuteriomethyl and 5-ethyl, 7,4′-dimethyl; and the second: luteolin 5-ethyl, 7, 4′-dimethyl, 3′-deuteriomethyl ether.

EXPERIMENTAL

Permethylations and perdeuteriomethylations were carried out on 0·05–1 mg of a flavonoid sample as previously described.[4] For GC, the following conditions were employed: 122 × 0·32 cm stainless steel columns containing 1·5% OV17 on chromosorb W HP, 80–100 mesh; helium flow rate of 30 ml/min (measured at 260°); temp. either 260° or 280° (isothermal). For detection of the eluted compounds, the columns were coupled to a FID for preliminary runs and to determine retention times. For GC–MS the columns were coupled to the mass spectrometers using a heated transfer line and a jet molecular separator at 300°. Three different mass spectrometers were employed for this study: a Dupont 21-491, and LKB 9000 and a Finnigan 1015C; in all cases the electron beam energy was 70 eV.

Hydrolyses of perdeuteriomethylated glycosides were performed by heating the samples at 100° in 2 N H_2SO_4 for 1 hr. In each case, after cooling, the aglycone precipitate was separated by centrifugation,* washed with sat. aq. $NaHCO_3$ and dried. After dissolving the aglycone precipitate in a minimum amount of MeOH, a few drops of diazoethane-ether[15] were added and the resultant solution was allowed to stand at about 5° overnight. The solution was taken to dryness, the residue dissolved in acetone and subjected to GC–MS

Acknowledgements—R D.S. thanks Deutsche Forschungsgemeinschaft for a fellowship. We are indebted to Professor J Oro, Department of Biophysical Sciences, University of Houston, for permission to work with the LKB-9000; to Dr. William Stavioha of the Pharmacology Department, University of Texas Medical School at San Antonio and Mr. Dennis Orr of the Finnigan Corporation for the use of the Finnigan 1015 GC–MS System, and to Dr. Daryl Nooner of the University of Houston and Mrs. Sue Weintraub of the University of Texas Medical School for technical assistance. This work was supported by the Robert A. Welch Foundation (Grant F-130), The National Science Foundation (Grants GB-29576X and GB-27152), and the National Institutes of Health (HD-04488 and 5TO1-GM-00789).

* The supernatant from a H_2SO_4 hydrolysis can be readily analyzed for sugars.[5,6]

[15] NUNÉZ-ALARCÓN, J., RODRIGUEZ, E., SCHMID, R. D. and MABRY, T. J. (1973) *Phytochemistry* **12**, 1451.
[16] ARNDT, F. (1943) *Org. Syn. Coll.* **2**, 165.