Phytochemistry, 1981, Vol. 20, pp. 532–534. Pergamon Press Ltd. Printed in England. 0031-9422/81/030532-03 $02.00/0

DIFFERENTIATION OF DIHYDROXY-MONOMETHOXYISOFLAVONES BY GAS CHROMATOGRAPHY–MASS SPECTROSCOPY

MICHAEL D. WOODWARD*

Division of Plant Industry, CSIRO, P.O. Box 1600, Canberra City, A.C.T. 2601, Australia

(*Received* 18 *April* 1980)

Key Word Index—Leguminosae; MS fragmentation; isoflavones; biochanin A; calycosin; theralin; texasin; retusin.

Dihydroxy-monomethoxyisoflavones can be distinguished by UV spectroscopy [1], ^{1}H NMR [2], Gibbs reaction [3] and other techniques if sufficient material is available. Detection of the minor isoflavone, 2′-hydroxyformononetin, from *Trifolium repens* L. in submicrogram quantities has been reported using gas chromatography–mass spectroscopy (GC/MS) with selected ion monitoring [4]. Numerous dihydroxy-monomethoxyisoflavones have been reported from various legume species [5] and radiolabelling and other studies indicate that at least three of these (biochanin A, 2′-hydroxyformononetin, calycosin) occur in *T. pratense* [6–9]. The purpose of this study was to compare the chromatographic and mass spectral properties of the trimethylsilyl (TMSi) ethers of seven naturally occurring dihydroxy-monomethoxyisoflavones—calycosin (**1**), isocalycosin (**2**), 2′-hydroxyformononetin (**3**), theralin (**4**), biochanin A (**5**), texasin (**6**) and retusin (**7**)—to determine if they could be differentiated by GC/MS using selected ion monitoring.

Mass spectra were obtained for the TMSi ethers of each of the seven isoflavones and the major ions and their relative intensities are given in Table 1. Isoflavones **1**, **2**, **4**, **6** and **7** all have the molecular ion ($M^{+\cdot}$) as the base peak. Isoflavone **3** has a medium intensity $M^{+\cdot}$ ion and isoflavone **5** has only a weak $M^{+\cdot}$ ion. Isoflavone **5** has the $[M-15]^+$ ions as base peak and this ion appears to be formed by loss of a methyl radical from the OTMSi group at C-5 and ring formation to give the stable ion **8**. The proximity of an OTMSi group *ortho* to the carbonyl group of alkylphenones has been shown [10] to result in enhanced methyl loss from the OTMSi group and ring closure, with the daughter ion being the base peak or the second most abundant peak in the mass spectrum. The base peak in the mass spectrum of isoflavone **3** is also the $[M-15]^+$ ion which probably arises from the loss of a methyl radical from the OTMSi group at C-2′ followed by the formation of an analogous stable ion (**9**). Compound **4** is a 2′-methoxyl isoflavone and shows an intense $[M-31]^+$ ion (**10**) which is characteristic of isoflavones with this substitution [11].

Isoflavones **1** and **2** have *ortho* methoxy-OTMSi substitution and each has an abundant $[M-30]^{+\cdot}$ ion. The mass spectra of the TMSi ethers of the methyl esters of

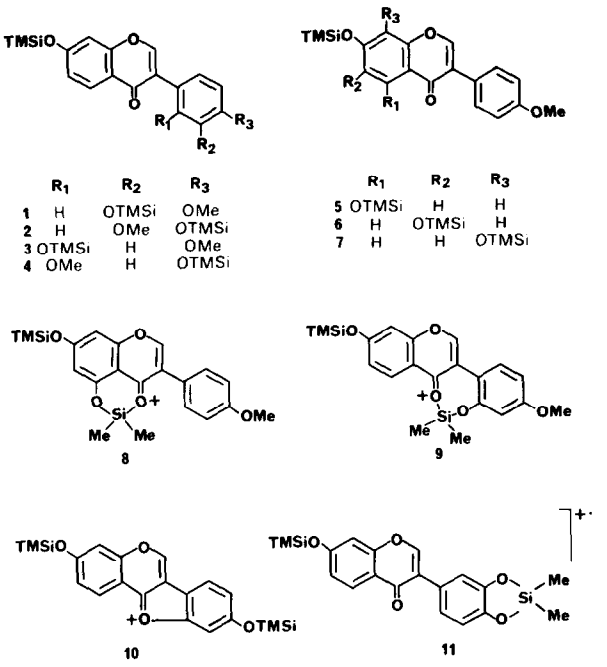
Table 1. Abbreviated mass spectra and retention indices of isomeric isoflavones

Isoflavone													
1 (1.70)*		**2** (1.80)		**3** (1.00)		**4** (1.52)		**5** (1.09)		**6** (1.76)		**7** (1.57)	
m/z	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%	*m/z*	%
428	100†	428	100	428	24	428	100	428	2	428	100	428	100
427	9	427	5	427	6	427	15	417	1	427	4	427	11
413	17	413	19	413	100	413	20	413	100	413	76	413	68
398	92	398	93	411	2	411	14	398	2	400	6	398	3
397	9	397	7	398	3	397	41	370	6	398	4	340	5
191.5	23	191.5	21	370	4	220	12	356	6	340	7	281	6
190	20	190	19	341	4	219	19	341	5	339	8	208	8
184	13	184	12	199	11	209	34	199	10	208	4	132	3
73	41	73	39	73	19	73	46	73	20	73	84	73	88

* Retention index values are relative to 6.75 min for isoflavone **3**.

† Relative abundances of ions at *m/z* 428, 427 and 413 for all compounds are averages of 10 repetitive scans over this mass range.

* Present address: Department of Chemistry, University of Colorado, Boulder, CO 80309, U.S.A.



ferulic and isoferulic acid (*ortho*-substituted methoxy-OTMSi isomers) have the $[M - 30]^{+\cdot}$ ion as base peak [12] and the loss of 30 mu was attributed to the sequential loss of methyl from the OTMSi group, ring closure and loss of methyl from the methoxyl group. By analogy, the $[M - 30]^{+\cdot}$ ion in the mass spectrum of isoflavones **1** and **2** can be represented by structure **11** obtained through the same fragmentation route.

Isoflavones **6** and **7** contain adjacent OTMSi groups and their mass spectra show prominent peaks for $[M - 15]^+$ and Me_3Si^+ ions, as does that of the di(TMSi) ether of pyrocatechol [13, 14]. The proposed fragmentation route to these ions [13, 14] is analogous to that indicated above for *ortho*-substituted methoxy-OTMSi compounds, except that the charge is retained by the smaller fragment in the second step. The abundance of the Me_3Si^+ ion in the mass spectra of compounds **6** and **7** is enhanced by comparison with the isomers that do not have adjacent OTMSi groups.

With the exception of isoflavone **4**, none of these isoflavones has significant ions due to retro Diels–Alder cleavage, which is an important fragmentation pathway for the underivatized isoflavones [15]. Isoflavans and isoflavanones show prominent ions due to retro Diels–Alder fragmentation both for the TMSi ethers [16] and for the underivatized compounds, indicating that a reduced C-2–C-3 bond may be important for this type of fragmentation in isoflavonoid TMSi ethers.

The retention indices of the seven isoflavones are shown in Table 1. Isoflavones **3** and **5** were found to elute from the column much earlier than the other five isomers. Both **3** and **5** have an OTMSi group near the carbonyl which reduces the polarity of the molecule resulting in the low index values. Isomer **4** has a methoxyl group in position C-2′ which slightly decreases the polarity of the molecule. Isomer **4** elutes ahead of isomer **2** in which the methoxyl group is in position C-3′. Isoflavone **7** has the next lowest index value, probably due to the proximity of the C-8 OTMSi group to the heterocyclic ring oxygen also resulting in a reduction in polarity.

The seven isomers studied were distinguishable both by their retention indices and by the relative abundance of the $M^{+\cdot}$, $[M - 1]^+$, $[M - 15]^+$ and other ions (Table 1). This will permit the use of selected ion monitoring for the detection and identification of these isoflavones in submicrogram quantities. The fragmentation pathways observed for these isomers and the retention index information obtained will provide a useful base for comparing other isomers and related compounds.

EXPERIMENTAL

TMSi derivatives were prepared with pyridine–BSTFA (1:1) at 60° for 5 min and chromatographed on a glass column (1.5 m × 2 mm) packed with 1.5% OV-101 on Gas-Chrom Q (80/100 mesh) at a He flow rate of 24 ml/min; injector temp. 230°; column 215°; jet separator and lines to spectrometer 240°; ion source 210°; ionization voltage 70 eV; accelerating voltage 4 kV; emission current 200 μA.

Acknowledgements—I am grateful to Dr. P. M. Dewick of the University of Nottingham for samples of calycosin, 2′-hydroxyformononetin, theralin, texasin and retusin, to Mr. I. Pavelic of the CSIRO Division of Entomology for obtaining the mass spectra and to Dr. M. J. Lacey of the Division of Entomology for assistance with spectral interpretations.

REFERENCES

1. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*, p. 165. Springer, New York.
2. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*, p. 253. Springer, New York.
3. King, F. E., King, T. J. and Manning, L. C. (1957) *J. Chem. Soc.* 563.

4. Woodward, M. D. (1979) *Physiol. Plant Pathol.* (in press).
5. Wong, E. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.) p. 743. Chapman & Hall, London.
6. Francis, C. M., Millington, A. J. and Bailey, E. T. (1967) *Aust. J. Agric. Res.* **18,** 47.
7. Dewick, P. M. (1977) *Phytochemistry* **16,** 93.
8. Dewick, P. M. and Ward, D. (1978) *Phytochemistry* **17,** 1751.
9. Biggs, D. R. and Lane, G. A. (1978) *Phytochemistry* **17,** 1683.
10. Smith, G. G. and Djerassi, C. (1971) *Org. Mass Spectrom.* **5,** 505.
11. Eguchi, S., Haze, M., Nakayama, M. and Hayashi, S. (1977) *Org. Mass Spectrom.* **12,** 51.
12. Horman, I. and Viani, R. (1971) *Org. Mass Spectrom.* **5,** 203.
13. Schwarz, H., Koppel, C. and Bohlmann, F. (1974) *Tetrahedron* **30,** 689.
14. Schwarz, H. (1978) *Top. Curr. Chem.* **73,** 231.
15. Mabry, T. J. and Markham, K. R. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.) p. 78. Chapman & Hall, London.
16. Woodward, M. D. (1979) *Physiol. Plant Pathol.* **17,** 17.