Phytochemistry, Vol. 21, No. 7, pp. 1733–1736, 1982.
Printed in Great Britain.

0031-9422/82/071733-04$03.00/0
Pergamon Press Ltd.

PISUM SATIVUM STRESS METABOLITES: TWO CINNAMYLPHENOLS AND A 2′-METHOXYCHALCONE

ROBERT E. CARLSON* and DAVID H. DOLPHIN

Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Y6

(*Received* 12 *May* 1981)

Key Word Index—*Pisum sativum*: Leguminosae; garden pea; stress metabolism; cinnamylphenols; 2′-methoxychalcone; HPLC.

Abstract—An HPLC study of copper(II) chloride treated *Pisum sativum* has shown that the cinnamylphenols obtustyrene [*E*-1-(4-hydroxy-2-methoxybenzyl)-2-phenylethylene] and xenognosin [*E*-1-(4-hydroxy-2-methoxybenzyl)-2-(4-hydroxyphenyl)ethylene] as well as the chalcone 4,4′-dihydroxy-2′-methoxychalcone accumulate as *de novo* metabolites in the stressed plant. This chalcone has not previously been identified from a plant source. The biosynthetic and stress response relationships of these compounds are discussed.

INTRODUCTION

A previous study [1] of the stress response of *Pisum sativum* L. has shown that most of the observed phenolic metabolites are related to the biosynthesis of the pterocarpinoid phytoalexins. However, three additional compounds are formed in response to copper(II) chloride stress which do not appear to fit the scheme of isoflavonoid biosynthesis. These compounds have been identified as the cinnamylphenols obtustyrene [*E* - 1 - (4 - hydroxy - 2 - methoxybenzyl) - 2 - phenylethylene], xenognosin [*E* - 1 - (4 - hydroxy - 2 - methoxybenzyl) - 2 - (4 - hydroxyphenyl)ethylene] and 4,4′ - dihydroxy - 2′ - methoxychalcone. The biosynthetic and stress response relationships of these compounds are discussed.

RESULTS

A representative chromatogram of the phenolics which accumulate in stressed pea is given in Fig. 1. The peaks corresponding to the non-isoflavonoid phenolics, which are the subject of this study (**1c**, **2a**, **2c**) as well as the phytoalexin pisatin, have been identified on the chromatogram.

The characteristic UV spectrum [2] of metabolite **1c** in combination with its molecular formula of $C_{16}H_{14}O_4$, which was derived from high resolution mass spectral measurements, suggested that the compound was a dihydroxymethoxychalcone. The mass spectrum had a hydroxy substituted cinnamoyl derived fragment and a hydroxy, methoxy substituted benzoyl derived fragment. The ^1H NMR aromatic couplings indicated that the benzoyl part of the molecule was 2,4-substituted while the cinnamoyl part was 4-substituted. Thus, the isolate was either 4,4′ - dihydroxy - 2′ - methoxychalcone (**1c**) or 2′,4 - dihydroxy - 4′ - methoxychalcone (**1d**). The identification of the metabolite as the 2′-methoxy isomer (**1c**) was based on the expected [2] effect of aluminium chloride on the UV spectrum of a 2′-hydroxychalcone and a comparison of its chromatographic properties with synthetic samples of both the 2′-hydroxy and the 2′-methoxychalcones. The synthesis of the 2′-hydroxy isomer followed a known aldol condensation route [3]. Unfortunately, it was

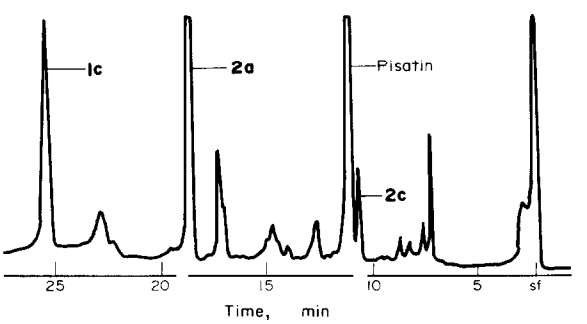


Fig. 1. A representative chromatogram of the *Pisum sativum* stress metabolites.

*Author to whom correspondence should be addressed. Present address: Gray Freshwater Biological Institute, P.O. Box 100, Navarre, MN 55392, U.S.A.

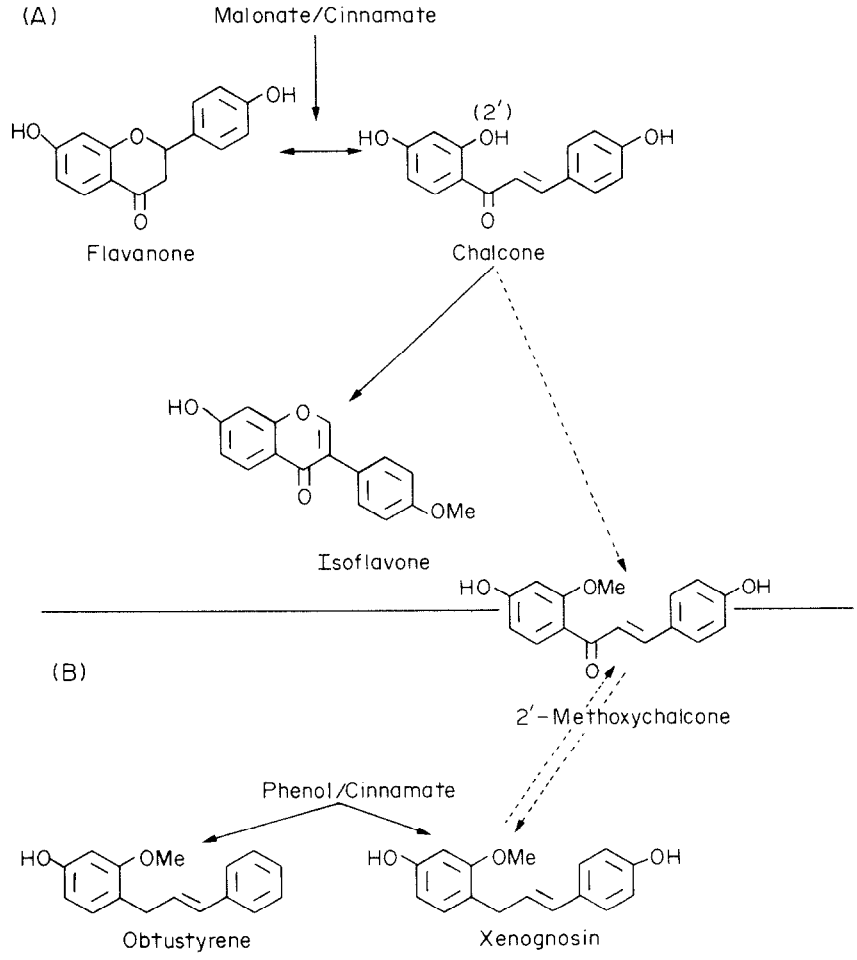


Fig. 2. Flavonid–isoflavonoid and cinnamylphenol biosynthesis. Potential interconnection (----).

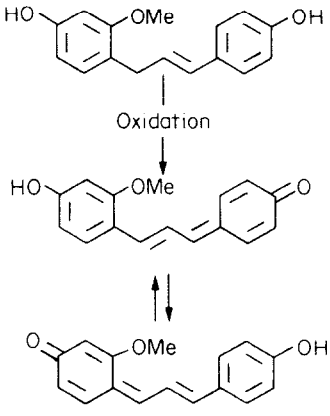
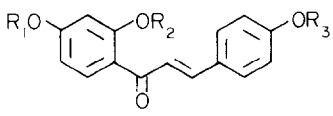


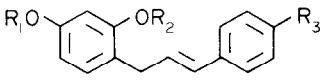
Fig. 3. Probable route to the formation of a quinone–methide from xenognosin.

found that the condensation of 4' - hydroxy - 2' - methoxyacetophenone with 4 - hydroxybenzaldehyde gave a poor yield of impure 4,4' - dihydroxy - 2' - methoxychalcone. However, this compound was obtained in good yield and purity by the sequential methylation and debenzylation of 4,4'-bis(benzyloxy)-2'-hydroxychalcone.

Metabolite **2a** had a high resolution molecular formula, determined by mass spectroscopy, of $C_{16}H_{16}O_3$. An ABX_2 coupling pattern in the ^1H NMR spectrum and an increase in mass of 28 after reaction with methyl iodide–potassium carbonate indicated that the compound did not contain a carbonyl oxygen and was therefore a dihydroxymethoxycinnamylphenol. Analysis of the mass spectrum, which had the tropylium fragments C_7H_7O and $C_8H_9O_2$, in combination with the similarity of the ^1H NMR aromatic couplings of this isolate and the previously identified chalcone indicated that the phenyl substituent patterns were 4-hydroxy and either 4-hydroxy-2-methoxy or 2-hydroxy-4-methoxy. The position of the double bond was determined by HPLC analysis of the products obtained from the ruthenium tetraoxide oxidation [4] of the isolate. This degradative procedure, which gave 4-hydroxybenzaldehyde as the only observed product, proved that the isolate was



	R_1	R_2	R_3
1a	CH_2Ph	H	CH_2Ph
1b	CH_2Ph	Me	CH_2Ph
1c	H	Me	H
1d	Me	H	H



	R_1	R_2	R_3
2a	H	Me	OH
2b	Me	H	OH
2c	H	Me	H
2d	H	H	OH
2e	Me	Me	Me

Fig. 4.

either *E* - 1 - (4 - hydroxy - 2 - methoxybenzyl) - 2 - (4 - hydroxyphenyl)ethylene (**2a**) or its 2 - hydroxy - 4 - methoxybenzyl isomer (**2b**). Chromatographic comparison of the isolate with synthetic samples of these compounds proved that it was the 4 - hydroxy - 2 - methoxybenzyl isomer (**2a**). This isolate is a rare natural product recently characterized for the first time from a exudate of *Astragalus* species and named xerognosin [5].

Metabolite **2c** was identified as obtustyrene [*E* - 1 - (4 - hydroxy - 2 - methoxybenzyl) - 2 - phenylethylene], which has been previously identified from *Dalbergia retusa* by Gregson *et al.* [6] by methods analogous to those used for **2a**.

DISCUSSION

The general scheme of flavonoid/isoflavonoid biosynthesis is given in Fig. 2(A). As illustrated, the formation of the isoflavone pyrane ring requires a free 2'-hydroxyl on the chalcone [7]. Thus the 2'-methoxychalcone does not appear to be an intermediate in flavonoid/isoflavonoid synthesis.

The cinnamylphenols have previously only been isolated from heartwoods. Consequently, the currently favoured route for cinnamylphenol biosynthesis, which is based on cinnamylphenol/3,3-diarylpropene co-occurrence in some plants and the *in vitro* observation of diarylpropene and cinnamylphenol formation in the condensation of cinnamyl alcohols with phenols [8, 9], requires that a cinnamyl pyrophosphate condense with a phenol (or its equivalent) to give a 3,3-diarylpropene and/or a cinnamylphenol [8] (Fig. 2B).

There is no evidence to indicate that the cinnamylphenols and the chalcones are biosynthetically related. However, the structural similarity of the 2'-methoxychalcone and the cinnamylphenol xenognosin suggests that they may be interconnected. The identification of both of these compounds as *de novo* metabolites of *P. sativum* provides a convenient system for further studies on their biosynthesis.

The role of these compounds in the plant's stress response is not immediately apparent because if they are antifungal it is likely that they would have been observed by Cruickshank and Perrin [10] in their initial TLC bioassays of stressed pea metabolites. However, we are currently investigating the possibility that xenognosin (**2a**) may function in the plant's disease response through its anticipated ability to form reactive quinone–methides [10] (Fig. 3). These compounds could participate in the resistance process by reaction with proteins and enzymes or by polymerization to tannins and lignans which can act as protein precipitants or as physical barriers to parasite expansion [11].

EXPERIMENTAL

The HPLC system and UV analysis procedures used in this study have been described previously [12]. The procedures for growth and stress of the pea plants (*Pisum sativum* cv Melting Sugar) have also been described previously [1]. MS (direct insertion probe; ionization voltage 70 eV). Chromatographic solvents were: petrol (PE), Et_2O (E), CH_2Cl_2 (MDC) and Me_2CO (A). The metabolites were isolated by HPLC as described previously [12]. Obtustyrene was identified by direct comparison of its HPLC, TLC, UV and MS characteristics with a synthetic sample [6].

4,4' - *Dihydroxy* - 2' - *methoxychalcone* (**1c**). *Identification.* The HPLC isolate was further purified by TLC on Si gel [PE–E (1:2); $R_f = 0.38$]. Analysis of the resultant sample gave UV λ_{max}^{MeOH} nm: 253, 349; NaOMe 257, 419; NaOAc 255sh, 358sh, 393; $NaOAc/H_3BO_3$ 351; $AlCl_3$ 235, 350; $AlCl_3$/HCl 235, 350; ^1H NMR [100 MHz, $(CD_3)_2CO$]: δ 7.50–7.64 (4H, *m*, C-β, C-2, C-6, C-6'); 7.28 (1H, *d*, $J = 18$ Hz, C-α); 6.91 (2H, *d*, $J = 8$ Hz, C-3, C-5); 6.59 (1H, *d*, $J = 2$ Hz, C-3'); 6.52 (1H, *dd*, $J = 2,8$ Hz C-5'); 3.92 (3H, *s*, -OMe); MS: Found: $[M]^+$ (HRMS), 270.0885. $C_{16}H_{14}O_4$ requires: $[M]^+$, 280.0892. *m/z* (rel. int.), 270 $[M]^+$ (100), 269 (23), 255 (38), 242 (31), 164 (38), 163 (18), 151 (80), 147 (33), 119 (25), 107 (89). *Synthesis.* 4,4'-Bis(benzyloxy) - 2' - hydroxychalcone [13] (**1a**) was methylated with MeI–K_2CO_3 to give 4,4' - bis(benzyloxy) - 2' - methoxychalcone (**1b**). The product was crystallized from Me_2CO–EtOH (5 mM scale; 82% yield). mp 94–95°, Found: C, 79.43; H, 5.85; $[M]^+$ (HRMS), 450.1808. $C_{29}H_{26}O_4$ requires: C, 79.42; H, 5.98; $[M]^+$, 450.1831; UV $\lambda_{max}^{CH_2Cl_2}$ nm (log ϵ): 236 (3.26), 340 (3.48); IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1} 3001, 1601, 1250, 1163, 1129, 1020, 830; ^1H NMR (100 MHz, $CDCl_3$): δ 7.18–7.76 (15H, *m*, C-α, C-β, C-2, C-6, C-6'–$CH_2\phi$); 6.50–6.64 (2H, *m*, C-3', C-5'); 5.01 and 5.03 (4H, $2 \times s$, $-C\underline{H}_2\phi$); 3.78 (3H, *s*, OMe); MS *m/z* (rel. int.) 450 $[M]^+$, (14), 359 (12), 197 (12), 92 (15), 91 (100).

The benzyloxychalcone (230 mg, 0.510 mmol) was debenzylated in conc. HCl–glacial HOAc (2:3, 25 ml) by stirring the reaction mixture for 2 hr in a H_2O bath at 48°. The product was extracted into Et_2O after the addition of H_2O (100 ml) to the reaction mixture. The Et_2O layer was successively extracted with H_2O, 5% aq $NaHCO_3$ and H_2O. After drying over dry Na_2SO_4 and filtration the solvent was

removed on a rotary evaporator. The residue was chromatographed on Si gel (15 g, 20 × 1 cm column) with MDC–A (4:1) and the resultant solid crystallized from aq. EtOH to give 70 mg (51%) of 4,4' - dihydroxy - 2' - methoxychalcone (**1c**) as orange microcrystals. This product was found to be identical with the isolate by HPLC, TLC, UV, ^{1}H NMR and MS. In addition it gave mp 210–212°, Found: C, 70.90; H, 5.11. $C_{16}H_{14}O_4$ requires C, 71.10; H, 5.22; UV λ_{max}^{MeOH} nm (log ϵ): 234 (4.00), 349 (4.25); IR ν_{max}^{nujol} cm^{-1}: 1560 *br*, 1330, 1315, 1250 *br*, 1218 *br*, 1170, 1120, 825.

E - 1 - (4 - *Hydroxy* - 2 - *methoxybenzyl*) - 2 - (4 - *hydroxyphenyl)ethylene* (**2a**) (*xenognosin*). *Identification.* The HPLC isolate was further purified by Si gel TLC (PE–E (1:1); R_f = 0.58). Analysis of the resultant sample gave UV λ_{max}^{MeOH} nm: 261.5; NaOMe 281; NaOAc, NaOAc–H_3BO_3, $AlCl_3$ and $AlCl_3$–HCl 261.5; ^{1}H NMR [100 MHz, $(CD_3)_2CO$]: δ 7.22 (2H, *d*, *J* = 9 Hz, C-2, C-6); 6.97 (1H, *d*, *J* = 8 Hz, C-6'); 6.76 (2H, *d*, *J* = 9 Hz, C-3, C-5); 6.38–6.50 (2H, *m*, C-3', C-5'); 6.16–6.32 (2H, *m*, C-α, C-β); 3.80 (3H, *s*, OMe); 3.36 (2H, *d*, *J* = 6 Hz, C–X of ABX_2); MS. Found: $[M]^+$ (HRMS), 256.1105. $C_{16}H_{16}O_3$ requires: $[M]^+$, 256.1100; UV λ_{max}^{MeOH} nm: 256 *m/z* (rel. int.) $[M]^+$ (100), 255 (19), 241 (17), 239 (17), 225 (21), 161 (11), 150 (15), 149 (23), 137 (63), 133 (26), 132 (23), 131 (41), 122 (36), 121 (61), 107 (56). *Synthesis.* *p*-Hydroxycinnamyl alcohol (0.750 g, 5 mmol) was dissolved with *m*-methoxyphenol (1.24 g, 10 mmol) in 20 ml 75% HOAc–H_2O and stirred for 15 min at 60°. The products were extracted into Et_2O after the addition of 100 ml H_2O. The Et_2O layer was extracted successively with H_2O, 5% aq. $NaHCO_3$ and H_2O followed by drying, filtration and removal of the solvent on a rotary evaporator. Chromatography on Si gel (100 g, 45 × 4 cm) using PE–E (1:1) gave 0.44 g of the mixed isomers **2a** and **2b**. Separation of these isomers by CC as above using PE–E (2:1) and MCD–EtOH (19:1) gave 190 mg of the faster moving and 205 mg of the slower moving isomers. Both isomers resisted all attempts at crystallization (though both were pure as measured by TLC) and both proved to be too unstable for satisfactory microanalysis. However, both isomers, when reacted with MeI in the usual manner, gave the expected trimethoxy product (**2e**). The second isomer to be eluted from the column was found by HPLC and TLC to correspond to the plant isolate. Its identification as **2a** was confirmed by TLC and HPLC comparison with the product obtained from the reduction of chalcone **1c** with AlH_3 [14]. The synthetic product was also found to be identical with the isolate by UV, ^{1}H NMR and MS. Found: $[M]^+$ (HRMS), 256.1105. $C_{16}H_{16}O_3$ requires: $[M]^+$, 256.1100; UV λ_{max}^{MeOH} nm: (log ϵ) 261 (4.0); ^{1}H NMR [270 MHz, $(CD_3)_2CO$]: δ 7.22 (2H, *d*, *J* = 9 Hz, C-2, C-6); 6.96 (2H, *d*, *J* = 8 Hz, C-6'); 6.76 (2H, *d*, *J* = 9 Hz, C-3, C-5); 6.45 (1H, *d*, *J* = 2 Hz, C-3'); 6.38 (1H, *dd*, *J* = 2,8 Hz, C-5'); 6.33 (1H, *d*, *J* = 16 Hz, C-β); 6.16 (1H, *td*, *J* = 6,16 Hz, C-α); 3.82 (3H, *s*, OMe); 3.36 (2H, *d*, *J* = 6 Hz, C–X of ABX_2).

Synthesis (**2e**). *p*-Hydroxycinnamyl alcohol (0.750 g, 5 mmol) was dissolved with resorcinol (1.10 g, 10 mmol) in 50 ml 75% HOAc–H_2O and stirred for 15 min at 70°. The product was isolated as above. CC purification of the residue on Si gel [100 g, 45 × 4 cm column, PE–E (1:3)] gave 0.449 g (37%) of **2d** as a glass. Found: $[M]^+$ (HRMS), 242.0925. $C_{15}H_{14}O_3$ requires: $[M]^+$, 242.0943; UV λ_{max}^{MeOH} (log ϵ): 262 (4.20); NaOMe 240 sh, 283; ^{1}H NMR [270 MHz $(CD_3)_2CO$]: δ 7.17 (2H, *d*, *J* = 9 Hz, C-2, C-6); 6.87 (2H, *d*, *J* = 8 Hz, C-6'); 6.72 (2H, *d*, *J* = 9 Hz, C-3, C-5); 6.37 (1H, *d*, *J* = 2 Hz, C-3'); 6.23–6.33 (2H, *m*, C-β, C-5'); 6.16 (1H, *td*, *J* = 6,16 Hz, C-α); 3.37 (2H, *d*, *J* = 6 Hz, C–X of ABX_2); MS 242 $[M]^+$, (100), 241(10), 225(17), 149(10), 147(40), 135(10), 133(32), 132(13), 131(28), 123(42), 119(29), 107(69). The trihydroxy product (108 mg, 0.44 mmol) was methylated with MeI–K_2CO_3. The product was purified by TLC on Si gel [2000 μm layer, 40 × 40 cm plate, PE–E (4:1), R_f = 0.75] and crystallized from aq. MeOH to give 72 mg (57%) of **2e** as white plates, mp 66–67°. Found: C, 75.60; H, 6.82; $[M]^+$, 284.1432. $C_{18}H_{20}O_3$ requires: C, 76.03; H, 7.09; $[M]^+$, 284.1412. UV $\lambda_{max}^{CH_2Cl_2}$ nm (log ϵ): 262 (4.35); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1611, 1506, 1465 *br*, 1296, 1252 *br*, 1184, 1165, 1127, 1045, 975, 845; ^{1}H NMR [270 MHz, $(CD_3)_2CO$]: δ 7.26 (2H, *d*, *J* = 9 Hz, C-2, C-6); 7.06 (2H, *d*, *J* = 8 Hz, C-6'); 6.80 (2H, *d*, *J* = 9 Hz, C-3, C-5); 6.44 (1H, *d*, *J* = 2 Hz, C-3'); 6.41 (1H, *dd*, *J* = 2, 8 Hz, C-5'); 6.33 (1H, *d*, *J* = 16 Hz, C-β); 6.19 (1H, *td*, *J* = 6 Hz, C-α); 3.80; 3.76; 3.75 (9H, 3 × s, OMe); 3.43 (2H, *d*, *J* = 6 Hz; C–X of ABX_2); MS 283 $[M]^+$ (100), 282(13), 269(11), 253(58), 151(18), 146(20), 145(42), 121(39).

Acknowledgements—This work was supported by the U.S. National Institutes of Health (AM 17989) and the Canadian National Science and Engineering Research Council. D. H. D. wishes to express his thanks to the Harvard Chemistry Department for their assistance in the preparation of this paper during his stay as a Visiting Professor, Autumn 1980.

REFERENCES

1. Carlson, R. E. and Dolphin, D. H. (1981) *Phytochemistry* **20**, 2281.
2. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids* p. 227. Springer, New York.
3. Pelter, A., Bradshaw, J. and Warren, R. (1971) *Phytochemistry* **10**, 835.
4. Nakata, H. (1963) *Tetrahedron* **19**, 1959.
5. Lynn, D. G., Steffens, J. C., Kamut, V. S., Graden, D. W., Shabanointz, J. and Riopel, J. L. (1981) *J. Am. Chem. Soc.* **103**, 1863.
6. Gregson, M., Ollis, W. D., Redman, B. T., Sutherland, I. O., Dietrichs, H. H. and Gottlieb, O. R. (1978) *Phytochemistry* **17**, 1395.
7. Jurd, L., Stevens, K. and Manners, G. (1973) *Tetrahedron* **29**, 2347.
8. Jurd, L. (1969) *Tetrahedron* **25**, 1407.
9. Jurd, L. (1969) *Tetrahedron Letters* 2863.
10. Cruickshank, I. A. M. and Perrin, D. R. (1961) *Aust. J. Biol. Sci.* **14**, 336.
11. Leatham, G. F., King, B. and Stahman, M. A. (1980) *Phytopathology* **70**, 1134.
12. Carlson, R. E. and Dolphin, D. (1980) *J. Chromatogr.* **198**, 193.
13. Kamkhalia, N. H., Mulahandani, N. B. and Kulkarni, A. B. (1965) *Indian J. Chem.* **3**, 30.
14. Gregson, M., Kurosawa, K., Ollis, W. D., Redman, B. T., Roberts, R. J., Sutherland, I. O., Braga de Oliveira, A., Eyton, W. B., Gottlieb, O. R. and Dietrichs, H. H. (1968) *J. Chem. Soc. Chem. Commun.* 1390.