

THE EFFECTS OF pH ON GLUCOSINOLATE DEGRADATION BY A THIOLUCOSIDE GLUCOHYDROLASE PREPARATION

V. GIL* and A. J. MACLEOD

Department of Chemistry, Queen Elizabeth College (University of London), Campden Hill Road, London, W8 7AH, U.K.

(Revised received 17 March 1980)

Key Word Index—Cruciferae; glucosinolates; thioglucoside glucohydrolase; isothiocyanates; nitriles.

Abstract—An active thioglucoside glucohydrolase extract was prepared from commercial mustard powder and its effect on the degradation of two pure glucosinolates was investigated. During reaction in a distilled water medium the pH of the solution decreased markedly and the ratio of products (isothiocyanate and nitrile) varied considerably. After 20–30 min, when the pH had fallen to ca 5.6, isothiocyanate production ceased whilst nitrile continued to be produced and in amounts which increased linearly with time for at least 40 min. This behaviour can be correlated with the changing pH of the medium. In controlled pH experiments it was confirmed that nitrile formation is favoured at lower pH levels and that the ratio of nitrile to isothiocyanate is directly related to the hydrogen ion concentration of the medium. No reason could therefore be found for the observed formation of nitrile in some natural systems at pHs greater than 7.

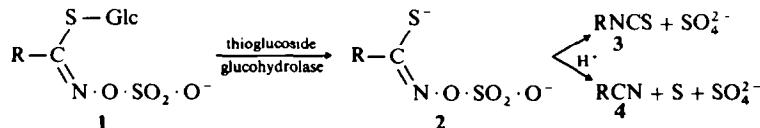
INTRODUCTION

Glucosinolates (1) are thioglucosides mainly located in the Cruciferae. It is generally accepted that they degrade enzymically as broadly outlined in Scheme 1 to give an aglucone (2), which can then either undergo a spontaneous Lossen rearrangement to give an isothiocyanate (3) or can form a nitrile (4), but only at low pH via a protonation mechanism. However, evidence has recently been presented that benzylglucosinolate of *Lepidium sativum* seeds naturally forms relatively large amounts of the nitrile predominantly at pH levels greater than 7 [1]. Nitrile formation at pH values at which isothiocyanate should be the sole product has been observed for other glucosinolates and other plant systems [2–8], and it has been pointed out that under genuine conditions of autolysis, whilst some Cruciferae are intrinsically isothiocyanate-producing others give nitrile as the major product even at a pH at which isothiocyanate is normally dominant [9]. It has been shown that in some natural systems the overall influence of pH on the mechanisms of enzymic degradation is certainly not as simple as suggested by current theories [1].

Because of these anomalies it was decided to re-evaluate the influence of pH on the enzymic degradation of glucosinolates, particularly with respect to the products

formed. For such work to be meaningful, as many extraneous influences as possible should be excluded, so the simplest, and reputedly one of the most active [10], thioglucosidase enzyme preparations was adopted, namely that in commercial mustard powder. The most relevant substrate for this particular enzyme preparation is allylglucosinolate and since this can be readily obtained commercially in a relatively pure form it was an obvious choice in this project. Clearly it is desirable to confirm, or otherwise, any results with a second glucosinolate and 2-phenethylglucosinolate was synthesized partly for this purpose [11].

It has often been assumed that ascorbic acid is a 'necessary' co-factor for thioglucoside glucohydrolase, and much work has been carried out in this belief [10, 12–19]. However, it has been shown with an *L. sativum* enzyme preparation that whilst ascorbic acid considerably promotes isothiocyanate formation, it is not essential [12]. Nitrile formation was erratic and unpredictable in its presence, but in its absence was produced linearly with time. It may well be, therefore, that ascorbic acid has different effects on the enzymic mechanisms of formation of isothiocyanate and nitrile, and in particular it might 'interfere' with the natural production of the latter. Since nitrile formation is a major consideration of this investigation and since ascorbic acid is not essential for



Scheme 1. Enzymic degradation of glucosinolates.

* Present address: Chotiravi College, Nakorn Sawan, Thailand.

enzymic degradation of glucosinolates to any of the major products [12], the co-factor was considered an unnecessary extraneous influence and was not employed in this work. Thus experiments were performed with the mustard enzyme preparation, the pure glucosinolate and, when necessary, a buffer solution to control the pH. This eliminated as far as possible the effects on the degradation of any non-relevant factors.

RESULTS AND DISCUSSION

In this work, we have been concerned mainly with those products formed from glucosinolates by normal catabolism (i.e. 3 and 4, Scheme 1); those produced by 'abnormal' catabolism (e.g. cyanoepithioalkanes) were not relevant and indeed were not detected. Experiments were first conducted in distilled water partly to provide a baseline and to ensure no unexpected activity of components of buffer solutions. However, results obtained from this system proved relevant to the influence of pH on glucosinolate degradation. Initially enzyme activity was assessed by the extent of substrate decomposition as measured by UV spectroscopy. The absorption maximum of allylglucosinolate is at 227 nm ($\epsilon = 6458$); this decreased in intensity during enzymic decomposition and an isosbestic point was formed at 259 nm. 2-Phenethylglucosinolate has λ_{max} at 213 nm with a distinct shoulder at 227 nm ($\epsilon = 5463$). During reaction with enzyme the absorption maximum decreased slowly and underwent a hypsochromic shift to 207 nm at which point it did not decrease any further. The shoulder also decreased during reaction, but more regularly, and an isosbestic point was formed at 253 nm. Decrease in absorbance at 227 nm was linear with time for the enzymic degradation of both glucosinolates up to *ca* 70 min. Since this is a non-logarithmic relationship, the rate of reaction is independent of the concentration of the undecomposed glucosinolate; it is therefore zero order in respect of the glucosinolate. These results confirmed that the enzyme preparation was active (in the absence of ascorbic acid) on both glucosinolates, but the detailed figures [20] show that the rate and extent of decomposition of both was similar (i.e. graphs of absorbance against time were coincidental).

Table 1. Variation in pH during the action of mustard thioglucosidase on allyl- and 2-phenethyl-glucosinolates in distilled water

Time (min)	Allylglucosinolate pH	2-Phenethylglucosinolate pH
5	6.62	6.83
10	6.36	6.50
15	5.99	6.18
20	5.69	6.07
25	5.00	5.90
30	4.12	5.58
35	3.78	5.12
40	3.58	4.61
45	3.46	4.27
50	3.40	4.07
55	3.33	3.89
60	3.27	3.76
65	3.22	3.65
70	3.20	3.58

for equimolar solutions). Therefore, in this instance, the nature of the substituent of the glucosinolate did not affect the activity of the thioglucoside glucohydrolase enzyme.

More interesting is the fact that during these reactions the pH of the solutions decreased markedly. Table 1 summarizes these data and Fig. 1 shows a graph of these figures for allylglucosinolate. The curve for 2-phenethylglucosinolate is similar, and so in neither case was this decrease in pH linear with time. As the pH dropped the ratio of nitrile and isothiocyanate products altered, as determined by direct GLC analysis. Results are

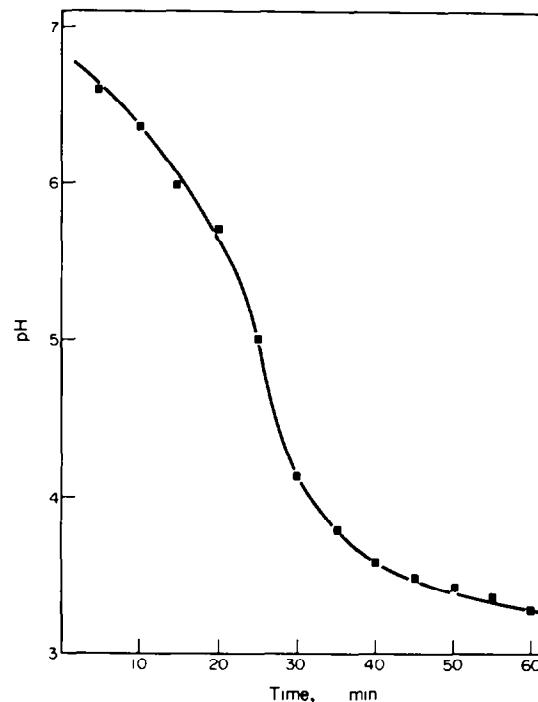


Fig. 1. Change in pH on enzymic decomposition of allylglucosinolate.

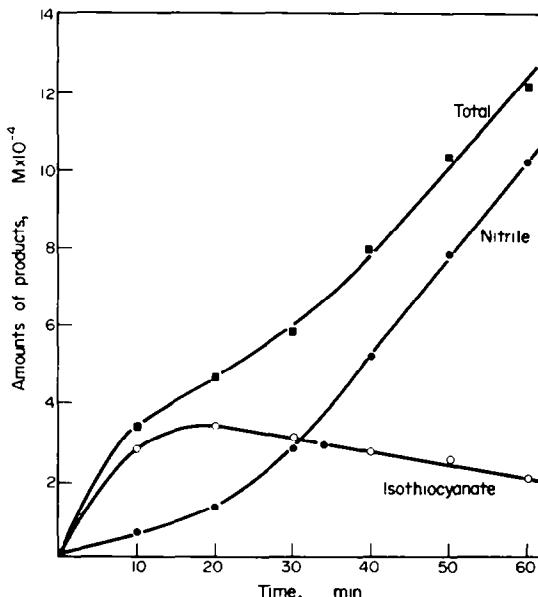


Fig. 2. Relative amounts of products formed on enzymic decomposition of allylglucosinolate in distilled water.

Table 2. Relative amounts of glucosinolate degradation products formed with time by the action of mustard thioglucosidase in distilled water medium

Time (min)	Amounts of products formed, M $\times 10^{-4}$					
	Allyl			2-Phenethyl		
	isothiocyanate	nitrile	total	isothiocyanate	nitrile	total
10	2.8	0.6	3.4	2.3	0.5	2.8
20	3.4	1.3	4.7	5.2	0.9	6.1
30	3.1	2.8	5.9	9.2	1.8	11.0
40	2.7	5.2	7.9	9.1	4.0	13.1
50	2.5	7.8	10.3	9.1	5.2	14.2
60	2.0	10.2	12.2	9.0	7.3	16.3
70				9.0	9.0	18.0

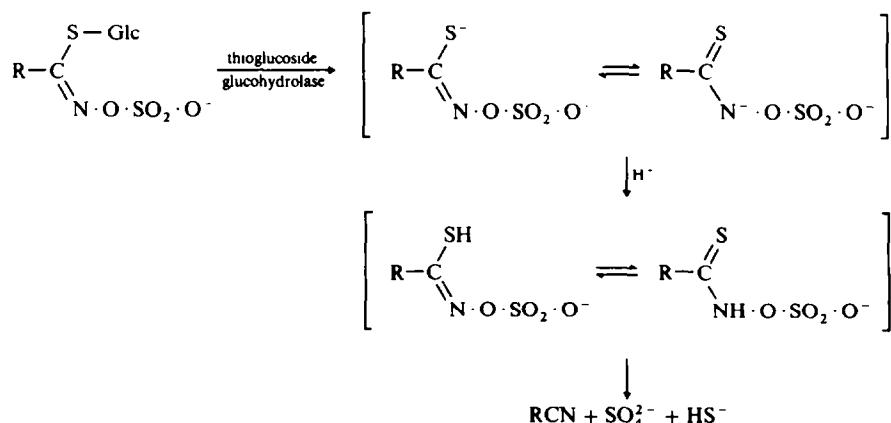
summarized in Table 2 and again data for allylglucosinolate are plotted (Fig. 2); with one exception the curves for 2-phenethylglucosinolate are again similar. For both glucosinolates isothiocyanate formation was very much favoured to begin with and relatively little nitrile was obtained. After some time isothiocyanate production slowed whilst that of nitrile accelerated eventually to overtake the concentration of isothiocyanate (after ca 30 min for allylglucosinolate but not until ca 70 min for 2-phenethylglucosinolate). The production of 2-phenethyl isothiocyanate ceased entirely after ca 30 min and a plateau level was maintained, whilst nitrile production was linear with time from ca 20 min. Very much the same behaviour was observed for allylglucosinolate except that an isothiocyanate maximum was formed and after ca 20 min concentration of isothiocyanate decreased slowly and regularly. Clearly it was reacting to give another product, but none was detected by GLC in this work.

A clue to this interesting behaviour of both glucosinolates is provided by comparing the initial part of the curve in Fig. 1 with that in Fig. 2 for total products. They are very similar in shape, suggesting that the variation in pH of the reaction medium was affecting the relative amounts of products formed (or vice versa). A reasonable explanation for these results would be as follows. In distilled water without any pH control (near neutral), a glucosinolate initially undergoes enzymic degradation to give predominantly isothiocyanate. At the same time

sulphate ions are released (Scheme 1) and in the aqueous, hydrolytic medium this is presumably the cause of the decrease in pH. As the pH falls (as more glucosinolate decomposes) the medium becomes more favourable for nitrile formation, and slowly this takes over from isothiocyanate production. Eventually the pH drops to a level where isothiocyanate cannot be formed. It is interesting that at about the time that allyl isothiocyanate production ceased (20 min) the pH had dropped to 5.7, whilst formation of 2-phenethyl isothiocyanate did not stop until about 30 min, at which time the pH was 5.6. This is a crude comparison and the good agreement may be fortuitous.

Scheme 2 shows in a little more detail the proposed effect of relatively high acidities on the mechanism of degradation of glucosinolates, where under such conditions the enzymically produced aglucone cannot readily undergo the Lossen rearrangement due to blockage by a proton.

From these results it follows that the enzymic degradation of glucosinolates in unbuffered aqueous media gives a relative product composition that varies appreciably with time. This has perhaps not been fully appreciated before and clearly must be taken into account in future experiments. It may well have some relevance to natural systems and could perhaps partly explain some of the previously mentioned anomalies. However, most plants possess certain degrees of natural buffering activity so the



Scheme 2. Formation of nitrile from glucosinolate at low pH.

subsequent experiments reported here determining enzyme activity and relative product distribution at a range of different pH levels also has some relevance.

Citrate-phosphate buffer solutions were employed and at pH 7 it was shown by UV spectroscopy that the rate of enzymic degradation of both glucosinolates was about the same, as had been observed in distilled water. Over the pH range 4–8.5 the rate was very similar with a slight peak of maximum activity at 5.95 (for both glucosinolates). Decreasing the pH to 3 significantly decreased the rate of reaction but the enzyme was still active even at these high acidities. The relative amounts of reaction products were fairly constant over the pH range 4–8.5 except towards the lower end. The trends observed there increased further at lower pH levels, at which the thioglucosidase became less active. Results for both glucosinolates are given in Table 3 and because the total amount of products formed decreased markedly at the very low pHs, relative percentages are also given to enable valid comparison of relative amounts of isothiocyanate and nitrile at any pH. Data for allylglucosinolate are shown in Fig. 3; the curves for 2-phenethylglucosinolate are very much the same. It can be seen that with the former an equal mixture of products was given at pH 3.65; for the latter this point was at 3.45, a very small difference. The influence of pH on the relative amounts of products from enzymic degradation of both glucosinolates was virtually identical.

The results agree very well with a previous survey conducted by Miller on potassium silver sinigrate (effectively the aglucone of allylglucosinolate with the carbohydrate moiety replaced by silver) [21]. He obtained 97% allyl isothiocyanate at pH 5 and 97% nitrile at pH 2, with equal amounts of both at pH 3.53. Miller [21], and others, have shown that the ratio of nitrile to isothiocyanate produced on enzymic decomposition of allylglucosinolate is proportional to the hydrogen ion concentration of the reaction medium. The results obtained for both glucosinolates agree, in that a straight line is obtained on plotting pH against the logarithm of the ratio of products. This suggests that the product-forming step does not depend on an enzyme, but originates from an intermediate whose fate is determined by the pH of the medium. This intermediate must be the aglucone, as shown in Scheme 2, so the quantitative relation between product

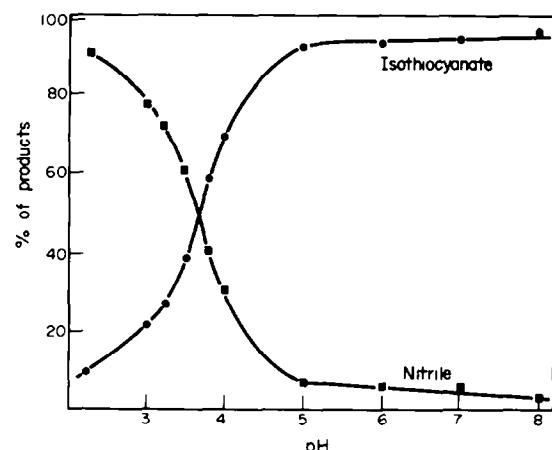


Fig. 3. Relative amounts of products formed on enzymic decomposition of allylglucosinolate at different pH.

ratio and pH corresponds simply to the existence of two competing first order reactions of the aglucone, one uncatalyzed and the other unimolecular in hydrogen ion concentration.

In some respects these results in buffer agree well with those obtained in distilled water, thus confirming the validity of both sets of experiments. For example, in distilled water, 2-phenethylglucosinolate degraded enzymically to give a mixture of exactly equal amounts of both products at 70 min (Table 2), at which time the pH of the medium had fallen to 3.58 (Table 1). In the second set of experiments equal amounts of products were obtained in buffer solution of pH 3.45 (Table 3). This agreement was not quite so good for allylglucosinolate, being pH 4.05 at 31 min compared with pH 3.65, but here the decrease in allyl isothiocyanate production with time in distilled water was a complication. Other points in the two sets of data agree quite well for both glucosinolates, but there is one major inexplicable difference. Thus, in distilled water isothiocyanate production ceased at 20 or 30 min when the pH of the medium had fallen to *ca* 5.6, but in the controlled pH experiments isothiocyanate was still very much the major product (>90%) even at pH 5. Presumably some

Table 3. Relative amounts of degradation products formed on enzymic degradation of allyl- and 2-phenethyl-glucosinolates at different pH values

pH	Amounts of products formed, $M \times 10^{-4}$ (%), in 50 min			
	Allyl		2-Phenethyl	
	isothiocyanate	nitrile	isothiocyanate	nitrile
2.25	0.1 (9)	1.0 (91)	0.2 (13)	1.3 (87)
3.00	1.1 (22)	3.9 (78)	2.2 (35)	4.1 (65)
3.25	1.5 (27)	4.2 (73)	3.8 (42)	5.3 (58)
3.50	2.6 (39)	4.1 (61)	5.9 (57)	4.4 (43)
3.80	4.8 (59)	3.3 (41)	9.6 (69)	4.2 (31)
4.00	7.0 (70)	3.0 (30)	11.5 (80)	3.0 (20)
5.00	9.0 (93)	0.7 (7)	14.0 (96)	0.6 (4)
6.00	9.5 (94)	0.6 (6)	13.6 (96)	0.6 (4)
7.00	9.5 (95)	0.5 (5)	14.0 (97)	0.4 (3)
8.00	9.6 (97)	0.3 (3)	14.0 (97)	0.4 (3)

influence other than pH must be operational in the experiments carried out in distilled water.

A further difficulty is that much previous evidence suggests that in some natural systems nitrile can be produced in relatively large amounts (if not to dominance) at pH levels at which, according to this confirmed theory, they should either not be formed at all or they should only be obtained in very small amounts [1-9]. The nature of the glucosinolate itself has little or no effect since, for example, allylglucosinolate of *Farsetia aegyptia* gave mainly isothiocyanate [3], whilst in *Brassica oleracea* it gave mainly nitrile (allyl cyanide) [22]. Regrettably, it seems necessary to invoke some unknown 'factor' to explain nitrile formation in these instances, which is not pH-dependent and which, incidentally, is not present in the extract prepared from mustard powder. It is present in some plants (e.g. *L. sativum* [1,2]) but not others (e.g. *Nasturtium officinale* [9]); see also [23] and [24]. It is conceivable that this 'factor' may be as simple as the metal ion content of the system. Tookey, for example, reported that an extract from *Crambe abyssinica* gave 95% isothiocyanate but in the presence of 10^{-4} M Fe^{2+} the same extract at the same pH produced 96% nitrile [17]; see also Youngs and Perlin [25] and Austin and Gent [26].

EXPERIMENTAL

Enzyme preparation. An active thioglucoside glucohydrolase extract was prepared from commercial mustard powder by the method devised by Schwimmer [13].

Assessment of enzyme activity by UV spectroscopy. Using 2 mm path-length quartz cells, absorbance was measured at 227 nm of known concn (ranging between 1×10^{-3} M and 2×10^{-3} M) of glucosinolate (allyl- or 2-phenethyl-). The reaction medium alone (either dist. H_2O or buffer soln of the correct pH prepared from appropriate amounts of aq. solns of 0.1 M citric acid and 0.05 M Na_2HPO_4) was used in the reference cell since the enzyme preparation showed no interfering absorbance. To the glucosinolate soln was added an aliquot of an aq. suspension of the enzyme prepn (10 mg in 5 ml H_2O) and absorbance readings were taken at 227 nm at 5 min intervals.

Assessment of products of enzymic degradation of glucosinolates by GLC. A soln of the glucosinolate (between 5×10^{-3} M and 3×10^{-3} M) in dist. H_2O or citrate-phosphate buffer of the appropriate pH was treated with an aliquot of the aq. enzyme suspension (10 mg in 5 ml H_2O) and the mixture shaken gently at room temp. At 10 min intervals 1 μl was removed and directly examined by GLC using a heated FID. For analysis of allylglucosinolate products a 1.5 m \times 4 mm glass column was used packed with 10% PEG 20 M coated on 100-120 BSS mesh acid-washed Diatomite C. It was operated isothermally at 100° with a N_2 carrier gas flow rate of 60 ml/min. For 2-phenethylglucosinolate products a 0.3 m column containing 3% PEG 20 M was used, at a temp. of 110° and a flow rate of 60 ml/min.

Identifications of degradation products in these simple systems were reliably accomplished by comparison of GLC RR_s with those of authentic standards and confirmed by GC-MS as previously described [3]. GLC peak areas were measured and compared with those of known amounts of pure standards of all glucosinolate products.

REFERENCES

1. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 1369.
2. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 1365.
3. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 227.
4. Daxenbichler, M. E., van Etten, C. H. and Wolff, A. I. (1966) *Biochemistry* **5**, 692.
5. van Etten, C. H., Daxenbichler, M. E., Peters, J. E. and Tookey, H. L. (1966) *J. Agric. Food Chem.* **14**, 426.
6. Daxenbichler, M. E., van Etten, C. H., Tallent, W. H. and Wolff, A. I. (1967) *Can. J. Chem.* **45**, 1971.
7. van Etten, C. H. and Daxenbichler, M. E. (1971) *J. Agric. Food Chem.* **19**, 194.
8. Daxenbichler, M. E., van Etten, C. H. and Spencer, G. F. (1977) *J. Agric. Food Chem.* **25**, 121.
9. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 1657.
10. Bjorkman, R. (1976) in *The Biology and Chemistry of the Cruciferae* (Vaughan, J. G., MacLeod A. J. and Jones, B. M. G., eds.) p. 191. Academic Press, London.
11. Gil, V. and MacLeod, A. J. (1980) *Tetrahedron* **36**, 779.
12. Gil, V. and MacLeod, A. J. (1980) *Phytochemistry* **19**, 2071.
13. Schwimmer, S. (1961) *Acta Chem. Scand.* **15**, 534.
14. Ettlinger, M. G., Dateo, G. P., Harrison, B. W., Thompson, T. M. and Thompson, C. P. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **47**, 1875.
15. Tsuruo, I., Yoshida, M. and Hata, I. (1967) *Agric. Biol. Chem.* **31**, 18.
16. Lein, K. A. (1972) *Angew. Bot.* **46**, 137.
17. Tookey, H. L. (1973) *Can. J. Biochem.* **51**, 1305.
18. Bjorkman, R. and Lonnerdal, B. (1973) *Biochim. Biophys. Acta* **327**, 121.
19. West, L. G., Badenhop, A. F. and McLaughlin, J. L. (1977) *J. Agric. Food Chem.* **25**, 1234.
20. Gil, V. (1979) Ph.D. Thesis, London University.
21. Miller, H. E. (1965) M.A. Thesis, Rice University, Houston, Texas, U.S.A.
22. Kaoulla, N., MacLeod, A. J. and Gil, V. (1980) *Phytochemistry* **19**, 1053.
23. Butterly, R. G., Guadagni, D. G., Ling, L. C., Seifert, R. M. and Lipton, W. (1976) *J. Agric. Food Chem.* **24**, 829.
24. Cole, R. A. (1976) *Phytochemistry* **15**, 759.
25. Youngs, C. G. and Perlin, A. S. (1967) *Can. J. Chem.* **45**, 1801.
26. Austin, F. L. and Gent, C. A. (1967) *Chem. Commun.* **2**, 71.