Phytochemistry, 1974, Vol. 13, pp. 2611 to 2615. Pergamon Press. Printed in England.

1-CYANO-3,4-EPITHIOBUTANE: A MAJOR PRODUCT OF GLUCOSINOLATE HYDROLYSIS IN SEEDS FROM CERTAIN VARIETIES OF *BRASSICA CAMPESTRIS*

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(*Received* 18 *March* 1974)

Key Word Index—*Brassica campestris*; Cruciferae; turnip rape; glucosinolate; 1-cyano-3,4-epithiobutane; 3-butenyl isothiocyanate.

Abstract—A new hydrolysis product derived from 3-butenylglucosinolate in seeds of certain strains of *Brassica campestris* Yellow Sarson is described. The structure, 1-cyano-3,4-epithiobutane is proposed. If the seeds are heated at 115° for 30 min before hydrolysis, 3-butenyl isothiocyanate is the main product.

INTRODUCTION

THE MAJOR glucosinolate in seeds of the important oilseed, turnip rape (*Brassica campestris* L), is 3-butenylglucosinolate (**1**).[1–3] 4-Pentenylglucosinolate and 2-hydroxy-3-butenylglucosinolate are also commonly present in smaller amounts. The glucosinolates of the yellow-seeded Indian form of *B. campestris*, Yellow Sarson, consist almost entirely of 3-butenylglucosinolate, with little or none of the other two compounds.[4,5]

When rapeseed is crushed for extraction of the oil the glucosinolates are brought in contact with a thioglucosidase present in the seed (thioglucoside glucohydrolase E.C. 3.2.3.1, trivial name—myrosinase). The myrosinase enzyme acts on the glucosinolate in the crushed seed or subsequently on moistening of the oil-free meal to hydrolyze the glucose residue. The sulphate moiety is also lost from the molecule, and what remains undergoes intramolecular rearrangements to give rise to isothiocyanates, thiocyanates or nitriles.[6] The predominant hydrolysis product from 3-butenylglucosinolate found hitherto has been 3-butenylisothiocyanate (**2**) and, in general, seed glucosinolates give rise mainly to the corresponding isothiocyanates on hydrolysis. However, when 2-hydroxy-3-butenyl glucosinolate, in seed of *Brassica napus* or *Crambe abyssinica*, is hydrolyzed under selected conditions, the main products are the nitriles, 1-cyano-2-hydroxy-3-butene and 1-cyano-2-hydroxy-3,4-epithiobutane (**3**);[7–9] under normal conditions the main product is 5-vinyloxazolidine-2-thione (derived by cyclization of 2-hydroxy-3-butenylisothiocyanate).

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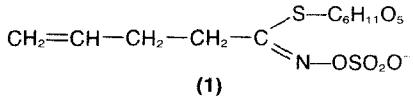
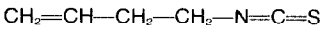
[2] JOSEFSSON, E. and MUHLENBERG, C. (1968) *Acta Agr. Scand.* **18**, 97.
[3] JOSEFSSON, E. and APPELQVIST, L.-A. (1968) *J. Sci. Food Agr.* **19**, 564.
[4] DOWNEY, R. K., CRAIG, B. M. and YOUNGS, C. G. (1969) *J. Am. Oil Chemists' Soc.* **46**, 121.
[5] KONDRA, Z. P. and DOWNEY, R. K. (1969) *Can. J. Plant Sci.* **49**, 623.
[6] VAN ETTEN, C. H., DAXENBICHLER, M. E. and WOLFF, I. A. (1969) *J. Agr. Food Chem.* **17**, 483.
[7] DAXENBICHLER, M. E., VAN ETTEN, C. H. and WOLFF, I. A. (1966) *Biochemistry.* **5**, 692.
[8] DAXENBICHLER, M. E., VAN ETTEN, C. H., TALLENT, W. H. and WOLFF, I. A. (1967) *Can. J. Chem.* **45**, 1971.
[9] DAXENBICHLER, M. E., VAN ETTEN, C. H. and WOLFF, I. A. (1968) *Phytochemistry* **7**, 989.

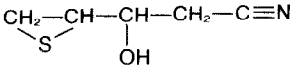
The toxicity of the various glucosinolate hydrolysis products greatly impairs the usefulness of rapeseed meal as a protein feedstuff for livestock. It appears that the nitriles are much more toxic than the oxazolidinethione, which is in turn more toxic than the isothiocyanates.[6,10] It is therefore very important to know to what extent nitriles may be present in different types of rapeseed meal. In this paper we report that the major product of glucosinolate hydrolysis in certain strains of *B. campestris* Yellow Sarson is a hitherto undescribed nitrile, 1-cyano-3,4-epithiobutane, (**4**).



(1)



(2)



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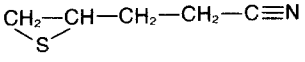
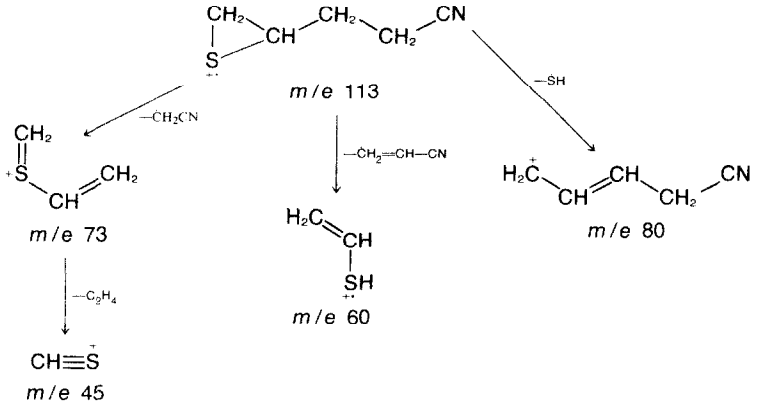


(4)

RESULTS

In the course of a survey of the glucosinolate composition of various cultivars of *Brassica campestris* L. by a method based on that of Youngs and Wetter[11] (involving GLC of the hydrolysis products) it was found that whereas in the typical Canadian variety, Echo, only the peaks of butenyl and pentenyl isothiocyanate were present, in certain Indian Yellow Sarson varieties a large new peak with retention time (on polyphenyl ether) nearly four times that of butenyl isothiocyanate, was observed.

It seemed likely either that this compound was an isothiocyanate derived from some other glucosinolate present in addition to 3-butenyl-glucosinolate, or that it was an alternative hydrolysis product formed from the 3-butenylglucosinolate. It has been observed that formation of isothiocyanates rather than other products is favoured by heat treatment of the seed or meal prior to hydrolysis.[6,12] We have found that if Yellow Sarson seed is heated for 30 min in an oven at 115° before enzymic hydrolysis is carried out then the new gas chromatographic peak almost entirely disappears and the 3-butenylisothiocyanate peak increases in size by a corresponding amount.



SCHEME 1. MS FRAGMENTATION OF **4**.

[10] RUTKOWSKI, A. (1971) *J. Am. Oil Chemists' Soc.* **48**, 863.
[11] YOUNGS, C. G. and WETTER, L. R. (1967) *J. Am. Oil Chemists' Soc.* **44**, 551.
[12] APPELQVIST, L.-A. and JOSEFSSON, E. (1967) *J. Sci. Food Agr.* **18**, 510.

Microanalysis and accurate mass measurement showed that the molecular formula of the compound is C_5H_7NS, indicating that it is an isomer of butenyl isothiocyanate. The IR spectrum showed a sharp intense band at 2250 cm^{-1} attributed to an unconjugated nitrile group. A strong band at 1050 cm^{-1} may be attributed to the wag vibration of the ring methylene group in structure (**4**), by analogy with a similar assignment made in the IR spectrum of ethylene sulphide.[13] In the UV the compound has an absorption peak at 255 nm in ethanol (ϵ = 49, approx.). Ethylene sulphide has an absorption peak at about 258 nm in EtOH, whereas trimethylene sulphide has a peak at about 273 nm, and tetramethylene sulphide has a shoulder at about 244 nm.[14] The UV spectrum is thus consistent with the presence of an ethylene sulphide ring.

The NMR spectrum of the compound showed the absence of *C*-methyl protons (no resonance below δ 1·3), and of any protons attached to an olefinic double bond (no resonance above δ 3·3). A single proton multiplet centred near δ 3·0 can be attributed to the ring proton on C-3 of the 1-cyano-3,4-epithiobutane. The remaining signals formed a very complex pattern between δ 1·3 and δ 2·75 which can be explained as a mixture of the resonances due to three different methylene groups, on C atoms 1, 2 and 4. Clarification of this complex pattern was achieved by using the NMR resolving agent, Tris (1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione) europium 3[15] (abbreviation—Eu-$(fod)_3$). A triplet at δ 2·46, δ 2·56 and δ 2·66 (in part) was assigned to the protons of the C-1 methylene. A set of four doublets (δ 2·20, δ 2·23; δ 2·29, δ 2·32; δ 2·50, δ 2·53; δ 2·63, δ 2·66 in part) centred around δ 2·4 was attributed to the ring methylene protons (C-4) of **4**. A similar identification was made by Daxenbichler *et al.*[9] in the case of the acetate of **3**. A very complex and spread-out pattern from δ 1·3 to δ 2·16, and perhaps as far as δ 2·5 was attributed to the two protons on C-2. These are next to an asymmetric carbon (C-3). For each of the two optical isomers present there will be three staggered conformers, and in each of these the environments of the two hydrogens on C-2 are non-equivalent;[16] this could account for the complexity of the NMR pattern.

The MS data are given in Table 1. The molecular ion had a MW of 113·02995; C_5H_7NS requires 113·02992. Metastable peaks (first or second field free region, or both)

TABLE 1. MASS SPECTRUM OF 1-CYANO-3,4-EPITHIOBUTANE

m/e	Composition	Relative intensity	*m/e*	Composition	Relative intensity
113	C_5H_7NS	61	59	C_2H_3S	11
112	C_5H_6NS	2	58	C_2H_2S	7
98	C_4H_4NS	2	54	C_4H_6	23
86	C_4H_6S	13	54	C_3H_4N	14
85	C_4H_5S	6	53	C_4H_5	24
81	C_5H_7N	24	47	CH_3S	16
80	C_5H_6N	12	46	CH_2S	12
79	C_5H_5N	4	45	CHS	40
73	C_3H_5S	27	44	CS	6
71	C_3H_3S	4	41	C_3H_5	100
67	C_4H_5N	16	41	C_2H_3N	8
60	C_2H_4S	26	39	C_3H_3	30

[13] GUTHRIE, G. B., SCOTT, D. W. and WADDINGTON, G. (1952) *J. Am. Chem. Soc.* **74,** 2795.
[14] DAVIS, R. E. (1958) *J. Org. Chem.* **23,** 1380.
[15] RONDEAU, R. E. and SIEVERS, R. E. (1971) *J. Am. Chem. Soc.* **93,** 1522.
[16] BOVEY, F. A. (1969) *Nuclear Magnetic Resonance Spectroscopy*, Chap. VI. Academic Press, New York.

were recorded for the following transitions: 113 → 98, 113 → 86 → 85, 113 → 80 → 53, 113 → 73 → 45, 113 → 60. Admixture of D_2O with the compound in the mass spectrometer's inlet system did not change the spectrum. Thus no hydrogen atom in the compound exchanged with D_2O, and structures containing an –SH group may be rejected.

The few epithio compounds whose mass spectra are available to provide a basis for mass spectrum—structure correlations include ethylene sulphide[17] and 12 of its simple alkyl or chloro derivatives.[18–20] Features common to the spectra of all of these compounds except 2,3-dimethyl-2,3-epithiobutane,[20] and to the spectra of many thiols, are the presence of an M–SH peak and a peak due to CHS^+ at *m/e* 45. Both of these features are shown by the spectrum of the compound. The peak at *m/e* 60 may be due to a reaction analogous to the "outside" McLafferty rearrangement of epoxides,[21] as may the peak of the same mass in the published spectrum of 1,2-epithiobutane.[19] The spectra of both ethylene sulphide[17] and epithiocyclopentane[20] have prominent M–Me peaks which must involve rearrangement, so the presence of the M–Me peak in the spectrum of the compound does not disqualify the methyl-free structure **4**. Possible structures for some of the above ions and neutral fragments are shown in Fig. 1. The MS is quite different from those of 3-butenyl-isothiocyanate and three other isomeric alkenylisothiocyanates.[22]

No optical activity was detected in a solution of the compound in isooctane suggesting that it is a racemic mixture of the two optical isomers of **4**.

DISCUSSION

The most plausible interpretation of the spectral data is that the new compound is 1-cyano-3,4-epithiobutane. Indeed a compound with this structure might reasonably have been expected to be formed under these conditions in seed meal containing 3-butenylglucosinolate, since the corresponding hydroxy compound (**3**) is already known to be formed in seed meal containing 2-hydroxy-3-butenylglucosinolate.[9] The formation of a racemic mixture is also to be expected since the transfer of the sulphur atom to the 3,4 double bond of the butenyl group is likely to be a non-enzymic reaction.

What remain a puzzle are the factors which determine whether the nitrile or the isothiocyanate is the major end product of glucosinolate hydrolysis. Heat treatment of the seed, which ensures isothiocyanate formation, may well, by its effects on proteins, lipids and other components, significantly alter the microenvironment in which the reaction occurs. Why untreated seeds of different strains of *Brassica campestris* should give different end products of 3-butenylglucosinolate hydrolysis is less clear. Of 24 *B. campestris* Yellow Sarson cultivars tested, 14 gave 3-butenyl isothiocyanate as the main product, one gave **4** as the main product, and nine gave comparable amounts of the isothiocyanate and **4**. For purposes of glucosinolate estimation by the GLC procedure of Youngs and Wetter,[11] if **4** is found to be present then, like **2**, it can be used as a measure of the amount of 3-butenylglucosinolate originally present. In view of its probable toxicity, the possible presence, or formation, of 1-cyano-3,4-epithiobutane in rapeseed meal intended for livestock feeding warrants investigation.

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[18] HOBROCK, B. G. and KISER, R. W. (1962) *J. Phys. Chem.* **66**, 1551.
[19] SIDHU, K. S., LOWN, E. M., STRAUSZ, O. P. and GUNNING, H. E. (1966) *J. Am. Chem. Soc.* **88**, 254.
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[21] BUDZIKIEWICZ, H., DJERASSI, C. and WILLIAMS, D. H. (1967) *Mass Spectrometry of Organic Compounds*, p. 454, Holden-Day, San Francisco.
[22] KJAER, A., OHASHI, M., WILSON, J. M. and DJERASSI, C. (1963) *Acta Chem. Scand.* **17**, 2143.

EXPERIMENTAL

Gas chromatographic detection of new hydrolysis product of butenyl glucosinolate. The procedure used was essentially that of Youngs and Wetter,[11] except that the seeds were not heat-treated, and a different pH and chromatographic column were used. Typically, about 15 mg of seed were crushed in a mortar and transferred to a 62 × 9 mm (i.d.) flat-bottomed specimen tube. 0·05 ml of 0·6% myrosinase in 0·2 M—Na acetate buffer, pH 4·0 was added, followed by 0·5 ml CH_2Cl_2. A small amount of allyl isothiocyanate was sometimes added to act as a marker. The tubes were stoppered and then incubated at 25°, with vigorous shaking, for 2 hr. The CH_2Cl_2 layer, containing the isothiocyanates and other breakdown products was removed for analysis. A 5 μl aliquot was injected into a 180 × 0·3 cm stainless steel column packed with 10% polyphenyl ether (6-ring) on Chromosorb at a column temperature of 175° and a helium flow rate of 60 ml/min, in a Hewlett–Packard 7620A gas chromatograph with a flame ionization detector.

Isolation of the new hydrolysis product of butenyl glucosinolate. Two varieties of *Brassica campestris* Yellow Sarson have been grown to provide seed for the isolation of the new compound: Yellow Sarson (Mysore)—originally obtained from the Central Food Technological Research Institute, Mysore, India; Yellow Sarson (Wagga)—from the seed collection at the Agricultural Research Institute, Wagga Wagga, N.S.W.

The standard procedure was to grind two lots of 3 g of seed each with 10 ml 60–80° petrol in a mortar. The combined homogenate was centrifuged and the petrol discarded. The meal was subjected to three further washes (by resuspension and centrifugation) with petrol (30 ml for each wash) and then allowed to dry in air. The dry, oil-free meal was distributed into two 250 cm^3 flasks. 10 ml of 0·6% myrosinase in 0·2 M Na acetate, pH 4·0, was added to each flask and allowed to soak into the meal. 25 ml CH_2Cl_2 was added to each flask which was then sealed with a ground-glass stopper. The flasks were incubated for 2 hr at 25° with shaking. The samples were centrifuged in glass tubes, and the upper aqueous layer was discarded. The CH_2Cl_2 layer was withdrawn from below the pellicle, with a Pasteur pipette. The pellicles were each shaken vigorously with 10 ml CH_2Cl_2 for 1–2 min, and then centrifuged, the supernatant being combined with the earlier CH_2Cl_2 fractions. The CH_2Cl_2 was clarified by a further centrifugation, dried over active anhydrous calcium sulphate and then reduced to 0·5 ml by distillation at 56°. Isolation of the new hydrolysis product was carried out by preparative GLC using the same equipment and conditions as described above. Yields were in the range of 1–2 mg per gm of seed.

Myrosinase preparation. A crude myrosinase preparation was obtained from white mustard (*Sinapis alba* L.) seed by the method described by Appelqvist and Josefsson.[12]

Physicochemical measurements. The UV spectrum was measured on a sample re-purified by a second passage through the GC column. IR spectra were taken with neat liquid between NaCl discs. NMR spectra were taken on a Varian A-60D 60 MHz instrument with samples dissolved in $CDCl_3$. In the experiments with $Eu(fod)_3$ a series of weighed amounts of the resolving agent were added to 0·5 ml of an approx. 3% (w/v) soln of the unknown in $CDCl_3$ to give 10, 20, 30, 50 and 80 mg of $Eu(fod)_3$ per 0·5 ml of $CDCl_3$ soln. Optical rotation was measured on a 0·27% (w/v) soln of the compound in isooctane in a 20 cm pathlength cell. MS were measured at 70 eV on an AEI MS 902 instrument with the aid of a Raytheon 706 computer programmed for on-line acquisition and processing of high resolution data. The compound was introduced through an all-glass heated inlet system. Elemental analysis was carried out by the Australian Microanalytical Service, Division of Applied Chemistry, CSIRO. Found C, 53·17; H, 6·20; N, 12·38; S, 28·3. C_5H_7NS requires: C, 53·06; H, 6·23; N, 12·38; S, 28·3.

Acknowledgements—We wish to thank Mr. C. Hurlstone for able technical assistance, and Mr. F. A. Pellegrino for carrying out the NMR measurements and in particular for suggesting the use of the europium derivative.