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Glucosinolates in Seeds of *Sibara virginica* (L.) Rollins: Two New Glucosinolates

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Seeds of the crucifer *Sibara virginica* (L.) Rollins contain three major glucosinolates.

On enzymic hydrolysis, one glucosinolate affords (partly racemic) (S)*5-phenyl-2-oxazolidinethione* (I), the enantiomer of which has previously been encountered in enzymically hydrolysed extracts of species of the genera *Barbarea* (Cruciferae) and *Reseda* (Resedaceae). The glucosinolate, whence (I) derives, most likely possesses the structure (II).

Similarly treated, the second glucosinolate yields (R)*8-methylsulphinyloctyl isothiocyanate*, characterised as the *levo*rotatory thiourea-derivative (Va), indistinguishable from a specimen previously obtained from a seed extract of the crucifer *Arabis hirsuta* (L.) Scop. Consequently, the second glucosinolate is formulated as (III).

The third glucosinolate affords the next lower homologue, (R)*7-methylsulphinylheptyl isothiocyanate*, again characterised as the thiourea-derivative (Vb). Hence, the parent glucosinolate is formulated as (IV) and represents a novel glucosinolate of natural provenance. Its finding completes the series, $(R)CH_3SO(CH_2)_nNCS$, $n=3-10$, of enzymic hydrolysis products deriving from ω-methylsulphinylalkylglucosinolates in higher plants.

Sibara virginica (L.) Rollins is a North American crucifer, the generic relationship of which has been the subject of considerable discussion.*** Through the kindness of Dr. M. G. Ettlinger, an authentic seed specimen of *S. virginica*, collected on the campus of Rice University, Houston, Texas, was generously placed at our disposal in 1963. Dr. Ettlinger further informed

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*** Synonyms for the species are: *Cardamine virginica* L. 1753; *Arabis virginica* Poiret; *Cardamine praecox* vel *pectinata* Raf.; *Sisymbrium Ludovicianum* Nutt.; *Arabis virginica* (L.) Trelease; *Cardamine Ludoviciana* Hook; *C. Engelmannia* Ind. sem. Berol.; *Arabis Ludoviciana* C. A. Mey; *Cardamine parviflora* L. subspec. *virginica* (L.) O.-E. Schulz; *Nasturtium Ludovicianum* O.-E. Schulz (1903). In 1912, Greene elevated the species to genus rank, as the monotypic *Planodes virginicum* (L.) Greene. By a more recent revision of the genus *Sibara* (anagram for *Arabis*), Rollins [1] transferred the species to the latter genus.

us about his chromatographic studies of the thioureas derived from the isothiocyanates in enzymically hydrolysed seed macerates and agreed on the further investigations being undertaken in the Danish laboratory. With the generous assistance of the Botanic Garden of the University of Copenhagen, the original seed sample was propagated in the following years to give sufficient material for a detailed study. We now report the results.

On paper chromatography in the solvent systems (A), butanol:ethanol:water (4:1:4), and (B), butanol:pyridine:water (6:4:3), seed extracts of *S. virginica* were found to contain at least four glucosinolates, possessing R_B-values (*i.e.* R_P-values relative to that of benzylglucosinolate) in solvent system (B) of: (1) 0.71; (2) 0.81; (3) 0.99; and (4) 1.10 (trace), and in system (A) of: (1) 0.59; and (2,3,4) 0.92, (2) and (3) obviously representing the major components. Preliminary chromatographic investigations of a chloroform extract of an enzymically hydrolysed glucosinolate solution revealed the formation of both isothiocyanates and an oxazolidinethione, the latter typically deriving from cyclisation of an initially produced 2-hydroxy-substituted isothiocyanate.*

A larger seed sample was extracted with 70 % methanol, and the purified glucosinolate fraction was subjected to enzymic hydrolysis. By column chromatography on silicagel, the residue from a chloroform extract of the hydrolysed solution was separated into (i) an oxazolidinethione, and (ii) a mixture of isothiocyanates.

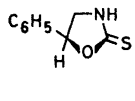
(i) On the basis of its composition, melting point, spectroscopical data, and chiroptical properties, the former was identified as partly racemic (+)-5-phenyl-2-oxazolidinethione (I), exhibiting a somewhat lower rotation (+59°) than that of a synthetic specimen (+71°), prepared in our laboratory several years ago.[3] The dextro*rotatory*, naturally derived 5-phenyl-2-oxazolidinethione possesses the (*S*)-configuration (I) as evident from the previously established correlation of (I) with (*S*)(+)-mandelamide.[3] From several analogies (*cf. e.g.* Refs. 4, 5), it seems safe to conclude that (I) derives from the previously unknown (*R*)2-hydroxy-2-phenylethylglucosinolate (II), present in the seeds of *S. virginica*. It is of interest that (II) represents an epimer of a glucosinolate formerly encountered in seeds of various species of the crucifer genus *Barbarea*,[6] as well as in leaves and inflorescences of *Reseda luteola* L. (Resedaceae).[3] On enzymic hydrolysis, the latter glucosinolate affords the enantiomeric, levo*rotatory* (*R*)5-phenyl-2-oxazolidinethione.[3] The occurrence in the plant kingdom of epimeric glucosinolates of the present type is not without precedent. Thus, Daxenbichler *et al.*[7] established a similar relationship between the major thioglucoside in *Crambe abyssinica* Hochst *ex* R. E. Fries (Cruciferae),** yielding (*R*)5-vinyl-2-oxazolidinethione, and a glucosinolate in various *Brassica* species (Cruciferae), producing (*S*)5-vinyl-2-oxazolidinethione (goitrin)[9] upon enzymic hydrolysis. From specifically designed experiments it was established that the oxazolidinethione-producing glucosinolate in *S. virginica* is represented by the spot (3) on the paper chromatograms (*vide*

* This finding is in keeping with a report from other side[2] stating the presence of volatile isothiocyanate(s) as well as oxazolidinethione(s), both unidentified, in enzyme-treated seed meal of *Arabis virginica* (L.) Poir. (synonym for *Sibara virginica* (L.) Rollins).

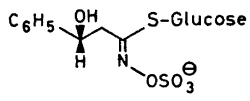
** In Ref. 7, the stereochemical specification of the *Crambe* glucosinolate is incorrectly given as (*R*); the necessary correction is, however, presented in a subsequent paper.[8]

supra). No racemisation experiments have been performed on the chiral 5-phenyl-2-oxazolidinethiones, but it seems unlikely that the mild conditions prevailing during the present isolation and enzymic hydrolysis could have caused significant epimerisation or racemisation. Consequently, *Sibara* seeds seem to contain unequal amounts of two epimeric glucosinolates solely differing in the chirality of the carbinol function of the side chain.

(ii) The mixture of isothiocyanates eluted from the column was subjected to mass spectrometry. A parent ion at *m/e* 233, and other features in the fragmentation pattern, were highly indicative of an 8-methylsulphinyloctyl isothiocyanate, $CH_3SO(CH_2)_8NCS$, being present. However, a weaker signal



(I)



(II)

at *m/e* 219, along with several other peaks displaced fourteen mass units towards the lower mass region, suggested admixture with the lower homologue, 7-methylsulphinylheptyl isothiocyanate, $CH_3SO(CH_2)_7NCS$. While the former had been previously characterised as the enzymic hydrolysis product of a glucosinolate present in *Arabis hirsuta* (L.) Scop.,* [10] the heptyl-derivative was novel. Attempts to separate the supposedly homologous isothiocyanates by various chromatographic methods were unsuccessful. On reaction with aniline, a *levo*rotatory mixture of very similar phenylthioureas was obtained as apparent from mass-spectrometrical analysis. Again, attempts to separate these were of no avail.

With a view to separating the thioureas, rather than the phenylthioureas, another seed portion (150 g) was employed for the production of a purified glucosinolate fraction by ion exchange.** After enzymic hydrolysis and separation of the phenyl-oxazolidinethione (I) (100 mg) from the isothiocyanates by chromatography on silicagel as above, the latter (350 mg) were converted into the corresponding thioureas on reaction with methanolic ammonia.*** Again, various attempts to separate more than analytical amounts of the thioureas by chromatography were unsuccessful. Finally, recourse was taken to a 60-plate counter-current distribution of the mixture, with chloroform and water serving as the solvent system.

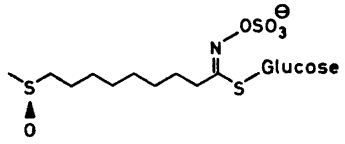
The contents of plates Nos. 19–38 were combined and yielded a total of 102 mg of a chromatographically homogeneous thiourea which, after recrystallisation, proved identical with a specimen of (*R*)(−)-1-(8-methylsulphinyloctyl)-thiourea (Va), m.p. 88°, previously obtained as a derivative of (*R*)8-

* (*R*)-Configuration was previously established for the naturally derived isothiocyanate.[11]

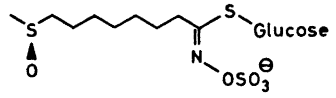
** The glucosinolate-free extract afforded isoferulic acid choline ester as a new natural product as described elsewhere.[12]

*** Paper chromatography, with water-saturated chloroform[13] or butanol:toluene:water (1:10:2) as the mobile phases, served as a convenient analytical method for separating the homologous ω-methylsulphinylalkyl-thioureas (M. G. Ettlinger, personal communication).

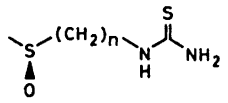
methylsulphinyloctyl isothiocyanate produced by enzymic hydrolysis of the glucosinolate (III), present in seeds of *Arabis hirsuta* (L.) Scop.[10] *



(III)



(IV)



(Va): n = 8

(Vb): n = 7

Similarly, plates Nos. 43 – 54 afforded a second thiourea (30 mg) which, after two recrystallisations, appeared as a chromatographically homogeneous, *levo*rotatory compound, $C_9H_{20}N_2OS$, m.p. 85.5°, possessing physical properties (UV, IR, MS, and optical rotation) so similar to those of the above thiourea (Va) that its structure as (*R*)(−)-1-(7-methylsulphinylheptyl)thiourea (Vb) can be considered established solely on this basis. In analogy with (Va), the thiourea (Vb) undoubtedly derives from a glucosinolate possessing the structure (IV) the presence of which in Nature has long been suspected (*cf.* Ref. 5).* The present finding completes a rather remarkable series of homologous ω-methylsulphinylalkylglucosinolates of identical chirality, containing anywhere from three to ten methylene-groups in their side-chains and occurring in various members of the family Cruciferae (for a recent review, *cf.* Ref. 5).

The co-occurrence of ω-methylsulphinylalkyl- and hydroxyaralkyl-glucosinolates in plant extracts has not been frequently observed. A recent case, however, is found in seeds of *Arabis hirsuta* (L.) Scop.,[14] belonging to a genus to which *Sibara* has an accepted, close affinity.[1] It should be noted here also that seeds of watercress (*Nasturtium officinale* R. Br.), in addition to 2-phenylethylglucosinolate, on the basis of chromatographic analyses,[5,15] appear to contain a mixture of (III) and (IV).

The amounts of the most lipophilic glucosinolate(s) in *S. virginica* seed extracts (spot (4) above) were insufficient for further studies.

* By paper-chromatographic separation of the glucosinolate mixture into bands, followed by elution of the individual bands, enzymic hydrolysis, and ammonia treatment of the liberated isothiocyanates, it was established that spot (2) (see above) represents the glucosinolate (III), and spot (1) the glucosinolate (IV).

EXPERIMENTAL

Melting points are uncorrected and determined in capillary tubes in an electrically heated bath. Mass spectra were recorded on a Perkin-Elmer model 270 mass spectrometer (ionising potential 70 eV). Rotations were measured on a Perkin-Elmer model 141 polarimeter; infra-red spectra (in KBr) were recorded on a Perkin-Elmer Infracord instrument, and UV-spectra on a Perkin-Elmer 402 UV-spectrophotometer. NMR-spectra were recorded on a Varian A-60 instrument in $CDCl_3$.

Paperchromatographic analyses. Defatted seed powder (from 1 g of seed) of *Sibara virginica* (L.) Rollins was extracted with 70 % methanol (10 ml). 10 µl aliquots of the filtered solution were chromatographed ascendingly on Schleicher and Schüll paper No. 2043b in the solvent system (A), butanol:ethanol:water (4:1:4) (upper phase); and (B), butanol:pyridine:water (6:4:3) (upper phase). Benzylglucosinolate (R_B-value by definition 1.00) was employed as a reference compound. The dried chromatograms were dipped through $AgNO_3$ in aqueous acetone (0.75 g of $AgNO_3$, dissolved in water (10 ml), and diluted with acetone to a total volume of 400 ml) and air-dried. They were then sprayed on both sides with a 5 % solution of NaOH in methanol, when the glucosinolates appeared as dark-brown spots. The paper strips were washed with very dilute HNO_3 and water, and finally dried at 100°.

In solvent (A), a major spot appeared with an R_B-value of 0.92, and a minor one with R_B 0.59. In the basic system (B), strong spots were present at R_B 0.81 and 0.99, a weak spot at 0.71, and a very weak spot at 1.10. By band chromatography, and elution of the individual zones, it was ascertained that the spots with R_B-values 0.81, 0.99, and 1.10 in solvent system B, migrated as one spot (R_B 0.92) in system A.

Enzymic hydrolysis. The remaining solution from the paperchromatographic analyses was concentrated to dryness. The residue was dissolved in citrate buffer (pH 6.4), and a drop of a myrosinase preparation and a trace of ascorbic acid were added. After standing for 4 h at room temperature, the reaction mixture was extracted with chloroform and subjected to two-dimensional thin layer chromatography on Kieselgel H (Merck). In the first solvent, chloroform:methanol (95:5), only one UV-absorbing spot appeared (R_F 0.53), which, however, split up into two (R_F 0.0 and 0.39) in the second solvent, ethyl acetate. The 0.39-spot gave a blue colour with Grote's reagent, and a yellowish colouration with $AgNO_3$ in ammonia, reactions which are characteristic for 2-oxazolidinethiones. The 0.0-spot produced a blue colour with Grote's reagent only after treatment with ammonia, indicating its character of one or more isothiocyanates.

Isolation and characterisation. A 100 g seed portion of *S. virginica* was finely ground and defatted with petroleum ether. The residue (85 g) was extracted twice with hot 600 ml-portions of 70 % methanol. The combined extracts were freed of methanol by evaporation *in vacuo*, and some extra water was added to the solution. A 20 % solution of lead acetate was added portionwise in order to precipitate impurities. Lead ions were removed from the filtered solution by precipitation with a 20 % Na_2HPO_4-solution. The filtered solution was subjected to enzymic hydrolysis by the addition of a few ml of a myrosinase preparation and about 10 mg of ascorbic acid. After 4 h at room temperature, the solution was extracted with chloroform and evaporated to dryness, leaving a residue (840 mg) which was dissolved in ethyl acetate (15 ml). The solution was passed through a column of silicagel (45 g), deactivated with 15 % of water, and elution was performed with ethyl acetate, 25-ml fractions being collected. Fractions Nos. 4—7 contained the oxazolidinethione, as established by thin layer chromatography. After total elution of the cyclic compound, the eluting solvent was changed to chloroform. The isothiocyanate(s) appeared in fractions Nos. 45—59.

(i) *The 5-phenyl-2-oxazolidinethione.* The residue from fractions Nos. 4—7 was recrystallised twice from ethyl acetate-petroleum ether to give colourless needles (295 mg), m.p. 123°. (Found: C 60.39; H 5.12; N 7.89; S 17.76. Calc. for C_9H_9NOS: C 60.31; H 5.06; N 7.82; S 17.88). The compound displayed a UV-spectrum (in EtOH) with a strong maximum at 246 nm (ε 20 000). The rotation, $[\alpha]_D^{27}$ +59° (*c* 1.8, MeOH), composition, and combined spectroscopic properties (UV, IR, NMR, MS) revealed the identity of the compound as partly racemic (*S*)5-phenyl-2-oxazolidinethione. For the pure enantiomer,

we formerly reported: m.p. 126°, $[\alpha]_D^{23}$ +71° ±1.5° (*c* 2.0 MeOH).[3]* On admixture with a synthetic, presumably optically pure specimen of the latter,[3] the *Sibara* derivative showed hardly any melting point depression (m.p. 122–123°), whereas admixture with an equal quantity of the (−) enantiomer (m.p. 126°) raised the melting point to 136–137°, the same as that of the synthetic, racemic modification, determined in the same bath (previously reported [6] m.p. 140°).

(ii) *The isothiocyanates.* The chromatographic fractions Nos. 45–59 were combined and evaporated to a small volume, followed by the addition of excess aniline. After 3 h at room temperature, the reaction mixture was concentrated to a small volume and ether was added, causing beginning crystallisation which was completed by careful addition of petroleum ether. After two recrystallisations from ethyl acetate-petroleum ether, colourless needles were obtained (75 mg), m.p. 126–128°, $[\alpha]_D^{28}$ −46° (*c* 0.9, $CHCl_3$), giving analyses, within experimental errors, for the composition $C_{16}H_{26}ON_2S_2$ and exhibiting an IR-spectrum very similar to, but not entirely coincident with that of an authentic specimen of (−)-1-(8-methylsulphinyloctyl)-3-phenylthiourea (m.p. 139°).[10] On mass-spectrometry, a molecular ion at *m/e* 326, expected for the latter derivative, was accompanied by a signal at *m/e* 312 suggesting admixture with a lower homologous phenylthiourea. This suspicion was confirmed by mass-spectrometric analysis of the parent isothiocyanate-fraction, again exhibiting, in addition to a molecular ion at *m/e* 233, a signal at *m/e* 219, probably the molecular ion of the lower homologue, 7-methylsulphinylheptyl isothiocyanate.

Attempts to separate the sulphoxide mustard oils by gas chromatography were unsuccessful, due to decomposition. Again, various efforts to achieve separation of the phenylthiourea-derivatives by chromatographic methods proved futile. Hence, a new extraction was made with a view to separating the simple thiourea-derivatives.

Production and separation of thiourea mixture. Another seed portion (150 g) was defatted and extracted with 70 % methanol as described above. After removal of methanol, the aqueous solution, diluted to 1 l with water, was passed through a column containing Dowex 1 × 1 ion exchange resin in the OH^--form. (The filtrate, after concentration and addition of ammonium thiocyanate, deposited a precipitate of isoferulic acid choline ester thiocyanate as described elsewhere).[12] The column was rinsed with water (1 l), and the glucosinolate fraction was eluted by passing a 5 % K_2SO_4-solution through the column; 250 ml-fractions were collected. According to paperchromatographic analysis, fractions Nos. 4–10 contained the glucosinolates. They were combined, concentrated to dryness, and the glucosinolates were extracted with hot 90 % ethanol from a residue of inorganic salt. Ethanol was removed from the extract, and the aqueous solution was buffered to pH 6.4 with a citrate buffer. After addition of myrosinase and a small amount of ascorbic acid, the mixture was set aside for 4 h at room temperature. The mixture was then extracted with three 50- ml portions of chloroform; the combined extracts were dried and concentrated to a small volume.

The residue was placed on top of a silicagel column (45 g), and elution with ethyl acetate began; 25-ml fractions were collected. Fractions Nos. 4–7 contained the (+)-5-phenyl-2-oxazolidinethione (100 mg), with the properties reported above.

After a total of 500 ml of ethyl acetate was passed through the column, elution was continued with chloroform. Fractions Nos. 45–49, containing the isothiocyanates according to thin layer chromatography, were combined and the solvent was removed. Part of the oily isothiocyanate mixture (350 mg) was used for a number of unsuccessful attempts at separation by gas and column chromatography. Finally, the residual isothiocyanate mixture was converted into a thiourea-mixture on reaction at room temperature for 2 h with a methanolic solution of ammonia. The residue (203 mg) was introduced into the first unit (each unit 20 ml) of a 60-plate, all-glass Craig counter-current apparatus, preloaded with water-saturated chloroform. Water was utilized as the mobile phase; after 60 transfers, paperchromatographic analysis served to locate the individual thioureas.**

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* Redetermination of the specific rotations of the original samples gave the values $[\alpha]_D^{26}$ +63.5° (*c* 1.6, MeOH) and −66.0° (*c* 1.9, MeOH) for the synthetic enantiomers.

** The observed R_{Ph}-values [13] for $CH_3SO(CH_2)_nNHCSNH_2$, $n=7$, 8, and 9, in water-saturated chloroform were 0.42, 0.70, and 0.86. In butanol:toluene:water (1:10:2), the corresponding values were 0.23, 0.50, and 0.88.

The contents of plates Nos. 19—38 were combined and yielded on concentration a crystalline residue (102 mg) which, after two recrystallisations from ethyl acetate, gave a crop of colourless crystals (65 mg), m.p. 87—88°, $[\alpha]_D^{22}$ −71.5° (*c* 1.0 abs. EtOH), λ_{max}(EtOH) 243 mμ (ε 13 000), indistinguishable, by IR, mixed m.p., and MS, from an authentic specimen of (*R*)(−)-1-(8-methylsulphinyloctyl)-thiourea (Va) isolated from seeds of *Arabis hirsuta*.[10]

Similarly, the contents of plates Nos. 43—54 were combined, evaporated to dryness (31 mg), and recrystallised twice from ethyl acetate to give colourless needles (about 10 mg), m.p. 85—85.5°, $[\alpha]_D^{21}$ −68° (*c* 1.0, abs. EtOH). (Found: C 45.90; H 8.46; N 11.35.* Calc. for $C_9H_{20}N_2OS$: C 45.75; H 8.53; N 11.86). The product proved homogeneous on paperchromatographic analysis. Its IR-spectrum was very similar to but not identical with that of (Va). The same applies to its mass spectrum; most of the observed ions were displaced 14 mass units towards lower mass numbers. The combined evidence shows that the isolate represents (*R*)(−)-1-(7-methylsulphinylheptyl)-thiourea (Vb).

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* Due to lack of material, the nitrogen determination was carried out on less than 1 mg.