A STUDY OF ISOTHIOCYANATE GLUCOSIDES OCCURRING IN RAPESEED

by

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ABSTRACT

A purpose of this research was to isolate thioglucosides which occur in rapeseed. Although sinalbin, glucoiberin and sinigrin have been isolated earlier, this work was repeated to make these substances available for further study. Plant extracts containing gluconapin were purified with anion exchangers. Gluconapin was not obtained in crystalline form. A purified fraction of gluconapin was acetylated and subsequently purified with the use of acid alumina. Gluconapin tetraacetate was obtained in crystalline form. Its infrared spectrum and elemental analysis were in agreement with the structural formula of gluconapin tetraacetate.

The anthrone method for estimation of thioglucosides was investigated and simplified for possible use in routine analysis of rapeseed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Rapeseed is produced mainly for production of oil for edible purposes. Rapeseed meal, which remains after removal of the oil from the seed, is used as a protein supplement for livestock and poultry. This use of the meal constitutes a problem in that an excess intake of rapeseed meal may cause goitre and growth depression in livestock and poultry. Goitre is understood to be caused partly by thio-oxazolidone, an enzymatic fission product of progoitrin. Progoitrin is one of six thioglucosides found in rapeseed (1), namely progoitrin, sinalbin, glucoiberin, gluconasturtin, gluconapin and glucobrassicanapin. The effects on animals of thioglucosides other than progoitrin are not well understood.

With increasing production of rapeseed in Canada the toxicity of the meal has been causing some concern and more attention has been given to the study of the goitre factors.

For a further study of the effects of individual thioglucosides on animals it would be desirable to have pure thioglucosides available for animal feeding experiments. If pure individual thioglucosides were made available, they would also become useful in the study of analytical methods for their determination in rapeseed.

Of the six known thioglucosides in rapeseed, sinalbin (2), glucoiberin (3), gluconasturtin (4) and progoitrin (5) have been earlier isolated. In addition, a probably impure preparation of gluconapin has been separated (6). However, these thioglucosides are not commercially available.

The isolation from plant materials of thioglucosides occurring in rapeseed constitutes one part of this thesis.

Sinalbin, glucoiberin and the acetyl derivative of gluconapin were isolated. The latter has not earlier been described in the literature. A thioglucoside, sinigrin, which does not occur in rapeseed was also isolated in order to provide material for further studies.

Another part of this thesis is concerned with the study of methods for determination of the total thioglucosides in rapeseed. Such a method is required for use in plant breeding experiments for the purpose of possible elimination of the thioglucosides from the seed. The method by Schultz and Gmelin (7) for estimation of thioglucosides with anthrone reagent was selected for study as it was considered the most promising method for this purpose.

REVIEW OF LITERATURE

I. Historical Development in the Study of Thioglucosides.

In 1831 Robiquet and Bourtons (8) isolated a crystalline sulphur containing constituent, sinalbin, from seeds of white mustard (Sinapis alba L.). This substance was later recognized as a thioglucoside. In 1840 Bussy (9) demonstrated the enzymatic formation of a mustard oil from black mustard seed (Brassica nigra, Koch) in the presence of water by isolation of the crystalline parent compound, subsequently named sinigrin, and a preparation of its hydrolysing enzyme, later named myrosinase. During the second half of the nineteenth and the beginning of the twentieth century, some studies on the chemical structure of thioglucosides appeared in the literature. Gadamer (10 - 12) proposed structural formulae for sinigrin and sinalbin in 1897. The common basis of these two structures was for many years used as the general structure of thioglucosides. Up to 1952 three thioglucosides and eight mustard oils with established structures had been recorded. In the following years a rapid progress was made in this field of study, largely because improved techniques such as paper chromatography became available. Ettlinger and Lundeen (13) revised Gadamer's structure in 1956, and their revised general structure

for thioglucosides has been accepted as the correct one. Up to 1960 (14) nine thioglucosides had been isolated in crystalline form and seven of these also as the acetyl derivatives. An additional six thioglucosides had been isolated only as the acetyl derivatives. Thirty isothiocyanates were known as the enzymatic fission products of naturally occurring thioglucosides. Although speculations about the biosynthesis of thioglucosides have appeared many times in the literature (15,16), it was not until 1962 that Wetter et al. (17) reported the first experimental study on the biosynthesis of a thioglucoside.

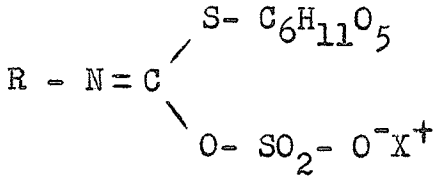
II. Elucidation of the General Structure of Thioglucosides.

Gadamer (lo - 12) in 1897 proposed structure (I) for sinigrin and sinalbin on basis of the following information:

(A) Will and Körner (18) had demonstrated in 1863 the formation of mainly allyl isothiocyanate, glucose and sulphate upon enzymatic decomposition of sinigrin.

(B) The general structure of isothiocyanates had been established as RNCS by Hofmann in 1868 (19).

(C) Gadamer found that reaction of one equivalent of silver nitrate with sinigrin or sinalbin resulted in the detachment of glucose and the appearance of a silver mercaptide which suggested that the glucose moiety was connected through a thioglucoside linkage in the original glucosides (10 - 12).



(I)

Sinigrin: R = CH_2= CH- CH_2 X= K

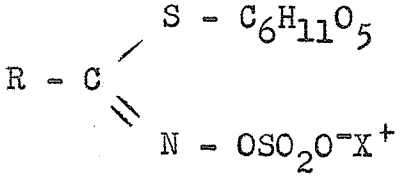
Sinalbin: R = (p)$HOC_6H_4CH_2$ X= Sinapine

Schneider and Wrede (20) later confirmed the presence of a thioglucoside linkage in sinigrin through reaction of the glucoside with potassium methoxide which yielded 1-thio-D-glucose. The configuration of the thioglucoside linkage was later determined by the same authors (21) as the β form in sinigrin and other thioglucosides.

It had long been known that enzymatic action on sinigrin and sinalbin produced, besides the main fission products, also smaller amounts of free sulphur, carbon disulphide and nitriles, which did not seem compatible with the formulae proposed by Gadamer. This led Ettlinger and Lundeen (13) to renewed investigations which on basis of the following evidence resulted in a revision of Gadamer's general structure for thioglucosides to (II):

(A) Hydrogenolysis of sinigrin with Raney nickel gave n-butylamine.

(B) Acid hydrolysis of sinigrin yielded vinylacetic acid and hydroxylamine.



(II)

All thioglucosides encountered thus far in nature are believed to have the same general structure as put forward by Ettlinger and Lundeen (13) for sinigrin. Thus the various thioglucosides differ only in the structure of the side chain and in the cation bound to the sulphate group.

The six thioglucosides known to occur in rapeseed have side chains and cations as shown in Table 1 (14).

TABLE 1

SIDE CHAINS (R) AND CATIONS (X^+) OF SIX THIOGLUCOSIDES FOUND IN RAPE SEED.

Thioglucosides	R-	X
Progoitrin	$CH_2=CHCH(OH)CH_2$-	Na
Gluconapin	$CH_2=CHCH_2CH_2$-	
Glucobrassicanapin	$CH_2=CHCH_2CH_2CH_2$-	
Glucoiberin	$CH_3SOCH_2CH_2CH_2$-	K
Sinalbin	$HOC_6H_4CH_2$-	Sinapine
Gluconasturtin	$C_6H_5CH_2CH_2$-	K

III. Preparation of Thioglucosides.

Thioglucosides have generally been difficult to isolate in crystalline form. Of more than thirty-five thioglucosides known to exist in plants, only nine have been brought to crystallisation. Among the nine crystalline thioglucosides only sinigrin, sinalbin and glucoiberin are considered to crystallize readily (14). Of the six thioglucosides known to occur in rape seed (1) gluconapin and glucobrassicanapin have not been obtained in pure crystalline form. Thioglucosides are readily extracted from plant materials with aqueous organic solvents such as methanol, ethanol and acetone. The solvent extracts usually contain impurities such as amino acids, tannins and pectins which interfere with crystallisation and should be removed. Some reported methods for purification of extracts of plant materials are briefly reviewed below:

For preparation of sinigrin from roots of horse radish (Cochlearia Armoracia L.) Stoll and Seebeck(22) removed sugars from the plant extract by fermentation followed by addition of calcium carbonate to precipitate the acid formed. The sugar-free solution was further purified by precipitation with lead acetate solution.

Schultz and Gmelin (23) isolated gluctropaeolin from

an extract of Lepidium sativum by employing adsorption chromatography on a column of cellulose powder with n-butanol: acetic acid: water as the developing solvent.

Electrophoresis with an acetic acid solution as buffer was employed by Schultz and Barthold (24) for purification of an extract of glucotropaeolin from Lepidium sativum. Although a pure thioglucoside fraction was not obtained by the electrophoretic procedure, a crystalline preparation of glucotropaeolin was obtained from the purified solution.

The electrophoretic behavior of glucotropaeolin led Schultz, Gmelin and Keller (25) to explore the applicability of anion exchangers for purification of thioglucosides contained in crude plant extracts. They found chromatography through various anion exchange columns to be very effective for this purpose. Lewatit M1, Amberlite IR-400 and Amberlite IR-4B in hydroxide, carbonate, acetate and chloride forms were examined, and the Amberlite IR-4B in chloride form was found the most suitable.

Schultz and Gmelin (26) later found that acid alumina functioned as an anion exchanger for thioglucosides and, further, that this adsorbent effected a good separation of thioglucosides from coloured material in the plant extracts. Schultz and Wagner (27) purified plant extracts

by lead acetate precipitation followed by chromatography on acid alumina, and other workers (28,29) have similarly with succes combined lead acetate precipitation and chromatography on anion exchange resins.

Greer (5) purified an extract of progoitrin from rutabaga seed (Brassica campestris L.) by chromatography on a column of alcohol-washed neutral alumina using 80 per cent alcohol as the eluting agent. Subsequent crystallisation of the progoitrin, however, occurred only after more than half a year. Greer (30) later isolated progoitrin more effectively with the use of Amberlite IR-4B in chloride form.

Counter-current distribution with a solvent system of n-butanol: water: pyridine was applied by Hietala (31) for fractionation of a mixture of thioglucosides. The crude extract had been previously chromatographed on ion exchange columns to remove amino acids and organic acids. While pure fractions of progoitrin and gluconapin were reported to have been obtained by this method, only progoitrin was obtained in crystalline form.

For isolation of sinigrin from an extract of horse radish leaves, Wetter et al. (17) employed a column of Amberlite IR-120 to remove amino acids and a column of Amberlite IR-4B to remove organic acids. The dimensions

of the latter column were such as to retain only organic acids and to allow sinigrin to pass through.

Nayar and Thorsteinson (6) found thin layer chromatography to effect a further purification of crude fractions of thioglucosides which had been previously chromatographed on neutral alumina.

Anion exchange resins have been used also for introducing cations such as sodium (30), tetramethylammonium (13) and rubidium (29) into thioglucosides.

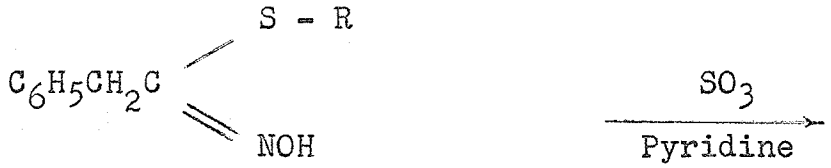
Schultz and Wagner (27) found that the poor crystallizing properties connected with some thioglucosides were greatly improved in the corresponding acetyl derivatives.

Besides isolation from natural sources thioglucosides may also be prepared synthetically. To date one thioglucoside has been synthesised. Ettlinger and Lundeen (16) synthesised glucotropaeolin by the following scheme:

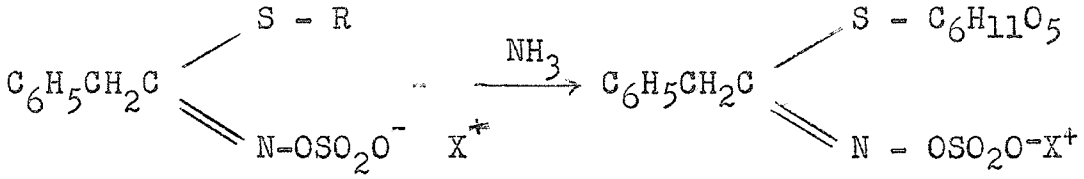
$$C_6H_5CH_2MgCl \xrightarrow{CS_2} C_6H_5CH_2CSS^- \xrightarrow{NH_3OH^+}$$

Benzyl magnesium chloride — Dithiophenyl acetate ion

Phenylaceto-thiohydroxamic acid



S-β-D-1-(Tetraacetyl glucopyranosyl)-phenylaceto-thiohydroxamic acid



X = $(CH_3)_4N$ Glucotropaeolin

R = Tetraacetyl-β-D-1-glucopyranosyl

Benn (32) used an alternative method to synthesize glucotropaeolin.

III Estimation of Thioglucosides.

Most of the available methods for determination of thioglucosides are based on the estimation of products formed by acid or enzymatic hydrolysis of the thioglucosides.

Acid hydrolysis of thioglucosides produces glucose, sulfate, carboxylic acids and free and substituted hydroxylamines. Quantitative yields are obtained of the former two products and these may be used for indirect determination of the thioglucosides. The latter two products are not produced in quantitative yields.

Enzymatic hydrolysis of thioglucosides gives rise to the three main products, glucose, sulfate and isothiocyanates, besides small amounts of byproducts such as carbon disulfide, elementary sulfur and nitriles (18). It is not certain at present whether any of the three main products may be obtained in quantitative yields.

Literature related to the estimation of thioglucosides through determinations of glucose, sulfate and isothiocyanates is briefly reviewed below:

(A) Determination of glucose.

Sandberg (33) determined the glucose content of an enzymatic hydrolysate of pure sinigrin by the method of Folin and Malmros (34), which is based on the reaction

of reducing sugar and ferricyanide to generate a blue color which is measured photometrically.

Nagashima and Uchiyama (35) determined the amounts of glucose produced during enzymatic hydrolysis of pure sinigrin by the use of 3,5-dinitrosalicylic acid according to Sumner (36). They pointed out an advantage with Sumner's method in that sinigrin exerted no reducing effect on the 3,5-dinitrosalicylic acid reagent, while it did have a reducing effect on the reagents used in other methods for determination of reducing sugars. In a similar study of enzymatic hydrolysis of sinigrin Reese et al. (37) obtained near quantitative yields of glucose as estimated by Sumner's method and they strongly recommended the determination of glucose rather than sulfate or isothiocyanates in enzymatic hydrolysates of thioglucosides.

Schultz and Gmelin (7) applied Dreywood's method (38) for determination of glucose and glucosides with anthrone reagent to the estimation of thioglucosides. Sulfuric acid contained in the reagent caused complete hydrolysis of the thioglucosides during heating and the glucose thus produced reacted with anthrone to form a blue color which was measured photometrically. For determination of the thioglucoside content in a plant material, they first purified the plant extract by chromatography on paper or on a column of acid alumina.

(B) Determination of sulfate.

Gadamer (39) determined the amount of sinigrin in solutions by hydrolysis with hydrochloric acid followed by precipitation of the sulfate as barium sulfate which was determined gravimetrically.

Sandberg and Holly (33) employed Hubbard's method (40) to precipitate sulfate with benzidine from a hydrolysate of sinigrin. The benzidine sulfate was subsequently determined colorimetrically. Alternatively, benzidine sulfate obtained as above has been determined titrimetrically by Nagashima and Uchiyama (35) by a modification of McKittrick and Smith's titrimetric method (41).

(C) Determination of isothiocyanates.

Schmid and Karrer (42) estimated isothiocyanates by reaction with an excess ammoniacal silver nitrate solution to form silver sulphide. The unreacted silver nitrate was determined by titration with potassium thiocyanate solution with ferric ammonium sulfate as indicator. Stoll and Jucker (43) in a similar study isolated the precipitated silver sulphide and determined it gravimetrically.

Astwood et al. (44) reported a method for spectrophotometric determination of the cyclic compound, 5-vinyl-2-thiooxazolidone, which is formed by spontaneous rearrangement of the isothiocyanate released from progoitrin by

enzymatic hydrolysis.

Wetter (45) reported a method, essentially as by Schmid and Karrer (42), for estimation of volatile isothiocyanates released from rape seed by enzymatic hydrolysis. He further used Astwood's method (44) for determination of the nonvolatile thiooxazolidone remaining in the sample after removal of isothiocyanates by steam distillation (46).

Kjaer (47) determined isothiocyanates by reaction with ammonium hydroxide to form thioureas which were subsequently determined spectrophotometrically.