phenazine compounds (Levitch and Stadtman, 1964; Millican, 1962; MacDonald, 1963).

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(*S*)- and (*R*)-1-Cyano-2-hydroxy-3-butene from Myrosinase Hydrolysis of *epi*-Progoitrin and Progoitrin*

M. E. Daxenbichler, C. H. VanEtten, and I. A. Wolff

ABSTRACT: Enzymatic hydrolysis of *epi*-progoitrin, the major thioglucoside from crambe seed, at pH 3.0 produced the previously characterized (*R*)-goitrin and an unknown nitrile. The nitrile was isolated and shown to be (*S*)-1-cyano-2-hydroxy-3-butene. Measurements were obtained for the yield of (*R*)-goitrin and (*S*)-1-cyano-2-hydroxy-3-butene as a function of the pH during the hydrolysis of *epi*-progoitrin. Hydrolysis of progoitrin from rutabaga seed formed the enantiomeric (*S*)-goitrin and (*R*)-1-cyano-2-hydroxy-3-butene. In the Cahn–Ingold–Prelog system *epi*-progoitrin has the (*S*) configuration at the asymmetric carbon atom of the aglycon and may, therefore, also be designated as a salt of 2-(*S*)-hydroxy-3-butenylglucosinolate.

The major thioglucoside in the seed of *Crambe abyssinica* Hochst ex R. E. Fries is *epi*-progoitrin[1] (Ia) (Daxenbichler *et al.*, 1965). Its structure was established in part through characterization of (*R*)-5-vinyloxazolidine-2-thione [(*R*)-goitrin] (IIIa) as the product obtained by mustard myrosinase hydrolysis of the thioglucoside at near neutral pH. In the course of our work, we observed that the yield of (*R*)-goitrin from *epi*-progoitrin by myrosinase hydrolysis at pH 3 was poor although other data showed complete hydrolysis of the thioglucoside. From early literature, as well as from recent reports by Virtanen and Saarivirta (1962) and Schwimmer (1960), it was presumed that a nitrile might be formed in addition to (*R*)-goitrin when the enzymatic hydrolysis of *epi*-progoitrin was carried out at an acidic pH. We have now characterized the nitrile as (*S*)-1-cyano-2-hydroxy-3-butene (IIa), and report here the amounts of IIa formed under different conditions of myrosinase hydrolysis of *epi*-progoitrin. The enantiomeric (*R*)-1-cyano-2-hydroxy-3-butene (IIb) from progoitrin (Ib) of rutabaga seed was also isolated and characterized.

Stereochemical interrelationships among the thioglucosides (I) and their derived nitriles (II) and oxazolidinethiones (III) are shown below. The reasonable assumption is made that no inversion of configuration occurs in these reactions at the asymmetric center of the aglycon. Greer (1956) and Astwood *et al.* (1949) showed the derivation of goitrin from progoitrin obtained from seeds of the *Brassica* genus. This goitrin was later assigned the absolute configura-

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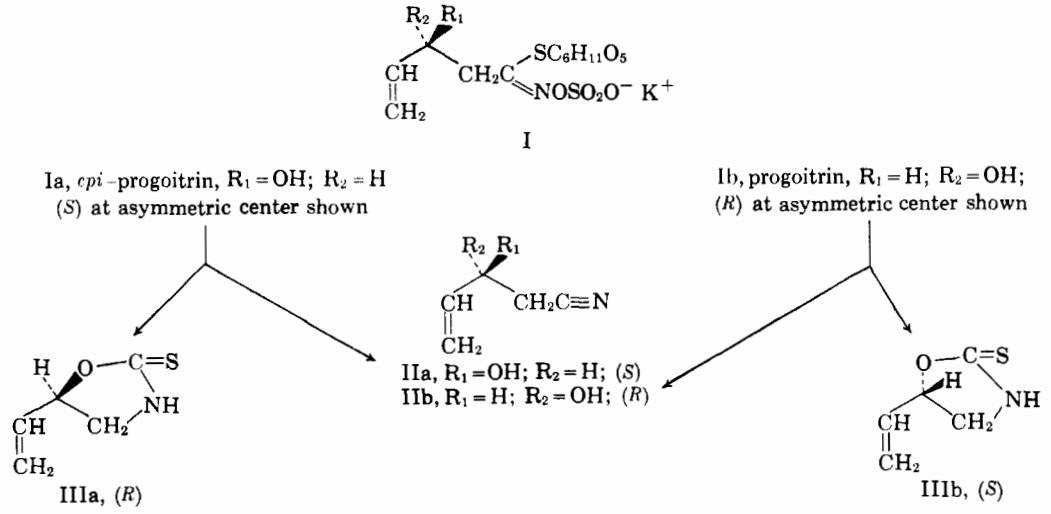
[1] Authors regret earlier erroneous use of (*R*) in referring to the *epi*-progoitrin molecule (Daxenbichler *et al.*, 1965).



I

tion (*S*) by Kjaer *et al.* (1959). The term *epi*-progoitrin, which we assign to the major crambe thioglucoside, shows its relationship to progoitrin. Presumption is that anomeric configuration at the glucosidic linkage is the same in the two thioglucoside compounds, since they are acted upon by the same enzyme systems; this has not, however, been rigorously proved.

Experimental Section

Preliminary Detection of Nitrile and Identification. Enzymatic hydrolyses of *epi*-progoitrin (estimated 90% purity) were carried out in a pH range from 2.9 to 7.5, and the amount of (*R*)-goitrin that formed in each hydrolysate was estimated. The hydrolysates were then analyzed for nitriles by modification of a method reported by Whitehurst and Johnson (1958), in which the ammonia formed from nitriles on boiling with alkali was distilled and titrated. The method was adapted to microsamples and hydrogen peroxide was not added to the alkali. Under conditions of the analysis only a trace of ammonia was obtained from (*R*)-goitrin and *epi*-progoitrin. These preliminary data indicated that from pH 5.5 to 3.0 nitrile formation increased with increased acidity while (*R*)-goitrin formation decreased.

Titration of sulfate ion liberated by enzyme activity by the method of VanEtten *et al.* (1965) showed no enzyme activity at pH 2.3 but quantitative thioglucoside hydrolysis at pH 3.0 in 24 hr at room temperature. Descending paper chromatography (Whatman[2] No. 1 filter paper with the upper phase of 1-butanol–ethanol–water (4:1:4) with alkaline silver nitrate detection reagent) on the pH 3 hydrolysate showed no *epi*-progoitrin and a large spot having an R_F identical with that of glucose. A chloroform extract was made of a pH 3 hydrolysate and the solvent removed to leave an oil. Thin layer chromatography (tlc)[3] of the oil on silica gel G, with ether–petroleum ether (3:1) used as the developing solvent and iodine vapor for detection, showed two major components. The slower migrating component moved at the same rate as (*R*)-goitrin. A small amount of the faster migrating component was prepared by removal of the appropriate area from several plates on which the material was chromatographed. An infrared curve of the component isolated in this manner showed typical bands for hydroxyl, nitrile, and terminal olefinic unsaturation. Carbon and hydrogen analyses supported the infrared data in tentative identification of the compound as 1-cyano-2-hydroxy-3-butene.

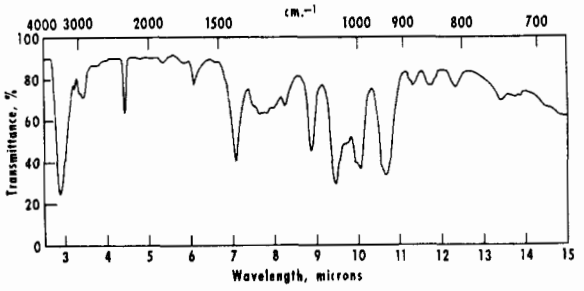


FIGURE 1: Infrared spectrum of 1-cyano-2-hydroxy-3-butene from *epi*-progoitrin. From film on sodium chloride.

Preparation and Identification of IIa. To 400-ml of 0.4 M glycine (adjusted to pH 3.1 with 0.4 M HCl) was added 5.0 g of crude crambe thioglucoside prepared as described by Daxenbichler *et al.* (1965) of 89% purity compared to *epi*-progoitrin as determined by the amount of (*R*)-goitrin that could be measured after hydrolysis. Myrosinase (400 mg) was added to the buffered thioglucoside solution and it was allowed to stand at room temperature. The myrosinase was prepared from white mustard seed according to Wrede (1941); at the final stage of the myrosinase preparation the product was lyophilized. After 24 hr (pH 2.9) the solids that precipitated in the hydrolysate were separated by centrifug-

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

[3] Abbreviations used in this work: tlc, thin layer chromatography; nmr, nuclear magnetic resonance.

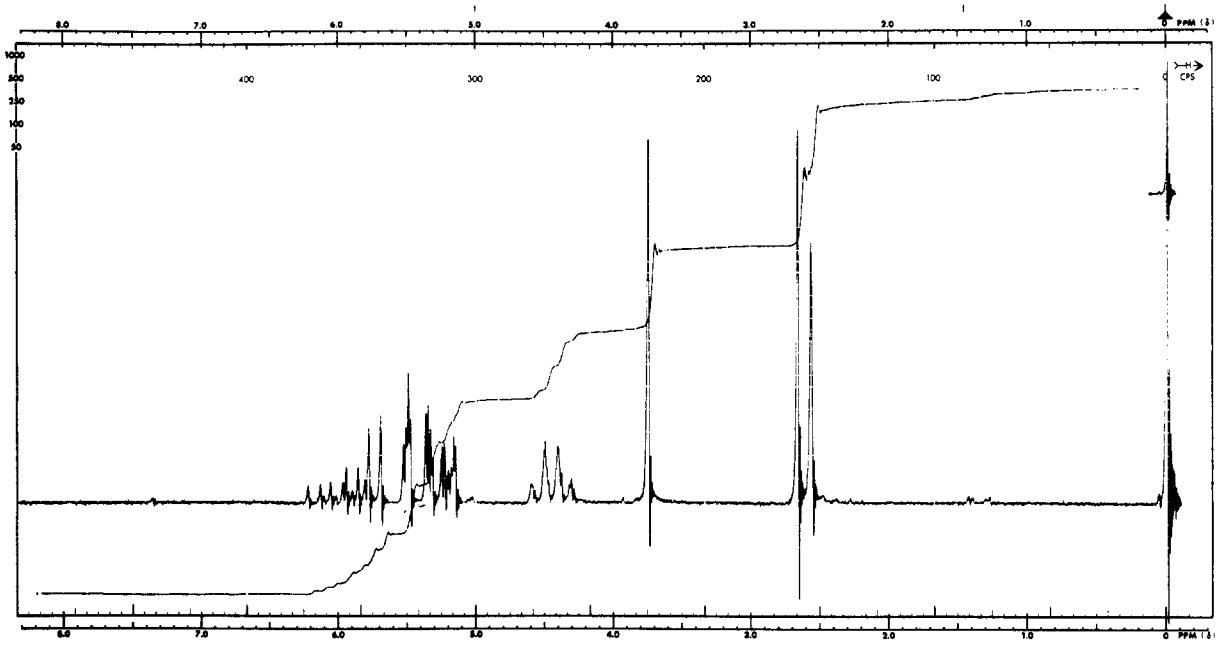


FIGURE 2: Nmr spectrum of 1-cyano-2-hydroxy-3-butene from *epi*-progoitrin in $CDCl_3$; internal standard, $(CH_3)_4Si$.

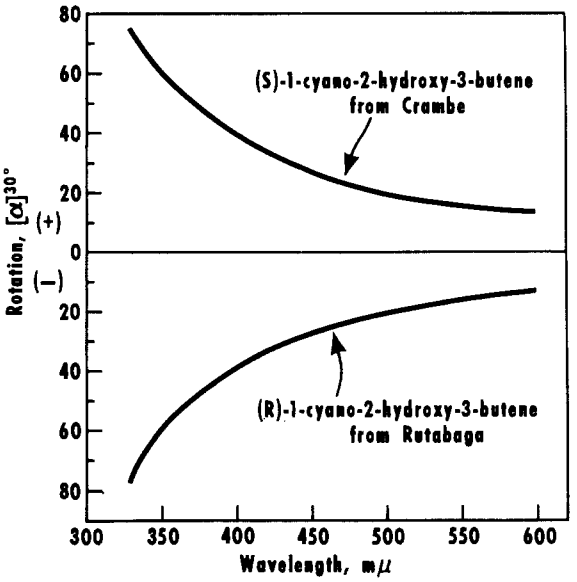


FIGURE 3: Rotatory dispersion curves. (upper) (+)-1-Cyano-2-hydroxy-3-butene (*c* 1.13, MeOH); (lower) (−)-1-cyano-2-hydroxy-3-butene (*c*, 1.04, MeOH).

ing. The turbid supernatant was clarified by filtration through analytical filter aid Celite (Johns Manville). The filtrate was extracted three times with chloroform (two–three volumes each extraction), and the combined extracts were dried over sodium sulfate. After filtering off the sodium sulfate, the extract was concentrated to 0.75 g of an oil.

A column (2 cm × 53 cm) prepared from 100 g of silica gel 60–100 mesh (Adsorbosil CAB6010; Applied Science Laboratories) was washed with 250 ml of ether–petroleum ether (3:1). The oil from the concentrated extract was applied to the column followed by 150–200 ml of ether–petroleum ether (1:1). The column was eluted with ether–petroleum ether (3:1) until 24 20-ml fractions were collected. The fractions from the column were concentrated to near dryness and evaluated by tlc. On the basis of tlc, fractions 12–19 were pure nitrile. These were dissolved in small volumes of ether and combined. The solvent was removed with a stream of nitrogen to give a residual oil dried under vacuum to constant weight of 0.46 g; $n^{20}D$ 1.4544, lit. (Bissinger *et al.*, 1947) for synthetic racemate $n^{20}D$ 1.4559.

Anal. Calcd for C_5H_7NO (97): C, 61.9; H, 7.2; N, 14.4; hydroxyl, 17.5; S, 0.0. Found: C, 61.4; H, 7.5; N, 14.3; hydroxyl, 17.7; S, 0.2.

An infrared spectrum of the oil was obtained as a film between sodium chloride disks (Figure 1). The spectrum included absorption at wavelengths (in μ) as follows: 2.9 (hydroxyl), 4.4 (nitrile), 6.1, 7.1, 10.1, and 10.7 (terminal olefinic unsaturation). An nmr (nuclear magnetic resonance) spectrum (Figure 2) of the compound in 25% concentration in deuteriochloroform showed an absorption pattern typical for vinyl group protons at δ 5.1–6.3, a quartet (J = 6 cps) for the β-proton at about δ 4.5, a peak for the hydroxyl proton at δ 3.8, and a doublet (J = 6 cps) for the methylene protons at δ 2.6. This spectrum was, therefore, in complete accord with the structure 1-cyano-2-hydroxy-3-butene (IIa and b).

The α-naphthylurethan derivative of the compound was obtained in poor yield. The nitrile (100 mg) weighed into a flask fitted with a microcondenser and drying

tube was dissolved in 3 ml of benzene to which was added 145 mg of α-naphthyl isocyanate. Dicyclohexylethylamine (10–20 mg) was added as catalyst and the mixture refluxed 15 min. Some dinaphthylurea that formed was filtered from the hot solution. Normal heptane was added to the filtrate and the flask was stored in the refrigerator for several days. After again heating the material, some additional urea was removed by filtering the hot solution. Crystals of the urethan derivative formed in the filtrate; yield 23 mg, mp 92°. After recrystallization from benzene–*n*-heptane the yield was 14 mg, mp 93°. Under a polarizing microscope equipped with a hot stage the material lost birefringence at 93–93.5°.

Anal. Calcd for $C_{16}H_{14}N_2O_2$ (266): C, 72.2; H, 5.3; N, 10.5. Found: C, 72.9; H, 5.5; N, 10.5.

Preparation of IIb. Crude progoitrin was prepared from seed of *Brassica napobrassica* Mill (rutabaga) in the same manner as was the *epi*-progoitrin from crambe seed. Myrosinase hydrolysis of 5 g of rutabaga thioglucoside at pH 3.0 gave a yield of 0.68 g of product which assayed 70% nitrile based on infrared measurements and 23% (*S*)-goitrin by ultraviolet analysis. By silica gel column separation 64% of the crude product was obtained as the nitrile. Infrared spectrum as a film on sodium chloride was identical with that of the nitrile from crambe.

Anal. Calcd for C_5H_7NO (97): C, 61.9; H, 7.2; N, 14.4. Found: C, 61.5; H, 7.3; N, 14.2; $n^{20}D$, 1.4543.

The α-naphthylurethan derivative was prepared from 100 mg of the starting nitrile. The crude isolate (48 mg), mp 82–87°, after four recrystallizations gave white crystals, which showed loss of birefringence at 89–92°.

Preparation of Racemic α-Naphthylurethan Derivative. Approximately equal amounts of the two urethan derivatives of the nitriles were mixed on a slide and melted. The temperature was allowed to rise to about 115°. The material recrystallized on the slide after standing overnight. When remelted under a polarizing microscope on a hot stage, the apparently racemic compound lost birefringence at 109–112°. According to Bissinger *et al.* (1947), the synthetic racemate melts at 112.5–113.5°.

Optical Rotatory Dispersion Measurements on the Nitriles. Measurements from 330 to 600 mμ (Figure 3) were obtained at 30° for the nitriles from crambe and from rutabaga in methanol at concentrations of 1.13 and 1.04%, respectively, with a 5-cm cell.

Quantitation of Infrared Measurement of Nitrile. The nitrile band (4.43 μ) was measured at six concentrations of the isolated crambe nitrile (1.024–32.78 mg/ml) in spectral quality chloroform with a 0.925-mm cell path. Chloroform was used in the reference cell to cancel a slight absorption near 4.4 μ due to the solvent. The net absorption from the compound was obtained by subtracting the minimum absorption near 4.5 μ from the peak absorption. The net absorptions for the concentrations measured ranged from 0.023 to 0.775 OD units. Concentrations plotted against net absorptions produced a straight line that passed through the origin. The specific absorptivity (Infrared Spectroscopy Committee, 1961) was calculated as 0.253. This information gave a convenient means of estimating the nitrile. However, the method is subject to error if other compounds containing C≡N groups are present.

pH Study. PREPARATION OF HYDROLYSATES. The same amount of thioglucoside (750 mg) and of enzyme (60 mg) and the same volume of buffer (60 ml) were used in all studies on the effect of pH on the end products from myrosinase hydrolysis. The hydrolyzing solutions stood for 24 hr at room temperature except the one at pH 2.3 which stood for 3 days. The buffer systems used were: glycine–hydrochloric acid, pH 2.3, 3.0 (for nitrile isolation); citric acid–dibasic sodium phosphate, pH 2.8, 3.3, 3.7, 4.2, 4.7, 5.9, 7.1; citric acid–sodium citrate, pH 3.1, 3.9, 5.4; acetic acid–sodium acetate, pH 4.8; monobasic potassium phosphate–sodium hydroxide, pH 7.2; boric acid–sodium borate, pH 8.0; boric acid–sodium hydroxide, pH 8.4, 9.2; sodium carbonate–sodium bicarbonate, pH 9.7; glycine–sodium hydroxide, pH 8.5, 9.9, 10.8, 12.0. Most of the buffer systems were prepared as described by Gomori (1955); except to increase the buffer capacity, the molarities of the solutions described were doubled. The monobasic potassium phosphate–sodium hydroxide buffer was prepared as described in Lange's Handbook (1961). Hydrolyses at the two pH extremes (2.3 and 12.0) were prepared by adjusting 0.4 M glycine to the desired pH value with either sodium hydroxide or hydrochloric acid.

WORK-UP OF PRODUCTS. Hydrolysates that were turbid or contained solids were centrifuged or filtered through Celite, or both, to clarify them before extraction with chloroform. The pH 12.0 hydrolysate was adjusted to near pH 7 just before extraction. The remaining hydrolysates were extracted at the final pH of the hydrolysate. The hydrolysates were extracted three times with two–three volumes of chloroform for each extraction. The alkaline hydrolysates when extracted with chloroform tended to form emulsions, which may have introduced some error in the product yields. The combined extracts from each hydrolysate were dried over sodium sulfate overnight. After filtering off the sodium sulfate, most of the solvent was removed under reduced pressure at 45°, and the resulting oils were taken to constant weight while purged with nitrogen.

ASSAY OF PRODUCTS FOR GOITRIN AND NITRILE. A weight of each product was dissolved in ethanol and the amount of (*R*)-goitrin present was determined by the absorption obtained at 244 mμ. The absorption at 244 mμ when multiplied by the factor 7.5 expresses the amount of goitrin in micrograms per milliliter. Our measurements of purified (*R*)- and (*S*)-goitrin in ethanol at 244 mμ agreed with the extinction coefficient reported by Kreula and Kiesvaara (1959), log ε 4.23 (H_2O, 240 mμ). No correction for nonspecific absorption was applied to the glucoside hydrolysis products in these assays because the products produced absorption in the range 220–280 mμ similar to the pure compound.

The amount of nitrile present in each product was

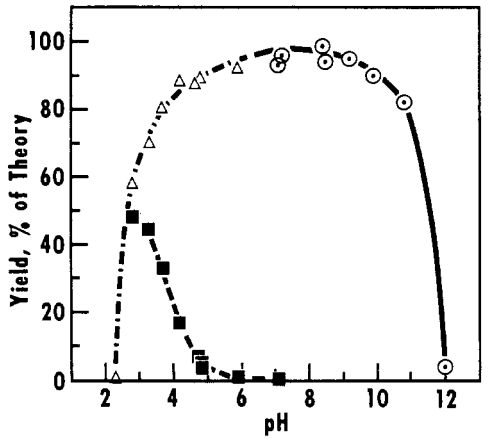


FIGURE 4: Yield of (*S*)-1-cyano-2-hydroxy-3-butene and of (*R*)-goitrin from *epi*-progoitrin *vs.* pH of myrosinase hydrolysis reaction: Δ, nitrile + (*R*)-goitrin; ⊙, (*R*) goitrin; ■, nitrile.

determined by dissolving a weighed amount in a small volume of spectroquality chloroform. The band near 4.4 μ was measured and the amount of nitrile was calculated as discussed above. At pH 5–7 where the minimum values for nitrile were obtained, as much as 167 mg of product/ml of chloroform was used for the measurement.

Uncorrected melting points were obtained with a polarizing microscope equipped with a hot-stage melting point apparatus. Instruments used for physical measurements were for ultraviolet, a Beckman Model DK-2A spectrophotometer; for infrared, a Perkin-Elmer Corp. Model 137B or Model 337; for nmr, a Varian Associates Model-A60 nmr spectrometer; and for rotatory dispersion measurements, a Cary Model 60 recording spectropolarimeter.

Results

The elemental analysis, infrared spectrum, nmr spectrum, refractive index, and optical rotation showed that the nitrile formed from *epi*-progoitrin by myrosinase hydrolysis at pH 3 was (*S*)-1-cyano-2-hydroxy-3-butene (IIa). Data on the isolate from myrosinase hydrolysis of progoitrin at pH 3 showed that a compound of like structure but equal and opposite rotation was formed. Optical rotatory dispersion curves of the nitriles (Figure 3) showed that they are enantiomers. Preparation of the crystalline α-naphthylurethan derivatives from the nitriles obtained from both progoitrin and from *epi*-progoitrin permitted determination of a mixture melting point of these two derivatives. Agreement of the mixture melting point with that reported for the synthetic racemate by Bissinger *et al.* (1947), which in this case was higher than either enantiomer, was good confirming evidence for their identity as optical antipodes.

Mustard myrosinase hydrolysis of purified *epi*-progoitrin as a function of pH is shown in Figure 4. As measured by titration of the sulfate ion liberated, less than 5% hydrolysis occurred at pH 2.3 and at pH 12 on standing for 24 hr. At pH values between these extremes the sulfate titration showed hydrolysis to be complete within experimental error of the method. Enzymatic hydrolysis occurs over a wide pH range under the conditions studied, favoring (*R*)-goitrin completely at pH 7 and above and a mixture of (*R*)-goitrin and (*S*)-1-cyano-2-hydroxy-3-butene at lower pH values. The greater the amount of nitrile obtained the lower was the combined yield of products. This result shows that under the conditions of hydrolysis at low pH, unidentified substance(s) are also produced from the aglycone. When the nitrile is formed, a precipitate containing large amounts of sulfur is also produced. Quantitation of infrared absorption of 1-cyano-2-hydroxy-3-butene at 4.43 μ, when the pure compound was used as a standard, provided a means of estimating the nitrile in this investigation.

Discussion

As part of the evidence for characterization of *epi*-progoitrin, a product from the mineral acid hydrolysis of the glucoside was identified as 2,4-pentadienoic acid (Daxenbichler *et al.*, 1965). It was assumed that the dienoic acid arose through dehydration and hydrolysis of an intermediate β-hydroxynitrile. By another route this nitrile has now been isolated. Ample evidence has been presented that the aglycon of *epi*-progoitrin contains a hydroxyl function which enters into the formation of an oxazolidinethione [(*R*)-goitrin] in the same manner as the formation of other oxazolidinethiones as discussed by Kjaer (1960). However, this is the first instance in which a derivative of the aglycon portion of an oxazolidinethione-forming mustard oil glucoside has been isolated with the hydroxyl function intact. Isolation of a nitrile as a product of enzymatic hydrolysis of a thioglucoside is in accordance with the recently assigned structure of plant thioglucosides established by synthesis of glucotropaeolin by Ettlinger and Lundeen (1957).

Based on work by Greer (1962) and by Greer and Deeney (1959), one would expect both (*R*)-goitrin and *epi*-progoitrin to have antithyroid activity. The toxicity of (*S*)- or (*R*)-1-cyano-2-hydroxy-3-butene has not been tested. However, since cyanohydrins and unsaturated nitriles are toxic (Mowry, 1948) toxicity tests should be made. Since the hydroxy nitriles are formed *in vitro* from isolated thioglucosides, their formation might also be expected by endogenous enzyme hydrolysis when the seed including the enzyme and the thioglucosides is eaten. Rat feeding of crambe meal at high levels causes early death of the animals (VanEtten *et al.*, 1965), which result strongly suggests that deleterious substances other than (*R*)-goitrin are present. Current study of the end products formed from endogenous enzyme hydrolysis of the thioglucosides in the presence of the defatted seed meal shows the end products to be more complex and varied than in the isolated system reported here and to be affected by variables in addition to the pH of

the system during hydrolysis. This work will be reported in a subsequent paper.

Acknowledgments

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The Hydrolysis of Piperazine-2,5-dione*

Brian D. Sykes, E. Brock Robertson, H. B. Dunford, and Dennis Konasewich

ABSTRACT: The hydrolysis of piperazine-2,5-dione or diketopiperazine to glycylglycine was studied in 0.1 M HCl and 0.1 M NaOH over a range of temperatures. The values of ΔH^* and ΔS^* were found to be 20.6 kcal/mole and -14.7 cal deg^{-1} mole^{-1}, respectively, in acid, and 8.7 kcal/mole and -38.5 cal deg^{-1} mole^{-1}, respectively, in base. No ^{18}O exchange could be detected in proton-catalyzed hydrolyses at 111°. Arguments are presented which indicate that the rate-controlling step in the proton-catalyzed hydrolysis may be ring opening which is concerted with water addition. In the hydroxyl ion catalyzed hydrolysis, the rate-controlling step appears to be OH^- addition to the carbonyl group.

The results of some nuclear magnetic resonance (nmr) experiments are consistent either with a planar structure or rapidly flipping boat structure for diketopiperazine in aqueous solution. Whether acetylglycine or diketopiperazine is a better model for an infinitely long polyglycine chain is discussed.

Piperazine-2,5-dione, commonly called diketopiperazine (DKP),[1] has a ring structure which contains two peptide linkages (Figure 1). It has peculiar solubility properties, that in although it contains highly polar groups, it does not have a high solubility in polar solvents. It exhibits the expected low solubility in solvents of low polarity. The formol titration (Edward and Meacock, 1957) was used to follow the rate of

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[1] Abbreviations used in this work: DKP, diketopiperazine or piperazine-2,5-dione; GG, glycylglycine; nmr, nuclear magnetic resonance.