ability (Stahmann and Woldegiorgis, 1975). The high values of pepsin pancreatin released lysine for the pepsin-hydrolyzed gluten samples enriched using N^{ϵ}-benzylidenelysine are comparable to or higher than the values for the whole egg and isoelectric casein standards. These results offer potential use of these high lysine gluten samples as good quality proteins.

These preliminary tests thus indicate good digestibility and availability of the lysine-enriched gluten samples, particularly those prepared with pepsin hydrolyzed gluten and N^{ϵ}-benzylidenelysine. It is possible that isopeptide links, which were not measured by these in vitro analyses, may be nutritionally available in the body. For example, ϵ-(γ-glutamyl)-L-lysine and ϵ-(α-glutamyl)-L-lysine have both been found to be available as lysine sources for the rat (Mauron, 1972). N^{ϵ}-Benzylidenelysine has also been shown to be virtually 100% utilized as a lysine source during growth trials on rats (Finot et al., 1977a). Finot et al. (1977b) tested various N-substituted lysine derivatives for their growth-promoting effect in rat assays. ϵ-N-(α-L-aminoacyl), α-N-ϵ-N-di-L-aminoacyl, and Schiff base type derivatives were all utilized efficiently as lysine sources for the rat. None of the α-N-acyl- and ϵ-N-acylglycyl derivatives were utilized at all. ϵ-N-Acyl and ϵ-N-(ω-L-aminoacyl) derivatives were generally not utilized, with the exception of ϵ-N-(γ-L-glutamyl)lysine and ϵ-N-formyl- and ϵ-N-acetyllysine. Kornguth et al. (1963) observed that papain, chymotrypsin, trypsin, leucine amino peptidase, and pronase did not attack the ϵ-(γ-glutamyl)lysine. However, this dipeptide is probably absorbed into the intestinal wall and hydrolyzed in the kidney since it can be found in the plasma of rats and chicks fed it in their diet (Waibel and Carpenter, 1972), and an ϵ-lysine acylase has been suggested to function in the mammalian kidney (Leclerc and Benoiton, 1968).

In vivo digestibility tests and animal feeding studies are recommended to check nutritional availability and to establish the absence of any toxic substances in the lysine-linked gluten. Investigation is also warranted on the feasibility and benefits of using the carbodiimide reaction for analogous attachment of threonine for further nutritional improvement of wheat gluten.

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Reactions of Hymenoxon: Base Conversion to Psilotropin and Greenein and Formation of "Michael Adduct" with Cysteine

Dennis W. Hill and Bennie J. Camp*

Studies on the reaction of hymenoxon in alkaline solution indicated that at pH 10–12 hymenoxon was converted to intermediates that formed psilotropin and greenein when the reaction medium was adjusted to pH 1. Hymenoxon was shown to react with the sulfhydryl group of cysteine. The second-order rate constant for the reaction was 504 L mol^{-1} min^{-1}.

Hymenoxon is a toxic α-methylene sesquiterpene lactone that has been identified as a component of *Hymenoxys odorata* (Kim et al., 1975; Ivie et al., 1975) and *Helenium hoopsii* and *Baileye multiradiata* (Hill et al., 1977). Under basic conditions, hymenoxon can be converted to isomeric dilactones, psilotropin, and greenein. This reaction has been used to confirm the structure of hymenoxon (Kim et al., 1975) and to produce derivatives that aid in the rapid screening of plants for the presence of hymenoxon (Hill

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et al., 1977). Plants that contain hymenoxon are toxic to sheep and other livestock (Kingsbury, 1964; Sperry et al., 1964; Rowe et al., 1973; Lewis and Dollahite, 1960).

Sesquiterpene lactones structurally related to hymenoxon have been isolated from composite plants. The toxicity of sesquiterpene lactones to animals has not been studied; however, similar lactones of plant origin have cytotoxicological action against tumors which is attributed to their reaction with the sulfhydryl groups of phosphofructokinase resulting in inhibition of the enzyme (Hanson and Lardy, 1970). Kupchan et al. (1971) observed that sesquiterpene lactones containing the α-exocyclic methylene group reacted with cysteine. The sulfhydryl group of cysteine formed a "Michael Adduct" with the methylene group of the lactone. A peri OH or O-acyl group to the methylene structure enhanced the rate of cysteine addition. However, the reaction rate was not a function of the cytotoxicity of the lactone. Sesquiterpene α-methylene lactones will react with other sulfhydryl containing compounds (Kupchan et al., 1970a).

In the present investigation, the effect of hydrogen ion concentration on the conversion of hymenoxon to greenein and psilotropin and the reaction mechanism of hymenoxon with cysteine were studied.

EXPERIMENTAL SECTION

Chemicals. Hymenoxon and psilotropin were prepared as previously described (Hill et al., 1977). Greenein was obtained from Dr. H. L. Kim, College Station, Texas. Flavone was obtained from K&K Laboratories, Inc., and cysteine hydrochloride was obtained from Fisher Scientific.

Instrumentation. Hymenoxon, greenein, and psilotropin were analyzed on a Barber-Colman gas chromatograph (GC) equipped with a 6 ft × $^1/_6$ in. glass column packed with 3% OV-17 on Gas-Chrom A (100–120 mesh). The operating temperatures were: column, 210 °C; injector, 235 °C; and flame ionization detector (FID), 250 °C. Nitrogen was the carrier gas at a flow rate of 45 mL/min.

pH Requirements for the Conversion of Hymenoxon to Psilotropin and Greenein. Ten milliliters of a 0.1 M phosphate buffer of pH 8.0, 9.0, 10.0, 11.0, or 12.0 was added to a series of glass-stoppered tubes containing 5 mg of hymenoxon. The pH of the contents of each tube was determined with a Beckman model Century SS expanded scale pH meter, and then the solutions stood at room temperature for 12 h. Duplicate, 1.0-mL aliquots were made acidic (pH <1) with 1.5 mL of 2 N HCl, and the sesquiterpene lactones were extracted from each tube with 3.0 mL of ethyl acetate containing 48.6 μg of flavone/mL. One milliliter of the ethyl acetate extract was evaporated to dryness under a stream of air. The residue was dissolved in 0.25 mL of ethyl acetate and analyzed on the GC.

Standard curves for hymenoxon and psilotropin were prepared from aqueous solutions of hymenoxon (0.150, 0.400, and 0.500 mg/mL) and psilotropin (0.125, 0.250, 0.375, and 0.500 mg/mL). One milliliter of each standard was mixed with 1.5 mL of 2 N HCl, and the solution was extracted with 3.0 mL of ethyl acetate containing 48.6 μg of flavone/mL. One milliliter of the extract was evaporated to dryness and the residue was dissolved in 0.25 mL of ethyl acetate. Two microliters of the concentrated solution was analyzed on the GC. The peak height of each lactone peak was divided by the peak height of the internal standard peak to give the relative peak height (RPH). The linear regression equation of RPH vs. concentration for each standard was calculated. The concentration of greenein was determined by using the regression equation for psilotropin and assuming equivalent detector response for the two isomers.

Reaction of Hymenoxon with Cysteine. The rate constant for the reaction between hymenoxon and cysteine was determined by mixing an excess quantity of hymenoxon with cysteine and measuring the decrease in hymenoxon concentration with time. A solution of cysteine (4.66 μmol/mL) was prepared in 0.15 M phosphate buffer (pH 7.4). The buffer was prepared with deionized and degassed distilled water. The cysteine solution was stored under nitrogen and used within 4 h.

One milliliter of the cysteine solution was added to a series of glass-stoppered test tubes and held under nitrogen during the reaction period. While mixing the cysteine solution on a vortex mixer, a 11.57 μmol/mL solution of hymenoxon in ethanol was added rapidly by blowing the solution from a 0.5-mL Oswald-Folin pipet that was calibrated to deliver 0.492 mL of ethanol. The reaction proceeded at room temperature for 0.5, 1.0, or 2.0 min and was terminated by the addition of solid Hg_2Cl_2. The reaction rate was measured in triplicate for each time period. A control mixture was prepared by adding solid Hg_2Cl_2 to the cysteine solution before the addition of hymenoxon. Following the addition of Hg_2Cl_2, 2 mL of 0.289 mg/mL of flavone in acetone was added to each test tube. A 2.0-μL aliquot of the solution was analyzed for hymenoxon on the GC. A standard curve for the assay was prepared by diluting the 11.57 μmol/mL hymenoxon solution to 2.0 with pH 7.4, 0.15 M phosphate buffer to give final concentrations of 8.66, 5.78, 2.89, 1.44, and 0.72 μmol/mL, respectively. Duplicate 0.5-mL aliquots of each standard were mixed with 1.0 mL pH 7.4, 0.15 M phosphate buffer and 2.0 mL of 0.289 mg/mL flavone solution. A 2.0-μL sample was analyzed on the GC. The linear regression equation of the RPH vs. mg/mL standard was calculated and the equation was used to calculate the concentration of hymenoxon in the reaction mixtures.

The carbon–sulfur bond between hymenoxon and cysteine was cleaved by a modification of the procedure of Kupchan et al. (1970b). A 1.0-mL aliquot of a 0.800 μmol/mL, pH 7.4 solution of the cysteine–hymenoxon complex was mixed with 0.5 mL of CH_3I and heated at 80 °C for 1 h. Upon cooling, 1 mL of 2 N NaOH was added and the mixture stood at room temperature for 30 min. The solution was made acidic (pH <1) by the addition of 2 mL of 2 N HCl and then extracted with 6.0 mL of ethyl acetate. The extract was assayed for psilotropin by GC. A control reaction was prepared by mixing 1.0 mL of 0.226 mg/mL aqueous solution of hymenoxon with 1.0 mL of 2 N NaOH and allowed to stand for 30 min. The solution was acidified with 2.0 mL of 2 N HCl, extracted with ethyl acetate, and assayed for psilotropin.

RESULTS AND DISCUSSION

Effect of pH on the Conversion of Hymenoxon to Psilotropin and Greenein. Figure 1 presents the effects of pH on the formation of psilotropin and greenein from hymenoxon. The total concentration of psilotropin and greenein was 1.87 mol/mL at pH 12.7. This concentration was equivalent to the assayed concentration of hymenoxon at pH 8.1. At pH 12.7, the ratio of psilotropin to greenein was 5.0.

The conversion of hymenoxon to intermediates of psilotropin and greenein was observed initially at pH 10 with maximum conversion at pH 12. The formation of psilotropin and greenein did not occur until the alkaline solutions (pH >10) were adjusted to pH 1. The alkaline intermediates were not detected by GC under the conditions employed to detect the sesquiterpene lactones.

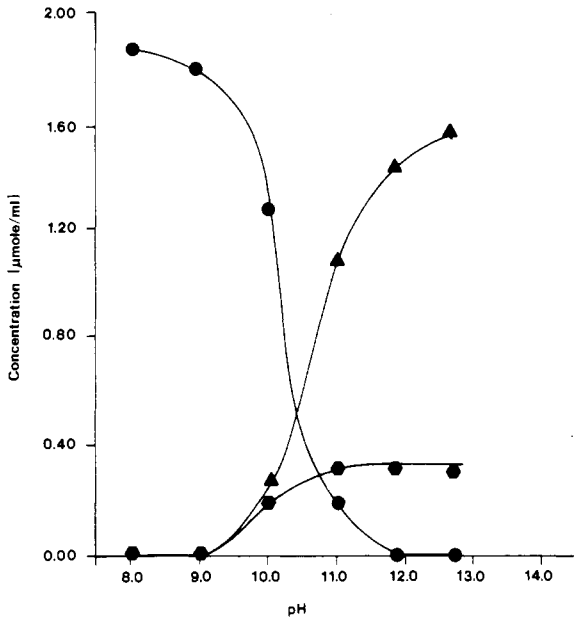
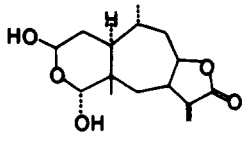
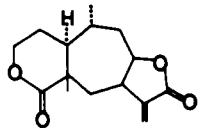


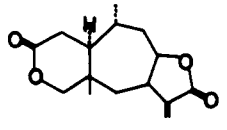
Figure 1. The effect of pH on the formation of psilotropin (▲–▲), and greenein (■–■) from hymenoxon (●–●).



HYMENOXON



GREENEIN



PSILOTROPIN

Figure 2. The stereochemical structure of three sesquiterpene lactones.

After the dilactones were formed, they could be detected by GC in solutions of pH 1–9. This reaction is important in forming derivatives of hymenoxon that contribute to the identification of hymenoxon in plant material (Figure 2).

The conversion of hymenoxon to psilotropin and greenein in the gastrointestinal tract could have an effect on the toxicity of hymenoxon. However, it is highly improbable that the pH of the gastrointestinal fluid of ruminant or monogastric animals is appropriate to induce the conversion of hymenoxon to psilotropin and greenein.

Reaction of Hymenoxon with Cysteine. The rate constant of a second-order reaction is defined by

$$\frac{(1/a - b) \ln [b(a - X)/a(b - X)]}{t} = k$$

where a = initial concentration of hymenoxon (M), b = initial concentration of cysteine (M), x = decrease in hymenoxon at time t (min), $a - x$ = concentration of hymenoxon (M) at time t (min), $b - x$ = concentration of cysteine (M) at time t (min), and k = second-order constant (1/mol min).

The reaction of hymenoxon with cysteine was shown to be second order by plotting the $(1/a - b) \ln [b(a - x)/a(b - x)]$ term against t. The linear relationship was tested by determining the linear correlation coefficient ($r = 0.991$). This relation is indicative of a second-order reaction (Barrow, 1961). From the second-order rate equation, the rate constant for the reaction was calculated as 504 L mol^{-1} min^{-1}.

Sesquiterpene lactones that possess an α-methylene group have been reported to form a "Michael adduct" with the sulfhydryl group of cysteine (Kupchan et al., 1970a; Kupchan et al., 1971). Kim et al. (1974) reported that cysteine given in conjunction with a crude extract of hymenoxon to dogs decreased the toxicity of the hymenoxon. In the present study when cysteine was added to an aqueous solution (pH 7.4) of hymenoxon, the concentration of extractable hymenoxon was reduced. Also, the sulfhydryl group of cysteine could not be detected with 5,5′-dithiobis(2-nitrobenzoic acid) when cysteine was reacted with a small excess of hymenoxon for 15 min. The inhibition of the reaction with Hg_2Cl_2 also indicated that the sulfhydryl group was involved in the reaction. These findings suggest that hymenoxon, like other α-methylene sesquiterpene lactones, binds to the sulfhydryl group of cysteine.

The hydrolysis of the hymenoxon–cysteine complex with CH_3I and base produced psilotropin in a quantity approximately equal to the concentration of hymenoxon added to form the hymenoxon–cysteine complex. This reaction is reported to cleave the carbon–sulfur bond and to regenerate the α-methylene group (Kupchan et al., 1970b).

The second-order rate constant (504 L mol^{-1} min^{-1}) for the reaction of hymenoxon with cysteine is twice the reported rate constant for sesquiterpene lactones that do not have an OH or *O*-acyl peri to the α-methylene group (Kupchan et al., 1971). However, the rate constant for hymenoxon is less than the reported rate constant for sesquiterpene lactones that do have an OH or *O*-acyl group. The rate of reaction of the sulfhydryl group of cysteine with the α-methylene group is not a function of the toxicity of the sesquiterpene lactones (Kupchan et al., 1971). Therefore, the rate constant will not predict the toxicity of hymenoxon relative to known sesquiterpene lactones.

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Quantitative Analysis of Hymenoxon in Plant Tissue

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Hymenoxon was successfully extracted with ethyl acetate from ground plant material and quantitatively analyzed on a 3% OV-17 gas–liquid chromatographic column. Dimethylhymenoxon or flavone was used as the internal standard. Recovery of fortified hymenoxon from plant tissue was 100–105%. The concentration of hymenoxon was determined in *Hymenoxys odorata*, *Helenium hoopsii*, and *Baileya multiradiata*, and the hymenoxon content was related to the relative toxicity of the three plants.

Hymenoxys odorata (bitterweed) is a major poisonous range plant that is indigenous to Texas, New Mexico, Colorado, Oklahoma, Arizona, and California. Kim et al. (1975) isolated a poisonous sesquiterpene lactone, hymenoxon, from bitterweed and Ivie et al. (1975) reported the isosolation of hymenovin, an epimeric mixture of hymenoxon, from this plant. Pettersen and Kim (1976) determined the stereochemistry of hymenoxon by X-ray crystallography. Hill et al. (1977) identified hymenoxon as a constituent of two other toxic plants of compositae, *Baileya multiradiata* (baileya) and *Helenium hoopsii* (sneezeweed). In the present study, a procedure was developed for the quantitative analysis of hymenoxon in these plants.

EXPERIMENTAL SECTION

Chemicals. Hymenoxon (mp 135–136 °C) was extracted from *H. odorata* and repeatedly recrystallized by the procedure of Kim et al. (1975). Flavone was obtained from K&K Laboratories Inc., and dimethylhymenoxon was prepared according to the method by Kim et al. (1975).

Instrumentation. A Varian 2100 or a Barber Colman 5000 gas chromatograph (GC) was used to analyze extracts of plant tissues. The Varian GC was equipped with a 4.8 ft × $^1/_8$ in. glass column packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) and helium was used as the carrier gas at a flow rate of 40 mL/min. The operating temperatures were as follows: column, 205 °C; injector, 215 °C; and flame ionization detector, 255 °C. The Barber Colman GC contained a 6 ft × $^1/_6$ in. glass column packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh). The operating temperatures were as follows: column, 210 °C; injector, 235 °C; and flame ionization detector, 250 °C. Nitrogen was the carrier gas with a flow rate of 45 mL/min.

Quantitative Analysis of Hymenoxon in Plant Tissues. Standard solutions of hymenoxon, dimethylhymenoxon, and flavone were prepared in ethyl acetate. To determine the linearity of detector response to these compounds, different concentrations of hymenoxon and dimethylhymenoxon standards were injected in triplicate on the Barber Colman GC, and different concentrations of hymenoxon and flavone standards were analyzed in triplicate on the Varian GC. The linear regression equation and the correlation coefficient of peak height vs. amount of standard applied to the column were calculated for each compound.

Standard solutions (0.161–0.241 mg/mL) of hymenoxon were made in ethyl acetate. Dimethylhymenoxon (50 μg/mL in ethyl acetate) or flavone (40 μg/mL in ethyl acetate) was added as the internal standard for the chromatographic procedure. To establish a standard curve, 1.0 mL of each hymenoxon standard was pipetted into a 15-mL centrifuge tube and mixed with 1.0 mL of the flavone internal standard or 1.0 mL of the dimethylhymenoxon internal standard. Each solution was evaporated to dryness under a stream of air in a water bath, and the residue was dissolved in 200 μL of ethyl acetate. A 2.0-μL sample of the concentrated solution was injected on the GC. The peak height of the hymenoxon peak was divided by the peak height of the internal standard peak to give the relative peak height (RPH). The linear regression equation and the correlation coefficient for the RPH vs. the milligrams of hymenoxon standard for each internal standard was calculated.

The ground, aerial portion of the plants were dried in a vacuum oven at 60 °C and stored in a desiccator. A 40–50-mg sample of the plants was weighed and transferred to a glass-stoppered test tube. Hymenoxon was extracted by mixing the plant sample with 8.0 mL of ethyl acetate for 2 min on a vortex mixer, and the particulate matter was removed from the ethyl acetate by centrifugation. A 1.0–4.0-mL aliquot of the ethyl acetate extract was transferred to a 15-mL centrifuge tube containing 1.0 mL of flavone internal standard or 1.0 mL of dimethylhymenoxon internal standard. The mixture was evaporated to dryness in a water bath under a stream of air and the residue was dissolved in 200 μL of ethyl acetate. A 2.0-μL sample of the concentrated extract was analyzed on the GC. The RPH of the unknown was determined and the amount of hymenoxon extracted was calculated from

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