lation was more than 10^8 organisms/g. As was true for shrimp, there was also good correlation between ammonia production and other quality indexes for fresh crab meat held on ice.

The direct enzymatic determination of ammonia and urea has a number of advantages over other common methods used to assess seafood quality. The practical advantage is that both compounds can be determined in the same sample by using the same technique. The technical advantage lies in the specificity of the enzymes for the substrates (Mondzac et al., 1965; Kaplan, 1969). While total volatile nitrogen analysis includes a number of amines in addition to ammonia, the enzymatic assay is specific for either ammonia or urea. No special apparatus is required with the exception of a narrow bandwidth UV spectrophotometer.

Registry No. Urea, 57-13-6; GDH, 9001-46-1; NADH, 58-68-4; urease, 9002-13-5; NH_3, 7664-41-7; N_2, 7727-37-9; TMA, 75-50-3.

LITERATURE CITED

APHA "Recommended Methods for Microbiological Examination of Food", 2nd ed.; American Public Health Association: New York, 1970.
Boehringer Mannheim Biochemicals "Methods of Enzymatic Food Analysis"; Boehringer Mannheim Biochemicals: Indianapolis, IN, 1980.
Burnett, J. L. *J. Assoc. Off. Anal. Chem.* **1965**, *48*, 624.
Chaney, A. L.; Marbach, E. P. *Clin. Chem. (Winston-Salem, N.C.)* **1962**, *8*, 130.
Cobb, B. F., III; Alaniz, I.; Thompson, C. A., Jr. *J. Food Sci.* **1973**, *38*, 431.
Conway, E. J.; Cooke, R. *Biochem. J.* **1939**, *33*, 457.
Fawaz, G.; Dahl, K. V. *Chem. Abstr.* **1964**, *61*, 2177.
Hillig, F.; Shelton, L. R., Jr.; Loughrey, J. H. *J. Assoc. Off. Anal. Chem.* **1959**, *42*, 702.
Kaplan, A. In "Methods of Biochemical Analysis"; Glick, D., Ed.; Wiley: New York, 1969; p 331.
Knight, C. B.; Toom, P. M. *Proc. Annu. Trop. Subtrop. Fish. Technol. Conf., 5th* **1980**, *5*, 181.
Mondzac, A.; Ehrlich, G. E.; Seegmiller, J. E. *J. Lab. Clin. Med.* **1965**, *66*, 526.
Seligson, D.; Hirahara, K. *J. Lab. Clin. Med.* **1957**, *49*, 962.
Stansby, M. E. In "Industrial Fishery Technology", 3rd ed.; Stansby, M. E., Ed.; Robert E. Krieger Publishing Co.: Huntington, NY, 1976; p 370.
Steinbrecher, K. *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 598.
Talke, H.; Schubert, G. E. *Klin. Wochenschr.* **1965**, *43*, 174.
Vyncke, W. *J. Food Technol.* **1978**, *13*, 37.
Ward, D. R.; Finne, G.; Nickelson, R., II *J. Food Sci.* **1979**, *44*, 1052.

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Characterization of the Cauliflower Peroxidase Isoenzyme

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Cauliflower peroxidases were separated into three isoenzymes by using hydrophobic chromatography. Some characters of the major isoenzyme, which consists of over 94% of the total peroxidase activity, were studied. Optimal pH and temperature with guaiacol as a substrate were found to be pH 6.5 and 40 °C. Kinetic studies indicated an apparent K_m of 3.1 mM for guaiacol at an optimal hydrogen peroxide concentration and a K_m of 8.7 mM for hydrogen peroxide at an optimal guaiacol concentration. The heat inactivation rate followed first-order kinetics with the activation energy, E_a = 46.4 kcal/mol. As this indicated, the isoenzyme preparation was very heat labile; at 50 °C, it took only 15 min to reduce its activity by 98%.

Peroxidase (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) is known to occur in most higher plants and is considered to have a direct relationship to the adverse changes in flavor and color of raw underblanched vegetables. Peroxidase is the most heat-stable enzyme in plants, and under certain conditions of limited heat treatment of vegetables it may regain activity during storage (Schwimmer, 1944; Lu and Whitaker, 1974). Any reactivation of peroxidase that does occur is probably due to the enzyme not being wholly inactivated by heat (Burnette, 1977). Recently an extensive review on peroxidase in fruits and vegetables was presented by Vamos-Vigyazo (1981).

Rosoff and Cruess (1949) were the first workers to report the presence of peroxidase in cauliflower. Since then a very limited number of papers on this vegetable and enzyme have been published. Bottcher (1975) reported that when cauliflower was heated at 95 °C for 2 min, less than 13% of residual activity was observed. When residual peroxidase activity ranged from 2.9 to 8.2% after hot water blanching, they observed that frozen cauliflower maintained its best quality. This and other reports were all based on peroxidase activity within cauliflower. In the present study, we isolated peroxidase in a homogeneous form from cauliflower and examined some of its chemical characters.

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MATERIALS AND METHODS

Persistent white cauliflower selections (Dickson and Lee, 1980) were grown during the 1981 and 1982 seasons at this experiment station. The peroxidases were extracted from cauliflower, harvested at maturity, by homogenizing with acetone. The isoenzymes were separated on a hydrophobic chromatographic column, phenyl-Sepharose CL-4B (Pharmacia), according to the methods of Flurkey and Jen

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(1978) and Wissermann and Lee (1980). The pooled fractions were dialyzed and freeze-dried. Throughout the experiment, the enzyme solution used was prepared in 0.2 M phosphate–0.1 M citric acid buffer at pH 6.5.

The peroxidase activity was determined on 50 μL of the enzyme solution in 2 mL of phosphate buffer, pH 6.5, containing 10 mM guaiacol and 10 mM hydrogen peroxide as substrates. The absorbance changes due to the oxidation of guaiacol were followed at 22 °C with a Varian Cary spectrophotometer (Model 219) at 470 nm, and 1 unit of peroxidase activity was defined as the amount of enzyme that caused 0.001 absorbance unit change per min. The specific activity was expressed as units of activity per milligram of protein.

The protein content was measured by the Coomassie Brilliant Blue G-250 (Eastman) dye-binding method (Bradford, 1976) using bovine serum albumin (Sigma) as a standard.

The pH optimum cauliflower peroxidase was assayed over the pH range 3.0–8.0 in McIlvaine's buffer solution (disodium phosphate–citric acid). The activity of the enzyme as a function of temperature was measured when the enzyme solution received a 2-min heat treatment within the range of 0–60 °C. Assay conditions were the same as those described above.

In order to check the purity of isolated peroxidase, starch gel electrophoresis was performed according to the methods of Evans (1968) and Shaw and Prasad (1970) using hydrolyzed potato starch (Sigma). After electrophoresis, the sliced gels were stained for peroxidase by using guaiacol and hydrogen peroxide.

Heat inactivation of cauliflower peroxidase isoenzyme was performed over a temperature range of 30–55 °C with the heating times varying from 0 to 60 min. After being heated for a given time period in a Haake water bath (Model FK), the solutions were rapidly cooled by immersing the tubes in an ice water bath; the samples were then assayed immediately. The percent peroxidase activity remaining after treatment was calculated from the initial activity.

For the kinetic studies, the rate constants were determined on concentrations of guaiacol from 0.1 to 50 mM in the presence of optimal levels of hydrogen peroxide in phosphate–citrate buffer at pH 6.5. Likewise, hydrogen peroxide as a substrate was studied at the optimal guaiacol concentration as determined from Lineweaver–Burk plots.

RESULTS AND DISCUSSION

Much of the difficulty in understanding peroxidase is due to the presence of multiple isoenzymes in various products. Therefore, in order to study the specific characters of peroxidase from a given product, it is essential to isolate first the individual isoenzymes. We were able to remove a large amount of the 280 nm absorbing materials and to separate the three isoenzymes using hydrophobic chromatography and step gradient elution. As illustrated in Figure 1, the isoenzymes were in two small and one large fractions. The combined activity of the two small isoenzyme fractions was less than 6% of the total activity. Because it appears that the overall cauliflower peroxidase activity is due to this single major isoenzyme, it was used as the source of cauliflower peroxidase.

The specific activity of the purified peroxidase isoenzyme was 44 600 units while that of the crude extract was 250 units, which is approximately a 180-fold increase in purification. When the isoenzyme was subjected to starch gel electrophoresis, a single peroxidase band was obtained, confirming the homogeneity of the purified isoenzyme.

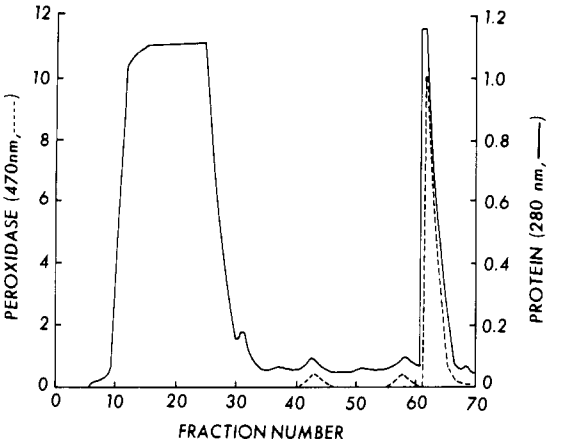

Figure 1. Elution profile from a phenyl-Sepharose CL-4B hydrophobic column for cauliflower peroxidase (- - -) and protein (—). Proteins (150 mg) in 200 mL were applied to a 40-mL bed volume column. Elution was made by stepwise decreasing gradients of ammonium sulfate–potassium phosphate buffer (Wissermann and Lee, 1980). Peroxidase activity was monitored by measuring absorbance at 470 nm of a 50-μL sample containing 4 mL of each 10 mM guaiacol and hydrogen peroxide.

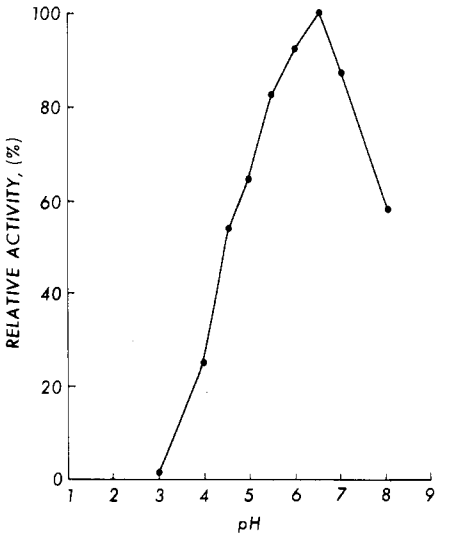

Figure 2. Cauliflower peroxidase isoenzyme activity as a function of pH. Guaiacol substrate at 22 °C.

By use of guaiacol as a substrate, the peroxidase isoenzyme showed a pH optimum of 6.5 at 22 °C (Figure 2). This differs from the optimum of 5.3 reported by Rosoff and Cruess (1949). Since their enzyme was a nonhomogeneous form of acetone powder, it is difficult to compare our results with their values. Various optimal pH values of peroxidases have been reported for vegetables, e.g., pH 7.0 for horse radish peroxidase (Wilder, 1962) and pH 5.5 for tomato peroxidase (Jen et al., 1980).

The optimum temperature of the isoenzyme was found to be 40 °C (Figure 3). At lower temperatures the enzyme still showed relatively high activity, retaining more than 35% of the maximum activity even at 0 °C. However, the enzyme activity decreased drastically as the temperature rose beyond 40 °C, being only 50% at 48 °C and nearly zero at 60 °C. It clearly shows that our peroxidase isoenzyme, unlike many other peroxidases from various sources, is very heat sensitive since inactivation occurred at relatively moderate temperatures.

Substrate concentration studies utilizing Lineweaver–Burk plots (Figure 4) showed an apparent K_m of 3.1 mM for guaiacol at 10 mM hydrogen peroxide concentration (optimum) and of 8.7 mM for hydrogen peroxide at 10 mM

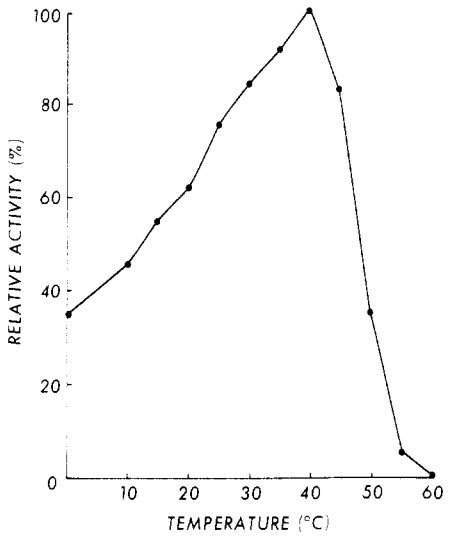

Figure 3. Cauliflower peroxidase isoenzyme activity as a function of temperature. Guaiacol substrate at pH 6.5.

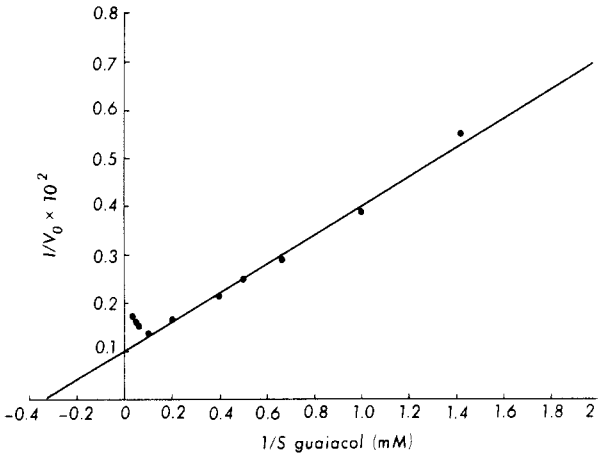

Figure 4. Lineweaver–Burk plot of cauliflower peroxidase isoenzyme on guaiacol substrate.

guaiacol concentration (optimum). The plots showed that guaiacol could provide substrate inhibition at approximately 15 mM.

The rate of heat inactivation of the peroxiddase isoenzyme followed first-order kinetics (Figure 5). As noted before, the isoenzyme was inactivated rapidly above 40 °C. When the enzyme was heated at 50 °C, approximately 50% of the activity was lost within 3 min and nearly 98% of the activity was destroyed in less than 15 min. This result was in a contrast to the many previous reports that had shown peroxidases from various sources to be very heat stable. When the logarithm of the first-order rate constant for inactivation was plotted against the reciprocal of the absolute temperature (Figure 6), the rate of activity loss clearly shows a temperature dependence. The activation energy, E_a for the inactivation, was found to be 46.4 kcal/mol. The complexity of peroxidase inactivation is due to its composition of isoenzyme species. Yamamoto et al. (1962) reported that the isoenzymes of sweet corn consisted of heat-labile and heat-resistant fractions. They found that the heat-resistant fraction, representing only 5% of total enzyme activity, requires additional heat treatment for inactivation. Winter (1969) studied the peroxidase in eight vegetables and found that most of them contained heat-sensitive and heat-resistant peroxidases. The heat-resistant peroxidase was detectable only in the range of 1–10% of the total activity but required an extra long heat treatment. In many occasions, the rate of thermal inac-

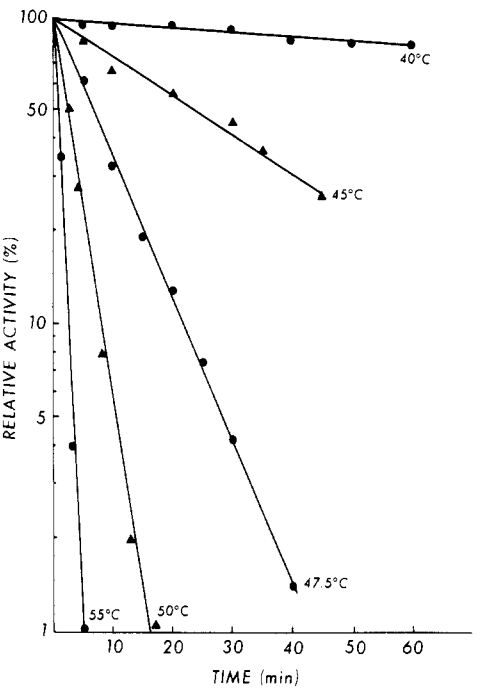

Figure 5. Rate of heat inactivation of cauliflower peroxidase isoenzyme.

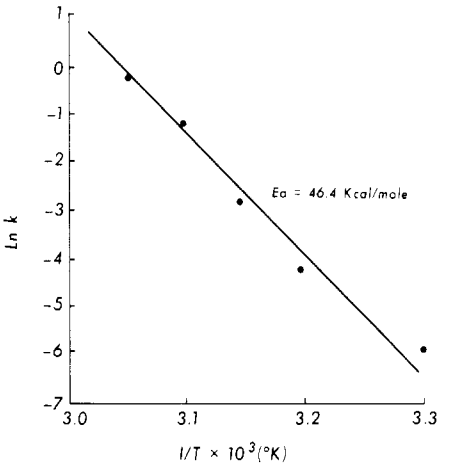

Figure 6. Arrhenius plot showing effect of temperature on the rate constant for the thermal inactivation of cauliflower peroxidase isoenzyme.

tivation of a composite peroxidase would not exhibit first-order reaction kinetics due to the presence of peroxidase isoenzymes of different stability (Lu and Whitaker, 1974; Ling and Lund, 1978). The cauliflower peroxidase inactivated at 80 °C reappeared during subsequent storage of cauliflower at −18 °C (Rosoff and Cruess, 1949). It thus appears that our peroxidase isoenzyme isolate happened to be a heat-sensitive fraction of the cauliflower peroxidase. Even though it consisted of over 94% of the total activity, it did not show any of the heat stability that had been observed by others. In the future a sufficient quantity of the other two small fractions will be isolated so that their heat inactivation characteristics can be studied. In this way we will be able to understand the complete heat stability properties of whole cauliflower peroxidases.

Registry No. Peroxidase, 9003-99-0; hydrogen peroxide, 7722-84-1; guaiacol, 90-05-1.

LITERATURE CITED

Bottcher, H. *Nahrung* **1975**, *19*, 173.
Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
Burnette, F. S. *J. Food Sci.* **1977**, *42*, 1.

Dickson, M. H.; Lee, C. Y. *J. Am. Soc. Hortic. Sci.* **1980**, *105*, 533.
Evans, J. J. *Plant Physiol.* **1968**, *43*, 1037.
Flurkey, W. H.; Jen. J. J. *J. Food Sci.* **1978**, *43*, 1826.
Jen, J. J.; Seo, A.; Flurkey, W. H. *J. Food Sci.* **1980**, *45*, 60.
Ling, A. C.; Lund, D. B. *J. Food Sci.* **1978**, *43*, 1307.
Lu, A. T.; Whitaker, R. *J. Food Sci.* **1974**, *39*, 1173.
Rosoff, H. D.; Cruess, W. V. *Food Res.* **1949**, *14*, 283.
Schwimmer, S. *J. Biol. Chem.* **1944**, *154*, 487.
Shaw, C. R.; Prasad, R. *Biochem. Genet.* **1970**, *4*, 297.
Vamos-Vigyazo, L. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49.
Wilder, C. *J. Food Sci.* **1962**, *27*, 567.
Winter, E. *Z. Lebensm.-Unters. -Forsch.* **1969**, *141*, 201.
Wissermann, K. W.; Lee, C. Y. *J. Chromatogr.* **1980**, *192*, 232.
Yamamoto, H. Y.; Steinberg, M. P.; Nelson, A. I. *J. Food Sci.* **1962**, *27*, 113.

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Synthesis of the 2-Methyl Ether of L-Ascorbic Acid: Stability, Vitamin Activity, and Carbon-13 Nuclear Magnetic Resonance Spectrum Compared to Those of the 1- and 3-Methyl Ethers

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Reaction of L-ascorbic acid (**4**) with dimethyl sulfate (1.6 equiv) at pH 10.5 and 60 °C gave 76% crystalline 2-*O*-methyl-L-ascorbic acid (**2**) along with 15% of a byproduct (**9**). The byproduct formed a lactone during isolation and was tentatively identified as methyl 2-*O*-methyl-γ-L-*lyxo*-hex-3-ulofuranosidonolactone (**7**). Compound **7** was quantitatively converted to **2** in boiling methanolic hydrogen chloride. 5,6-*O*-Isopropylidene-L-ascorbic acid (**5**) was 2-methylated by dimethyl sulfate at pH 10.5 and 60 °C, and removal of the 5,6-acetal blocking group gave **2** in 85% yield from **4**. The 2-methyl ether (**2**) showed only marginal vitamin C activity in the tobacco hornworm and guinea pig. The structures of the 1-, 2-, and 3-methyl ethers (**1**, **2**, and **3**) were confirmed by ^{13}C NMR and UV spectroscopy. Under aerobic conditions at pH 7, the order of stabilities of the methyl ethers was $2 \gg 3 > 1$.

The occurrence of 2-*O*-methyl-L-ascorbic acid (**2**) (Figure 1) in the urine of guinea pigs and rats (Blaschke and Hertting, 1971; Gazave et al., 1975) suggested that this compound might be an important metabolite that would be more stable than L-ascorbic acid (**4**). In this paper we describe the synthesis of crystalline **2** starting from either L-ascrobic acid (**4**) or 5,6-*O*-isopropylidene-L-ascorbic acid (**5**, Figure 2). The stability of **2** in water under aerobic conditions at pH 2, 7, and 10 was compared with that of **4**, 3-*O*-methyl-L-ascorbic acid (**3**), and 1-*O*-methyl-L-ascorbic acid (**1**) at pH 7 and 10. The structures of **1**, **2**, and **3** were verified by UV and ^{13}C NMR spectroscopy. We also tested the growth promoting activity of **2** in vertebrate and invertebrate animal species.

EXPERIMENTAL SECTION

General. Melting points were determined with a Thomas-Hoover Unimelt apparatus. Solutions were evaporated under reduced pressure below 50 °C. TLC was performed on plates coated with silica gel G (Brinkman Instruments, Inc., Westbury, NY). Components were located by using an ultraviolet lamp, by spraying with 1% ferric chloride in 95% ethanol (Vestling and Rebstock, 1946) or by spraying with 50% aqueous sulfuric acid and charring on a hot plate. A Beckman DB-G (Beckman Instruments Inc., Palo Alto, CA) or a Cary 14 recording spectrophotometer (Varian Associates, Palo Alto, CA) was used to record UV spectra. ^{13}C NMR spectra were recorded on a Varian Model XL-100-15 spectrometer interfaced to a Nicolet 1180 digital computer and to a Nicolet 1093B pulse Fourier transform system with quadrature phase detection. In aqueous solution dioxane was used as an internal reference and the shifts were calculated relative to tetramethylsilane (Me_4Si): $\delta_{Me_4Si} = \delta_{dioxane} + 67.40$ ppm. In organic solutions Me_4Si was added as an internal reference standard.

High-performance liquid chromatography (HPLC) was carried out at 25 °C using a Waters Model 6000A pump (Waters Associates, Inc., Milford, MA) fitted with a loop injector and a Beckman Model 100-10 UV detector. Retention times and integrations were recorded using a computing integrator (Waters Data Module, Waters Associates Inc.). The stainless steel column (250 × 4.0 mm) contained a reverse-phase packing (BioSil ODS-10, Bio-Rad Laboratories, Richmond, CA). Samples (20 μL) were injected and components were eluted isocratically with 0.05 M phosphate buffer (pH 3) at a flow rate of 1.0 mL min^{-1}. Peaks were detected at 255 nm. The retention times of compounds **4**, **2**, and **3** were approximately 4.3, 6.1, and 9.8 min, respectively.

1-*O*- and 3-*O*-Methyl-L-ascorbic Acid (1 and 3). Methylation of L-ascorbic acid by diazomethane (a hazardous chemical: handle with caution) was done essentially as described by Reichstein et al. (1934) and Haworth et al. (1934). To a rapidly stirred solution of **4** (2.5 g) in dry methanol (<0.1% water, 30 mL) maintained at 0–5 °C was added dropwise, until a yellow color persisted, a solution of diazomethane (2.7–2.9 g, 0.07 mol) in ethyl ether (125 mL). The diazomethane had been generated previously (DeBoer and Backer, 1967) from *N*-methyl-*N*-nitroso-*p*-

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