A New Intermediate in the Metabolism of Ethanol*

Robert J. Bloom and W. W. Westerfeld

ABSTRACT: The incubation of ethanol-1-^{14}C with rat liver or kidney homogenates gave a previously unrecognized metabolite which was isolated and identified as 5-hydroxy-4-ketohexanoic acid (I). Periodate oxidation of the labeled product yielded equimolar quantities of acetaldehyde and succinic acid; nearly all of the ^{14}C was present in C_1 of the acetaldehyde (C_5 of I). Large amounts of I were prepared by incubating acetaldehyde plus α-ketoglutarate with beef kidney homogenates. The isolated I was a viscous oil, soluble in water, alcohol, $CHCl_3$, and Et_2O; insoluble in petroleum ether (bp 30–60°); pK = 4.6; $[\alpha]_D$ −17.9°; dehydroabietylamine salt, mp 98–99°; 2,4-dinitrophenylosazone, mp 278–279°; phenylosazone (cyclized), mp 188–189°; *p*-phenylphenacyl ester, mp 131–132°. Elementary analysis and nuclear magnetic resonance (nmr) and infrared spectra were consistent with these structures. Compound I was synthesized from acetoin by acetylation and bromination to give 3-acetoxy-1-bromo-2-butanone (II), bp 57.5–58° at 0.15 mm. Compound II was condensed with diethyl malonate to form ethyl 5-acetoxy-2-carbethoxy-4-ketohexanoate (III), bp 119.5–120° at 0.05 mm. Compound III was converted to I by autoclaving in 1 N HCl. Synthetic I was purified by the same chromatographic procedures used for isolating the natural product, and gave the same derivatives.

The evidence for the metabolic conversion of ethanol to acetaldehyde and acetate has been reviewed previously (Westerfeld, 1955, 1961). There is no reason to doubt that such a pathway exists in mammalian tissues, especially in view of the recent demonstration of free acetate in the blood leaving the liver during ethanol metabolism (Lundquist *et al.*, 1962b). However, three points of evidence have kept open the question of an alternate metabolic pathway for ethanol, *i.e.*, (1) the better utilization of ethanol, as compared with acetate, for the synthesis of cholesterol, fatty acids, and acetylsulfanilamide (Synder *et al.*, 1964); (2) the different rates at which each carbon of ethanol, as compared with acetate, is converted to CO_2 (Russell and Van Bruggen, 1964); and (3) the presence in tissue homogenates of an unidentified metabolite produced from labeled ethanol, but not from acetate (Westerfeld and Schulman, 1959). The first two points listed above do not necessarily indicate different pathways, and in fact, recent studies from this laboratory (Snyder *et al.*, 1964) have explained at least a part of these differences as being due to a compartmentation of the acetyl-CoA formed from these substrates at different sites within the cell.

A unique pathway for ethanol metabolism is of importance because of the possible role that such intermediates might play in the pharmacological or pathological effects of ethanol. A major portion of the ethanol need not traverse such a pathway for it to be of importance in this regard. Acetaldehyde formed from the ethanol would be the most likely substrate to enter into a metabolic pathway that was unavailable to acetate or acetyl-CoA, and such has now been found to be the case. Previous studies with acetaldehyde (Lundquist *et al.*, 1962a; Westerfeld, 1949) or ethanol (Lundquist *et al.*, 1963) did not detect this pathway for reasons which are understandable in retrospect.

This new metabolite from ethanol was detected in the course of other studies on a comparison of ethanol and acetate metabolites in rat liver homogenates (Bloom and Westerfeld, 1966). Radioactive ethanol or acetate was incubated with a rat liver homogenate, and the nonvolatile labeled products were separated by column chromatography and identified. In general, both ethanol and acetate were incorporated into the components of the citric acid cycle in the same manner, as previously reported by Dajani and Orten (1962). In these studies, a Dowex 1-X10 column was used to adsorb and separate the radioactive citric acid cycle components. Two fractions were obtained from this column prior to the elution of the Krebs cycle acids: (1) a forerun which was not adsorbed to the column (fraction A), and (2) a fraction which was eluted rapidly by formic acid (fraction B). Both of these fractions contained radioactive materials when the substrate was either ethanol-1-^{14}C or acetate-1-^{14}C. However, fraction B accumulated more ^{14}C from ethanol than from acetate, and this was the first indication of a possible difference in their metabolic pathways. Fraction B was, therefore, separated into its constituents by chromatography on a Dowex 1-X4 AG column. The second major compound eluted from this column by acetic acid (fraction B-2) contained the radioactivity which was derived preferentially from ethanol.

The yield of fraction B-2 from ethanol-1-^{14}C was

* From the Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, New York. *Received April 26, 1966.* This study was aided by Grant No. M-1947 from the National Institute of Mental Health of the National Institutes of Health, U. S. Public Health Service.

improved by the use of rat kidney instead of rat liver homogenates, the addition of glutathione to the incubation medium, and by incubation with oxygen instead of air. With these modifications, it was possible to obtain enough of fraction B-2 to identify it tentatively as 5-hydroxy-4-ketohexanoic acid (HKH)[1] since it formed acetaldehyde and succinic acid on periodate oxidation. The HKH appeared to be formed by a decarboxylation–condensation reaction between α-ketoglutarate and acetaldehyde, and sufficient HKH was then prepared from these substrates to complete the identification and prepare suitable derivatives.

$$HOOCCH_2CH_2C(=O)COOH + CH_3CHO \longrightarrow HOOCCH_2CH_2C(OOH)CHCH_3 + CO_2$$

For additional proof of structure, 5-hydroxy-4-ketohexanoic acid was synthesized by standard chemical procedures and proved to be identical with the isolated product. So far as we can determine, this product has not been described previously in either the chemical or biological literature, and nothing is yet known about its physiological effects or its significance as an intermediate in ethanol metabolism.

Experimental Section

Preparation of HKH from Ethanol-1-^{14}C. The incubation system consisted of 20 ml of a 1:5 rat kidney homogenate in 1.155% KCl containing 0.01 M cysteine (pH 7.1), 4 ml of 0.0002 M cytochrome *c*, 2 ml of 0.1 M $MgCl_2$, 6 ml of 0.01 M ATP, 8 ml of 0.5 M KCl, 6 ml of 0.1 M potassium phosphate buffer, pH 7.1, 4 ml of 1.5% glutathione, 6 ml of a mixture containing 0.054 M sodium citrate and 0.054 M oxalacetate adjusted to pH 7.1, 0.14 ml of ethanol-1-^{14}C (3.7 μmoles), and sufficient water to make a total volume of 60 ml. No unlabeled ethanol was added. The radioactive ethanol provided a total of 3.36 million cpm and a final concentration of only 0.28 mg of alcohol/100 ml. Eight such 60-ml batches were placed in 500-ml flasks, gassed with oxygen, and incubated with shaking for 4.5 hr at 38°. $HClO_4$ (6 ml of 50%) was then added to each flask; the protein precipitate was removed by centrifugation and washed with 5% $HClO_4$. The supernatant and wash were combined, neutralized with 20% KOH, chilled overnight, and filtered. The filtrate from the $KClO_4$ precipitate was then chromatographed sequentially on Dowex 1-X10, Dowex 1-X4 AG, and Celite columns as described below. All fractionations in the studies with ethanol-1-^{14}C were followed by measuring the radioactivity.[2] About 5% of the original radioactivity was recovered in the HKH, and the total yield was subsequently estimated to be 6–10 mg of HKH from the combined eight 60-ml portions of incubation mixture.

Chromatography. The following chromatographic procedures have been described for the filtrate obtained from a single 60-ml batch. For larger amounts, all three columns were scaled up ten times or more in exactly the same proportions as described without changing the results.

Dowex 1-X10 Column. Dowex 1-X10 in the chloride form (J. T. Baker Co.) was washed free of fine particles and converted to the formate form as previously described (Busch *et al.*, 1952). A 1.5-cm diameter tube was filled to a height of 15 cm with about 30 ml of the resin. The sample (100 ml of the $KClO_4$ filtrate at pH 7) was passed through the column at 2 ml/min; it was followed by 50 ml of water, and the column was then eluted with 100 ml of 1 M formic acid. When the effluent was collected in 10-ml fractions, fraction B peaked in tube 22, and the entire fraction was collected in tubes 20–24. This was evaporated to dryness in a flash evaporator or by a stream of warm air.

Dowex 1-X4 AG Column. Fraction B was rechromatographed on a Dowex 1-X4 AG column (one-half chloride, one-half acetate) as described by Berl *et al.* (1962). A 20-ml sample at pH 7 was passed through a 1.5 × 22 cm column of the resin at 2 ml/min. This was followed by 35 ml of 0.05 N, 80 ml of 0.1 N, and 60 ml of 0.3 N acetic acid. When the effluent was collected in 5-ml fractions, the desired material (fraction B-2) peaked in tube 32, and was collected in tubes 29–35. This solution was evaporated to a small volume by repeated addition and distillation of H_2O until it was essentially free of acetic acid.

Celite Column (Ueno *et al.*, 1960; Phares *et al.*, 1952). Celite (Johns-Manville No. 535) was washed with ether and dried at 100° for 5 hr. Five grams was thoroughly mixed with 2 ml of 0.2 N H_2SO_4 and transferred to a 1 × 40 cm column with water-saturated $CHCl_3$. The sample (1 ml), acidified with a few drops of 5 N H_2SO_4, was mixed with about 1 g of additional Celite, and added to the top of the column. The column was plugged with cotton and eluted with 50-ml portions of 1, 5, and 10% butanol in $CHCl_3$; both solvents were saturated with water after mixing. When 5-ml fractions of the effluent were collected, the HKH usually peaked in tube 19 and was collected in tubes 16–24. The solvents were evaporated to a small volume; the HKH was extracted into water and lyophilized; the resulting viscous liquid was dried *in vacuo*.

Periodate Oxidation of HKH Obtained from Ethanol-1-^{14}C. Although criteria for the purity of the HKH fraction were lacking at the time of these studies, all of its radioactivity could be accounted for in the 2,4-

[1] Abbreviations used: HKH, 5-hydroxy-4-ketohexanoic acid; nmr, nuclear magnetic resonance; ATP, adenosine triphosphate; TMS, tetramethylsilane; DMF, dimethylformamide.

[2] An aliquot of each tube was dried on a tared sample pan under an infrared lamp and weighed, and the radioactivity was determined on a Nuclear-Chicago D47 micromil gas-flow counter and corrected to infinite thinness.

dinitrophenylosazone derivative which was prepared from this fraction and subsequently shown to be the HKH derivative (mp 277–278°; 33,180 cpm/μmole of derivative; original HKH = 32,558 cpm/μmole, based on the amount of acetaldehyde released by periodate oxidation). Periodate oxidation of the HKH obtained from ethanol-1-^{14}C provided the original clues to the structure of this molecule, the mechanism by which it was formed, the location of the ^{14}C in the molecule, and the location of the carbonyl and hydroxyl groups on C_4 and C_5, respectively, of the HKH. Equimolar quantities of acetaldehyde and succinic acid were obtained as the major products, and this established the carbonyl group on C_4 and the hydroxyl group on C_5 of the HKH. No formaldehyde or formic acid was produced, and no unreacted HKH remained. However, two additional minor products (approximately 20% of the total) were obtained in equimolar quantities; one was identified as acetic acid and the other was probably succinic semialdehyde. These contaminants apparently were derived from an isomer of the major component, *i.e.*, from 4-hydroxy-5-ketohexanoic acid, but this point was not established unequivocally.

Procedure. A 3-ml sample of HKH which contained a total of 2–5 million cpm was mixed with 6 ml of 1% periodic acid and allowed to stand at room temperature for 5 min. The solution was then neutralized to pH 7 with NaOH and aerated into 1 ml of 4% $NaHSO_3$ for 3–4 hr to trap any volatile aldehydes; the aeration was conducted at room temperature initially, and then with heating. The contents of the reaction flask were concentrated and chromatographed on a Celite column to separate the acids which were formed; the chromatography was followed by titrating and then determining the radioactivity in each tube. A volatile acid identified as acetic acid was collected in tubes 3–14; an unidentified acid, probably succinic semialdehyde, was recovered from tubes 14–22, and succinic acid in tubes 27–37. In two such experiments, periodate oxidation gave an average of 61 μmoles of acetaldehyde by a colorimetric analysis of the bisulfite solution; titration of the eluates from the Celite column showed 127 μequiv or 63.5 μmoles of succinic acid. About 18 μequiv each of acetic acid and succinic semialdehyde were formed simultaneously. The volatile radioactivity released with the acetaldehyde during periodate oxidation accounted for 75–80% of the radioactivity in the original HKH; some 18% was present in the acetic acid, while the succinic acid and semialdehyde accounted for only 1–2% each. The data were consistent with the formation of the major product in this fraction (HKH) by a condensation of highly radioactive acetaldehyde with relatively unlabeled ketoglutarate.

Identification of Periodate Reaction Products. The aldehyde trapped in the bisulfite gave the typical acetaldehyde color reaction, and this was utilized for its quantitative determination (Stotz, 1943); no greenish color characteristic of formaldehyde could be detected visually or spectrophotometrically. A 2,4-dinitrophenylhydrazone derivative was prepared in the usual way from about 60 μmoles of the acetaldehyde present in the bisulfite, and recrystallized from ethanol, mp 167–168°; no depression on mixing or recrystallizing with an authentic derivative.

Another aliquot of the bisulfite solution which contained 50 μmoles of acetaldehyde and 1.5 million cpm was treated with iodine in alkaline solution to produce iodoform. This had the same melting point (119–123°) and mixture melting point as the iodoform prepared from authentic acetaldehyde, but it possessed less than 500 cpm of total radioactivity; the radioactivity present in the filtrate from the iodoform chromatographed like formic acid on the Celite column. Hence C_6 of the HKH was unlabeled, and all of the radioactivity in the acetaldehyde portion of HKH was in C_5.

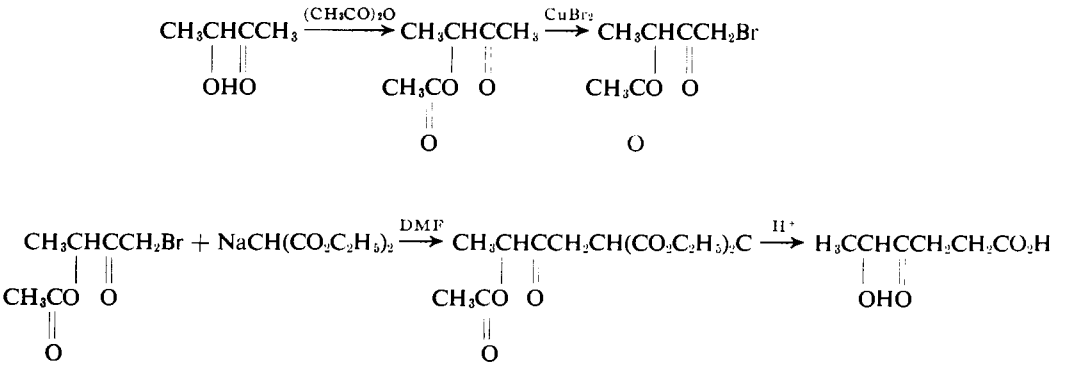
In one experiment, the 2,4-dinitrophenylhydrazone derivative of the acetaldehyde contained 17,920 cpm/μmole. This acetaldehyde was obtained from a periodate oxidation of the HKH that was isolated from a reaction mixture which originally contained 126,800 cpm/μmole of ethanol-1-^{14}C. Hence, the specific activity of the acetaldehyde portion of the HKH molecule was diluted out by unlabeled endogenous precursors; only 14% of the acetaldehyde found in the HKH molecule was derived from the labeled ethanol. Subsequent studies have shown that pyruvate can act as a source of acetaldehyde for the formation of HKH (Bloom *et al.*, 1966).

The material eluted from the Celite column in the succinate position (tubes 27–37) also chromatographed like succinate on Dowex 1-X10 and on paper with two different solvent systems (Roberts *et al.*, 1957). The *p*-phenylphenacyl derivative of this fraction was prepared and recrystallized from benzene–ethanol, mp 213–124°; no depression on mixing with an authentic succinate derivative. The small amount of radioactivity in this derivative (374 cpm/μmole) remained constant on recrystallization, and was attributed to the incorporation of some ^{14}C from the ethanol into α-ketoglutarate prior to its condensation with acetaldehyde.

The acid in tubes 3–14 was identified as acetic acid by adding 242 μequiv of unlabeled acetate to 20 μequiv of the acetate fraction obtained from the Celite column, and preparing the *p*-phenylphenacyl derivative. After two recrystallizations from 50% ethanol, the melting point was the theoretical 110–111°. The radioactivity in this derivative (13,840 cpm/mg) was unchanged by five additional recrystallizations, and a value of 13,750 cpm/mg was calculated for this derivative from the original titration, counting, and dilution values. The specific activities of the acetate and the acetaldehyde, which were produced by periodate oxidation, were comparable when obtained from the same HKH preparation.

The fraction eluted in tubes 14–22 was treated with 2,4-dinitrophenylhydrazine, and the derivative was extracted into ethyl acetate, passed into sodium carbonate, and reextracted with ethyl acetate after acidifying the carbonate solution. Chromatography of this derivative on paper with 1-butanol–ethanol–H_2O (40:10:50) gave the same R_F (Schepper *et al.*, 1958) as an authentic 2,4-dinitrophenylhydrazone of succinic semialdehyde.

SCHEME I: Synthesis of 5-Hydroxy-4-ketohexanoic Acid.



Large-Scale Preparation of Unlabeled HKH. Once the nature of the product was recognized, much larger quantities of unlabeled HKH were prepared by the following simplified procedure. Approximately 500 g of beef kidney was homogenized with 1 l. of water containing 22.5 ml of 1 M phosphate buffer, pH 7.1. Acetaldehyde (0.455 M, 25 ml), 25 ml of 0.274 M α-ketoglutarate (pH 7.1), and 700 ml of water were added, and the mixture was incubated at 37° for 3 hr. An additional 25 ml each of the acetaldehyde and the α-ketoglutarate were then added, and the incubation was continued for an additional 3 hr. The reaction was stopped by adding sufficient $HClO_4$ to give a final concentration of 5%, and the protein precipitate was filtered off with the aid of Celite; excess $HClO_4$ was removed from the filtrate and the resulting solution was chromatographed on the Dowex and Celite columns as previously described. These purification procedures were followed by a color reaction for HKH (Bloom *et al.*, 1966).

Approximately 2 g of HKH was produced in the above reaction. After purification on three columns, the viscous liquid was reasonably pure, but was carried through the following additional steps as a precaution. Approximately 600 mg of the dry oil was leached with 10 ml of dry $CHCl_3$. An occasional small amount of insoluble material was discarded. The $CHCl_3$ was evaporated, and the oil was redissolved in 5 ml of ethyl ether. This was fractionally precipitated with low boiling (30–60°) petroleum ether; one-half of solids were precipitated by 28% petroleum ether. The oil which came out of solution between 28 and 55% petroleum ether (200 mg) was collected by centrifugation and dried over P_2O_5 at room temperature. This "center cut" was arbitrarily assumed to be the purest fraction. Attempts to crystallize the free acid or its sodium or barium salt were unsuccessful.

Properties of the Isolated HKH. The nmr spectrum in $CDCl_3$ with 2% TMS standard gave a broad absorption near τ = 2.8 for the acidic H and OH, quartet centered at 5.7 (J = 7 cycles per second) for CH, singlet at 7.28 for CH_2CH_2, and doublet centered at 8.6 ppm (J = 7 cycles per second) for CH_3. The integral ratios were 2:1:4:3. There were weak impurities absorbing near τ = 7.4 and 7.8 ppm.

The infrared spectrum (liquid film) showed a broad absorption between 3600 and 2500 for the OH and acid OH, 2990 and 2450 for CH_2 groups, CH, and CH_3, and 1720 cm^{-1} for the free acid carbonyl.

The oil (121 mg) required 8.90 ml of 0.08 N NaOH to titrate the acid to a pH of 7. The titration curve obtained with a pH meter was typically sigmoid, and the midpoint or approximate pK was 4.6. For an acid with a theoretical neutralization equivalent of 146, the preparation was 86% pure.

An aqueous solution containing 197 mg of the oil/ml gave a rotation of −6.713° in a 2-dm tube at room temperature. This preparation was estimated to be 96% pure on the basis of a colorimetric analysis, $[\alpha]_D$ − 17.9°.

Synthesis of 5-Hydroxy-4-ketohexanoic Acid (Scheme I)

3-Acetoxy-1-bromo-2-butanone. 3-Acetoxy-2-butanone (Bergmann and Ludewig, 1924) (130.1 g, 1.0 mole) was brominated by the procedure of King and Ostrum (1964) to give 26.2 g (12.5%) of 3-acetoxy-1-bromo-2-butanone as a pale yellow oil of bp 57.5–58° at 0.15 mm. *Anal.* Calcd for $C_6H_9BrO_3$: C, 34.45; H, 4.34. Found: C, 34.55; H, 4.33.

The nmr spectrum of this compound in $CDCl_3$, with ~2% TMS as an internal standard, gave absorption peaks which were assigned as follows: the quartet centered at τ = 4.69 ppm (J = ~7 cycles per second) was attributed to the >CH, a singlet at τ = 5.90 ppm to the $C(=O)CH_2Br$, another singlet at τ = 7.87 to the $OC(=O)CH_3$, and a doublet centered at τ = 8.53 ppm (J = ~7 cycles per second) to the CH_3. The respective integral ratios were 1:2:3:3.

Ethyl 5-Acetoxy-2-carbethoxy-4-ketohexanoate. Diethyl malonate (17.6 g, 0.11 mole) was alkylated in DMF with 3-acetoxy-1-bromo-2-butanone (25.1 g, 0.12 mole) by the procedure of Zaugg *et al.* (1961). Sodium hydride dispersion in mineral oil was used. It was found that the mineral oil codistilled with the product, and a separation was effected by extraction of an acetonitrile solution of the distillate with *n*-pentane. Redistillation gave 11.0 g (34.6%) of ethyl 5-acetoxy-2-carbethoxy-4-ketohexanoate as a pale yellow oil of bp 119.5–120° at 0.05 mm. *Anal.* Calcd

for $C_{13}H_{20}O_7$: C, 54.16; H, 6.99. Found: C, 54.02; H, 7.04.

The infrared spectrum ($CHCl_3$) showed absorptions at 2995 and 2945 (aliphatic), 1740 (br, carbonyl), and other strong absorptions at 1375, 1250, 1180, 1160, 1100, 1033, and 865 cm^{-1}.

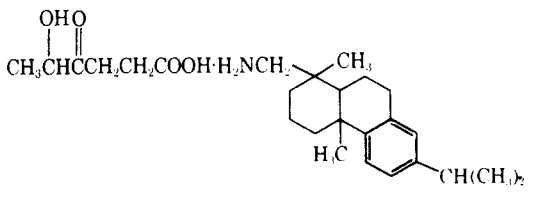
The nmr spectrum in $CDCl_3$, with ~2% TMS as an internal standard, gave the following absorption peaks: a quartet centered at τ = 4.89 ppm (J = ~7 cycles per second) for the >CHO, a quartet centered at τ = 5.81 ppm (J = ~7 cycles per second) attributed to the OCH_2, doublets centered at τ = 6.07 and 6.20 ppm (J values = ~7 cycles per second) for the C(=O)-CH(=O)C, an apparent doublet centered at τ = 6.91 ppm (J = ~7 cycles per second) for the $C(=O)CH_2$, a singlet at τ = 7.90 ppm for the $CH_3(=O)C$, and an apparent quartet (overlapping triplet and doublet) centered at τ = 8.68 ppm (J = ~7 cycles per second) for the terminal methyl groups of the ester functions. The respective integral ratios were 1:4:0.5:0.5:2:3:9.

5-Hydroxy-4-ketohexanoic Acid. The preceding ester (8 g) was stirred with 50 ml of 1 N HCl at room temperature for 2 weeks to dissolve the compound. Complete hydrolysis and the removal of one carboxyl group was effected by autoclaving aliquots of this solution for 2 hr. The solution was evaporated to dryness, neutralized to pH 7, and passed through the Dowex 1-X10, Dowex 1-X4 AG, and Celite columns as previously described for the natural product. The resulting viscous liquid (yield = 45% of theory) was soluble in $CHCl_3$ and was fractionally precipitated from ether by petroleum ether as previously described for the isolated product.

The nmr and infrared spectra of the synthetic HKH were comparable with the spectra for the isolated HKH, but also showed impurities absorbing near 5.1 and in the τ = 7.0–8.0-ppm region of the nmr and at 1780 cm^{-1} in the infrared region.

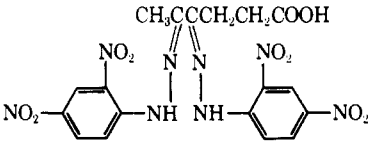
Derivatives

Dehydroabietylamine Salt. The isolated or synthetic HKH (50–100 mg) was dissolved in 5 ml of ethyl ether and mixed with an equal volume of ether which contained one to two times the stoichiometric amount of dehydroabietylamine. Colorless needles precipitated rapidly and were washed with ether; yield = 80–96% of theory; mp 98–99°. There was no melting point depression on mixing the synthetic and natural derivatives. *Anal.* Calcd for $C_{26}H_{41}NO_4$: C, 72.35; H, 9.58; N, 3.85. Found (natural): C, 72.71; H, 9.82; N, 3.55. Found (synthetic): C, 72.23, H, 9.57; N, 3.63.



The infrared spectrum of the synthetic or natural dehydroabietylamine salt (KBr pellet) showed absorption near 3400 cm^{-1} for OH and 3050 cm^{-1} for aromatic CH groups, in the 3000–2600-cm^{-1} region for CH_2 groups, CH_3 groups, and CH, 1715 cm^{-1} for a hydroxyl ketone carbonyl, 1620 and 1570 cm^{-} for aromatic C=C, and 1515 and near 1400 cm^{-1} for the carboxylate carbonyl.

2,4-Dinitrophenylosazone. Natural or synthetic HKH (55 mg) was dissolved in a small volume of water and mixed with 100 ml of the reagent containing 400 mg of 2,4-dinitrophenylhydrazine dissolved in 2 N HCl. After refluxing for 3 hr, the orange precipitate was filtered and washed with 2 N HCl and water; yield = 92% of theory. Several recrystallizations from 1 ml of nitrobenzene gave short, stubby needles, mp 278–279°. (There was no depression on mixing the two derivatives.) *Anal.* Calcd for $C_{18}H_{16}O_{10}$: C, 42.86; H, 3.20; N, 22.22. Found (natural): C, 43.00; H, 3.25; N, 22.37. Found (synthetic): C, 43.50; H, 3.40; N, 21.93.



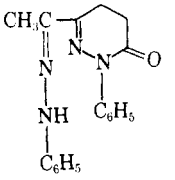
The nmr spectrum of the natural or synthetic 2,4-dinitrophenylosazone derivative in dimethyl sulfoxide-d_6 with an external TMS standard showed an apparent singlet at τ = 1.38 ppm and doublets centered at τ = 1.7 and 2.2 ppm (J = 10 cycles per second) for the aromatic CH groups, and poorly resolved absorption in the τ = 7–8 ppm region with a singlet at τ = 7.8 ppm for the CH_2CH_2 and CH_3. The integral ratio of aromatic:aliphatic protons was 6:7.

The infrared spectrum (KBr pellet) showed absorptions near 3420 cm^{-1} for OH, 3320 cm^{-1} for NH, near 2950 cm^{-1} for CH_2 groups and CH_3, 1715 cm^{-1} for free acid carbonyl, 1615 and 1600 cm^{-1} for C=C and C=N, and 1500 and 1340 cm^{-1} for NO_2.

The derivative formed from HKH as well as all of the other 2,4-dinitrophenylosazones tested (acetoin, 3-hydroxy-4-ketohexane, methylglyoxal, propionylmethylcarbinol) gave a bright purple color with 10% alcoholic KOH. Such osazones are usually too insoluble in water to give any color with aqueous alkali. Of those listed above only the HKH derivative gave a bright purple color with aqueous NaOH, and this was undoubtedly due to the presence of a carboxyl group in this derivative. Simple 2,4-dinitrophenylhydrazone derivatives of aldehydes, ketones, or keto acids gave orange, red, or brown colors with aqueous or alcoholic alkali.

Phenylosazone. Phenylhydrazine hydrochloride (400 mg) and 600 mg of sodium acetate·$3H_2O$ were dissolved in 4 ml of warm water. Natural or synthetic HKH (50–75 mg) was dissolved in 1 ml of water and added, and the solution was heated in a boiling water bath for 1 hr. After cooling, HCl was added to a pH of 2, and the supernatant was discarded. The precipitate (65% of theory) was washed well with water and recrystallized from 4-ml portions of hot ethanol to give cubic

crystals with a constant melting point of 188–189°. (There was no depression on mixing the derivatives.) This derivative was insoluble in alkali and the analytical data were consistent with a cyclized structure.



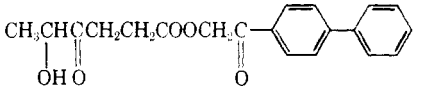
Anal. Calcd for $C_{18}H_{18}N_4O$: C, 70.56; H, 5.92; N, 18.29. Found (natural): C, 70.81; H, 6.09; N, 18.31. Found (synthetic): C, 69.07; H, 5.83; N, 18.95.

The nmr spectrum of the natural or synthetic derivative in $CDCl_3$ with 2% TMS standard showed a multiplet in the τ = 2.1–3.3-ppm region for the aromatic CH and NH groups, asymmetrical multiplets centered at τ = 6.9 and 7.3 ppm for CH_2CH_2, and a singlet at τ = 7.89 ppm for the CH_3. The integral ratios were 10:2:2:3.

The infrared spectrum (KBr pellet) showed absorption at 3360 cm^{-1} for NH groups, 1685 cm^{-1} for the free acid carbonyl, 1600, 1570, and 1495 cm^{-1} for the C=N and aromatic C=C, and 755 and 695 cm^{-1} for the monosubstituted phenyl groups.

In one experiment in which the sodium acetate and then the phenylhydrazine were dissolved directly in an aqueous solution of the HKH, and the heating was limited to 20 min, the open-chain derivative was apparently obtained, mp 179°, neut equiv 317 (calcd 324). However, this product was unstable and could not be obtained consistently, presumably because it changed to the cyclized structure on repeated recrystallization.

p-Phenylphenacyl Ester. An aqueous solution containing 100 mg of the natural HKH was neutralized to a pH of 6.8 with NaOH and diluted to 5 ml; 200 mg of *p*-phenylphenacyl bromide, dissolved in 15 ml of hot ethanol, was added, and the solution was refluxed for 1 hr. The chilled precipitate was washed with water, and recrystallized from ethanol–benzene to give 92 mg of long thin needles, mp 131–132°. *Anal.* Calcd for $C_{20}H_{20}O_5$: C, 70.57; H, 5.92. Found (natural): C, 70.65; H, 5.86.



The nmr spectrum of the phenylphenacyl ester in a $CDCl_3$–dimethyl sulfoxide-d_6 solution with TMS as a standard showed a multiplet in the τ = 2.0–2.8-ppm region for the aromatic CH groups, a singlet at τ = 4.7 ppm for $OCH_2C(=O)$, a quartet centered at τ = 5.67 ppm (J = 7 cycles per second) with a broad absorption under it for the CH and OH, multiplet centered at τ = 7.2 ppm for CH_2CH_2, and a doublet centered at τ = 8.7 ppm (J = 7 cycles per second) for the CH The integral ratios were 9:2:2:4:3.

The infrared spectrum (KBr pellet) showed absorption at 3460 cm^{-1} for OH, 2990 and 2940 cm^{-1} for CH_2 group, CH_3, and CH, 1752 cm^{-1} for the ester carbonyl, 1715 cm^{-1} for the ketone carbonyl, 1605 cm^{-} for the aromatic C=C, 835 cm^{-} for *para*-substituted phenyl, and 770 and 700 cm^{-} for the monosubstituted phenyl.

Discussion

The procedure employed in obtaining labeled HKH from ethanol-1-^{14}C utilized the ethanol in a trace concentration of high specific activity, and gave a relatively small amount of highly radioactive product. This was of some advantage in devising the purification procedures, since the product was followed by means of its radioactivity. The procedure was practical only because other endogenous substrates provided enough additional acetaldehyde to give a workable yield of the product. Although not designed for that purpose, the experiments clearly showed that this reaction took place when only trace amounts of ethanol or acetaldehyde were present in the homogenate.

Acknowledgments

We thank Mr. R. B. Babel, Mr. W. F. Minor, and Dr. L. C. Cheney of the Research Division, Bristol Laboratories, Syracuse, N. Y., for designing and carrying out the first three steps of the synthesis. We also thank Mr. A. L. Vulcano of Bristol Laboratories Research for the infrared and nmr spectra and their interpretation.

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