ation of sulfur has been reported (Iismaa, 1959) but the small oxygen volume limited the plant sample size to about 100 mg. In the work reported 1 g of dry plant material is combusted in a closed 5-l. oxygen-filled flask (Gutenmann and Lisk, 1960) followed by turbidimetric determination of sulfur as barium sulfate.

EXPERIMENTAL PROCEDURE

One gram of dry plant material was pressed into a pellet using a Parr pellet press having a 0.5 in. bore. The pellet was wrapped in a 1.5-in. square of Whatman No. 41 filter paper and placed in the platinum holder of the 5-l. combustion flask. Gases were absorbed in the flask using the solution of Lysyj and Zarembo (1958). This consisted of 100 ml of a solution containing 6% hydrogen peroxide and adjusted with 0.02 *N* sodium hydroxide to the methyl red endpoint. Combustion was conducted as previously described (Gutenmann and Lisk, 1960). The flask was rinsed with 50 ml of distilled water, which was then combined with the original absorbing solution and made to a total volume of 150 ml with water. An aliquot of this solution up to 50 ml was taken for analysis. The determination of sulfur was performed by the published method (Standard Methods, 1965).

DISCUSSION

The method was applied to the analysis of sulfur in a variety of plant materials. In past years these samples had been repeatedly analyzed by the A.O.A.C. (Official Methods, 1965) method for sulfur in plant material involving magnesium nitrate ashing followed by the turbidimetric determination of sulfur. Table I lists duplicate analyses of sulfur in plant material by the method described and the corresponding percent sulfur determined following the A.O.A.C. (Official Methods, 1965) ashing procedure. Ten replicated analyses of the same sample of Ladino clover showed that the relative standard deviation of the method was 4.04%. We believe the method described should be useful for the rapid determination of sulfur in plant material.

Table I. Percent Sulfur in Plant Material

Plant material	Ashing procedure	
	A.O.A.C.	Oxygen flask
Brome grass	0.22	0.19, 0.21
Ladino clover	0.27	0.25, 0.24
Orchard grass	0.24	0.21, 0.22
Sugar beet leaves	0.61	0.59, 0.59
Timothy	0.24	0.23, 0.24
White pine needles	0.13	0.14, 0.13

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A Potent Juvenile Hormone Mimic, 1-(4′-Ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene, Labeled with Tritium in either the Ethylphenyl- or Geranyl-Derived Moiety

Reduction of citral with sodium borotritide, conversion of the alcohol product to the bromo derivative, formation of the ether by reaction with 4-ethylphenol, and epoxidation yields 1-(4′-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene-*1*-^{3}H. Alternatively, tritiation of 4-ethylphenol with tritium water in sulfuric acid, reaction of the recovered phenol with geranyl bromide, and epoxidation yields 1-(4′-ethylphen-^{3}H-oxy)-6,7-epoxy-3,7-dimethyl-2-octene. The products have a high specific activity (33 to 654 mCi per mmol) and are useful in studies on the degradation and mode of action of this potent juvenile hormone mimic.

Interest in the potential use of juvenile hormones or related compounds eliciting a similar biological response (juvenoids) for insect control has led to the synthesis of several types of materials having a relatively simple structure compared to the natural product, but which also have equal or higher morphogenetic activity. There is a need for information on the biotransformations which occur with juvenoids of interest in various organisms and in the environment, and on the binding characteristics of these compounds at pertinent hormone receptor sites. These studies are greatly facilitated by the availability of radio-labeled preparations.

1-(4′-Ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene (compound I, Figure 1; code R-20458 of Stauffer Chemical Co., Mountain View, Calif.) combines a relatively simple structure with very high morphogenetic activity (Pallos *et al.*, 1971). It is desirable to have separate preparations of this

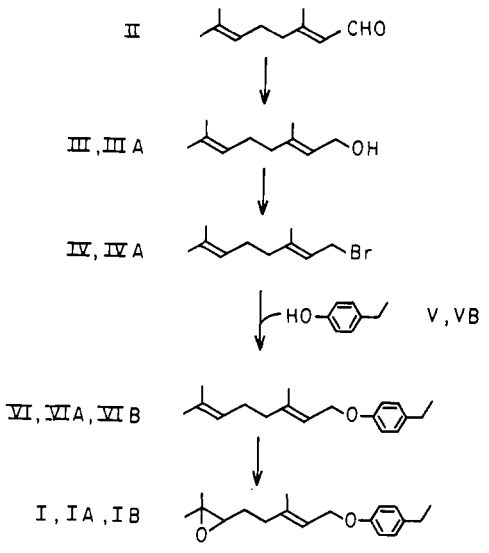

Figure 1. Reaction scheme used for preparation of 1-(4′-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene (I) labeled with tritium in the 1 position of the aliphatic chain (series A) or in the phenyl group (series B). The intermediates are geranial (II), geraniol (III), geranyl bromide (IV), 4-ethylphenol (V), and 1-(4′-ethylphenoxy)-3,7-dimethyl-2,o-6ctadiene (VI)

juvenoid with a radiolabel in the ethylphenyl- and geranyl-derived moieties because cleavage of the ether group is a possible reaction during metabolism or photodegradation. This paper describes two routes for the synthesis of such preparations. One route introduces tritium in the 1 position of the aliphatic chain and the second route labels the phenyl group with tritium. The reaction scheme is given in Figure 1, along with compound designations.

MATERIALS AND APPARATUS

Natural citral (a mixture of geranial, II, and its cis isomer, neral; Aldrich Chemical Co., Milwaukee, Wis.) was partially purified by thin-layer chromatography (tlc) on chromatographic alumina (Wako Chemical, Japan) by development with petroleum ether–ether mixture (10:1). Geraniol (III, Stauffer Chemical Co.) was distilled to obtain material showing only a single peak by gas–liquid chromatography (glc). 4-Ethylphenol (V, mp 47°C, 96% purity) and 3-chloroperoxybenzoic acid (Aldrich Chemical Co.) were used without further purification. The isotopes used were: sodium borotritide (100 mCi, 7 Ci per mmol, Amersham-Searle Co., Arlington Heights, Ill.) and tritium water (35 Ci per ml, *ca.* 1.2% ^3H_2O, Laboratory of Chemical Biodynamics, University of California, Berkeley, Calif.) and deuterium water (99.5% D_2O, Matheson Coleman & Bell, Norwood, Ohio).

Tlc was accomplished, unless noted otherwise, with silica gel F_{254} chromatoplates (Merck, Darmstadt, Germany) of 20-cm length and 0.25-mm gel thickness for analysis or 2-mm gel thickness for preparative separations. The primary solvent system used was a petroleum ether (bp 30 to 60°C)–ether mixture (10:1); it gave R_f values of 0.3 for I, 0.7 for II, and for neral (or citral), 0.3 for III and for nerol, 0.1 for V, and 0.7 for VI. The more difficult separation of the cis and trans isomers of the epoxy ether (IA) was achieved on silica gel HF chromatoplates (20 × 20 cm, 0.25-mm gel thickness, Analtech, Inc., Newark, Del.) by developing six times, three in the first dimension and three in the second dimension, all developments being with benzene–ether mixture (30:1), giving R_f values of 0.68 for cis and 0.63 for trans. The compounds were detected by their ability to quench gel fluorescence when viewed briefly under ultraviolet light or, when radioactive, by autoradiography. A Varian Aerograph Hy-Fi gas chromatographic apparatus, model 600B, equipped with a hydrogen flame detector and a 5-ft × 1/8-in. glass column packed with 5% (w/v) SE-30 on 60 to 80 mesh Chromosorb W, and operated at 160 or 205°C and a nitrogen gas flow of 20 to 25 ml per min, was used for glc. Although retention times (Rt) are only reported for the SE-30 column, all glc studies were confirmed with an OV-1 column [3% (w/v) OV-1 on Gas Chrom P under the same operation conditions]. A Beckman LS-150 liquid scintillation spectrometer was used for radioactivity counting and a Varian T-60 spectrometer was used for nuclear magnetic resonance (nmr) determinations, employing tetramethylsilane (TMS) as the internal standard.

METHODS OF SYNTHESIS

1-(4′-Ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene-*1*-^3H (IA). Citral (2.2 mmol) in methanol (1.0 ml) at −5°C was reduced with unlabeled $NaBH_4$ (0.26 mmol) for 15 min, then with NaB^3H_4 (100 mCi, 7 Ci per mmol) for several min until it reacted completely forming geraniol-^3H, and immediately thereafter with unlabeled $NaBH_4$, using a total of 0.5 mmol of reducing agent. The final reaction mixture was held for 15 min at −5°C and 2 hr at 25°C and then water (1.0 ml) was added; the resulting oily layer was recovered and combined with a pentane extract (2 ml × 4) of the remaining aqueous solution. (Although 93% of the radioactivity was recovered in the organic phase, some was in a readily exchangeable form.) After drying the pentane extract with sodium sulfate and removing the solvent by evaporation, preparative tlc gave a gel region corresponding to geraniol (IIIA) and its cis isomer (nerol); the oil recovered on extraction of this gel region with ether was equivalent in weight to 1.51 mmol of the alcohol(s), or 75.6% recovery (based on the $NaBH_4$ used).

A portion of the alcohol product (IIIA, 1.4 mmol containing a small amount of cis isomer impurity) was treated with phosphorus tribromide (0.61 mmol) in petroleum ether at −18°C for 1 hr. (These conditions were selected to minimize rearrangement.) Ice (2 g) was added, and the mixture was extracted with petroleum ether (12 ml × 6), the extract was washed with water and bicarbonate solution, and concentrated under a nitrogen stream. The geranyl bromide (IVA) residue (about 1.3 mmol) was reacted with a stirred solution of 4-ethylphenol (V, 1.2 mmol) and potassium carbonate (24 mmol) in dimethoxyethane (0.5 ml) at 25°C for 18 hr and then at 70 to 80°C for 7 hr. The reaction mixture containing no residual geranyl bromide was filtered, concentrated under nitrogen, and subjected to preparative tlc to give the diene ether product (VIA, 0.69 mmol or 49.4% recovery based on the alcohol used). The specific activity of the diene product (VIA) was 33 mCi per mmol *vs.* a theoretical value of 50 calculated on the basis of complete reaction of the NaB^3H_4 and no loss of tritium by exchange.

The diene ether (VIA, 0.45 mmol) was epoxidized by treatment with 3-chloroperoxybenzoic acid (0.44 mmol) in dichloromethane (4 ml) at 2°C for 20 min and the tritiated product (IA) was isolated by concentrating the reaction mixture, filtering off precipitated 3-chlorobenzoic acid which was washed with petroleum ether, concentrating the combined filtrate, purifying the product by preparative tlc, and extracting the appropriate gel regions with ether to give the desired epoxide product (IA, 0.27 mmol, 90% recovery, based on the reacted diene, 33 mCi per mmol) and the unreacted diene (VIA, 0.15 mmol).

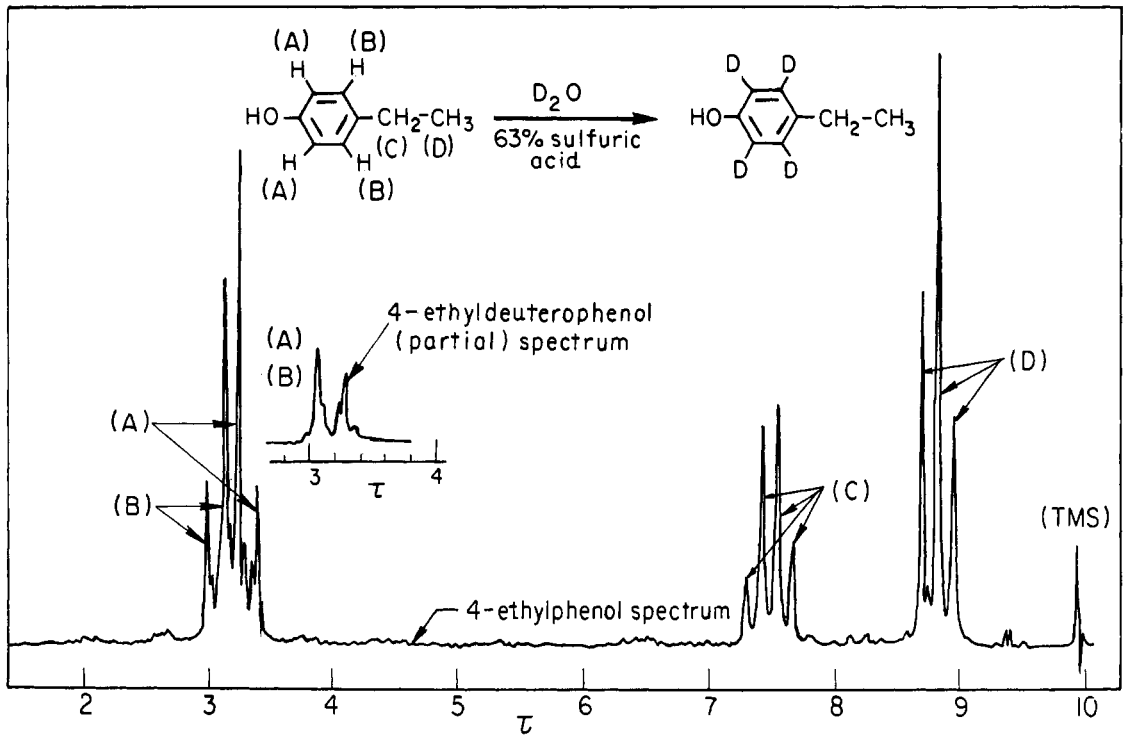

Figure 2. Nmr spectrum of 4-ethylphenol, shaken with D_2O, and partial nmr spectrum of 4-ethyldeuterophenol (offset insert) in CCl_4 solutions, showing the only region of change resulting from 72% deuteration of the aromatic protons

1-(4′-Ethylphen-^3H-oxy)-6,7-epoxy-3,7-dimethyl-2-octene (IB). Conditions appropriate for tritiation of 4-ethylphenol were determined by heating the phenol with sulfuric acid or potassium hydroxide in deuterium water in a closed reaction vessel at 80 to 100°C for intervals varying from several hours to 3 days. The extent of deuterium exchange was calculated from the nmr integration curve using the three protons of the terminal methyl group as a standard. Almost no deuterium incorporation occurred under basic conditions, but 33 to 72% deuteration was obtained under the acidic conditions used. Other acidic reagents, such as phosphoric acid and boron trifluoride, were tried but without improvement. As shown in Table I, sulfuric acid at 70% or higher concentration reduced the phenol recovery, possibly by sulfonation of the phenol. With 65% sulfuric acid, the percentage deuteration increased with reaction time and, even after 40 hr, the phenol recovery was 49%. The 63% sulfuric acid concentration was selected as an adequate compromise for high deuterium incorporation and satisfactory yield; under this condition, the yield was 60% and the deuteration of all aromatic protons was 72% complete without any exchange of methyl- or methylene protons (Figure 2). Conversion of this deuterated phenol to the diene ether and epoxy ether products was found, by nmr analysis, to result in no significant deuterium loss; in this determination the two methylene protons adjacent to the ether oxygen were used as a standard.

In the preparation of 4-ethylphenol-^3H (VB), fuming sulfuric acid (0.8 ml, approximately 23% free SO_3) was added, dropwise, to tritium water (1.0 ml, total activity of 35 Ci), with cooling in an ice bath, to give a final acid concentration of approximately 63% sulfuric acid. 4-Ethylphenol (244 mg) was suspended in the diluted sulfuric acid and, after closing the 20-ml flask with a glass stopper, the mixture was held at 83 to 85°C for 47 hr. The reaction mixture was cooled in an ice bath, diluted with water, and extracted with benzene; the benzene extract was washed with water (to remove the easily exchangeable tritium), dried by passing through a column packed with anhydrous sodium sulfate, and concentrated under nitrogen to give a dark brown oil (about 680 mCi). Preparative tlc, using chloroform for development, was used to isolate the phenol (R_f 0.16 to 0.42) which was recovered from the gel by extraction with ether. The ether solution, when concentrated under nitrogen, gave a pale brown oil which solidified at −20°C (*ca.* 70 mg, *ca.* 600 mCi per mmol). While being heated, the reaction mixture containing tritium rapidly turned dark brown and a tar was formed, but this does not occur with the deuterated product. The reduced yield and larger tar formation with the tritium reaction probably resulted from the high level of radioactivity involved.

Geranyl bromide [IV, 0.87 mmol, prepared from pure geraniol (III) as previously described] was heated with 4-ethylphenol-^3H (VB, the total amount of product prepared) in dimethoxyethane (0.5 ml) containing potassium carbonate

Table I. The Effect of Acid Concentration and Reaction Time on the Degree of Deuteration of the Phenyl Group of 4-Ethylphenol (126 mg) with Deuterium Oxide (2.5 ml) in Sulfuric Acid

Conditions varied		4-Ethylphenol recovered	
Sulfuric acid, %	Reaction time, hr	Deuteration, %	Phenol recovery, %
50	15	45	96
62	15	55	49
70	15	52	12
80	15		0
65	3.5	33	99
65	6	45	78
65	12	45	76
65	24	60	52
65	40	65	49

(1.2 mmol) at 80 to 88 °C for 8 hr, with stirring. Preparative tlc yielded the diene ether (VIB, 0.437 mmol, 654 mCi per mmol) and unreacted 4-ethylphenol-^3H (VB, 0.06 mmol). Epoxidation of the entire amount of the diene ether prepared and tlc isolation gave the desired epoxy ether product (IB, 0.17 mmol, 654 mCi per mmol) and unreacted diene ether (0.05 mmol). The slight increase of the specific activity of the products (VIB and IB) over that anticipated from the tritiated phenol (VB) probably resulted from the removal of an unlabeled impurity such as a solvent in the subsequent reaction and purification steps; this also established that exchange of the tritium is not likely to occur under the usual handling procedures.

DISCUSSION

The epoxy ether product labeled in the geranyl-derived moiety (IA) is not isomerically pure because the synthesis started with citral, a mixture of the cis and trans isomers, and complete resolution of isomers was not achieved in the subsequent synthesis and purification steps. The citral used gave two peaks on glc in a 2:3 ratio of cis and trans at Rt of 3 and 4 min, respectively, at 160°C. The diene ether (VI) obtained from the above source gave two peaks in a 9:16 ratio of cis and trans at Rt of 25 and 29 min, respectively, at 205 °C. Each of two peaks from the diene ether (VI) in glc gave an identical spot on tlc with the *trans*-diene ether, which co-gas chromatographed with the peak of the longer retention time. The epoxide ether product (I) was not successfully gas chromatographed under these conditions and a peak did not appear, even after 1 hr at 205°C. However, the isomeric composition of the epoxide ether product was probably similar to that of the diene ether intermediate. This assumption was indirectly supported by the finding that the epoxidation reaction is not preferential for one of the isomers; thus, the isomer ratio of the diene ether remaining unreacted after epoxidation with oxidant:diene ether ratios of 0.5:1 to 2:1 was the same as that of the starting material, as determined by glc. Finally, the radioactive epoxide ether (IA) was shown on tlc to be a mixture of cis and trans isomers in a 9:16 ratio, by extracting each spot and counting the radioactivity, but the condition was not used for a preparative resolution. The final desired product (IA), with the high specific activity, gives a nmr spectrum superimposable throughout with that for the authentic material prepared from pure geraniol (pure by glc criterion). Further, the radio-labeled product (IA) shows essentially identical potency in the *Tenebrio* assay with the authentic unlabeled compound, and it induces normal development of allatectomized *Schistocerca americana* (Hammock *et al.*, 1971).

The ring-labeled compound (IB) consists almost entirely of the desired trans isomer but the question arises here of possible tritium loss by exchange during etherification and epoxidation. This was not a problem, based on nmr studies with deuterium compounds and specific activity determinations with the tritium compounds.

The two labeled preparations of the epoxy ether juvenoid (IA and IB) have adequate specific activities for use in metabolism and photodegradation studies. When they were subjected to separate but identical experiments, more than eight products were formed from each labeled preparation of the juvenoid (I) on exposure to sunlight for 8 hr as a deposit on a silica gel tlc plate, and at least 16 metabolites were produced by a selected rat liver enzyme preparation (Singh *et al.*, 1971). Each of these 16 metabolites, produced in greater than 2% yield, is detected with both the chain-labeled and ring-labeled preparations; so the ether group is not cleaved to an important extent in the metabolism system used and the labeled preparations are appropriate for investigation of metabolism and photodecomposition reactions.

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