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SYNTHESIS OF RADIOLABELED JUVENILE HORMONE ANALOGS AND CHIRAL HOMOLOGS

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ABSTRACT

Radiolabeled juvenile hormone (JHs) and labeled JH derivatives are required for the study of JH metabolism and JH-protein interactions as well as JH radioimmunoassay in insects. The progress in the synthesis of these labeled compounds for the last five years is reviewed, including the preparation of photoaffinity labels, enantiomerically enriched JH I and JH II with ^{3}H labels, and radioiodinated analogs of JH I, methoprene and phenoxyphenyl ether insect growth regulators (IGRs). Experimental details are given for the efficient synthesis of two ^{3}H-labeled photoactivatable JH analogs, EFDA and EFTP, their photolysis in methanol, and their competitive binding assays performed on the Manduca sexta JH binding proteins (JHBP).

INTRODUCTION

Juvenile hormones (JHs) are synthesized and secreted in insects by the pair of neurosecretory organs, the corpora allata, and have a variety of biological effects during the insects' lives.[1] The two primary effects of juvenile hormones are (i) morphogenetic effect, i.e., the regulation of the growth and development of larvae and prevention of metamorphosis, and (ii) gonadotrophic effect, i.e., the regulation of reproduction, particularly vitellogenesis, in many adult insects. The mode of action of the hormones is not well understood on a molecular level, and its study forms one of the main themes in our current research on juvenile hormone bioorganic chemistry.

The history of JH research can be traced back to Wigglesworth's discovery of the "inhibitory hormone" in the mid-1930's.[2] He presented evidence that a blood-borne, hormonal factor from the corpora allata of the blood-sucking bug Rhodnius prolixus served to prevent metamorphosis of the larvae. The term was soon altered to "juvenile hormone" after the active role of the hormone in producing larval characters in adults was recognized.[3] Based on the pioneering work of Williams, who reported high levels of JH activity in the abdomens of adult male Hyalophora cecropia moths,[4] and the prediction of Bowers et al. on the structural similarity of natural JH to synthetic methyl 10,11-epoxyfarnesoate,[5] the structure of JH I (1) was determined by Röller et al. in 1967 to be methyl (2E,6E)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate.[6] It was not long before the two major homologs were characterized : JH II (2) by Meyer et al.,[7] and JH III (3) by Judy et al.[8] The other minor homologs, JH 0 (4)[9] and 4-Me-JH I (5)[10], were recently identified by Bergot et al.

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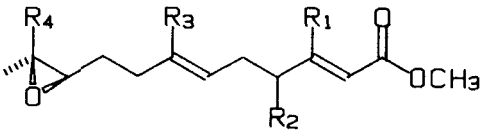
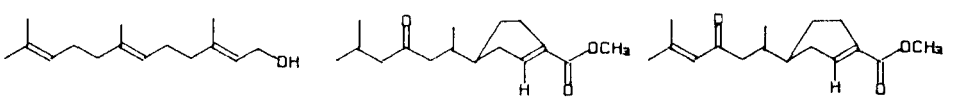
It should be noted that JH III had been synthesized by Bowers et al.[5] in advance of its identification at the most common juvenile hormone. Indeed, JH III is the only JH in insects outside the order Lepidoptera.[11] However, the presence and proportion of these homologs in an insect within this order is still species dependent.



JH 0	(1)	R_1=R_3=R_4=CH_2CH_3, R_2=H
JH I	(2)	R_1=CH_3, R_2=H, R_3=R_4=CH_2CH_3
JH II	(3)	R_1=R_3=CH_3, R_2=H, R_4=CH_2CH_3
JH III	(4)	R_1=R_3=R_4=CH_3, R_2=H
4-MeJH I	(5)	R_1=R_2=CH_3, R_3=R_4=CH_2CH_3

FIG. 1.

While the investigation on the structures of the hormones was under way, numerous JH analogs (JHAs) or juvenoids were characterized and synthesized [12a,b,c]. Farnesol (6) was the first pure compound identified[13] in 1961 to possess the similar biological effects as those of juvenile hormones found by Wigglesworth. Later, the famous "paper factor"[14] was related to juvabione (7)[15] or dehydrojuvabione (8),[16] as shown in FIG. 2.



Farnesol (6) Juvabione (7) Dehydrojuvabione (8)

FIG. 2.

Some of the JHAs found were more active and more stable than the natural hormones. Thus, compounds marketable as insect growth regulators and pest control agents were produced, although these have fallen short of being the much-heralded "third generation of pesticides" predicted by Williams.[17] Anti-juvenile hormone analogs (AJHAs) were also explored because of a less critical timing in application, a shorter response time, high efficacy in control of larval pests, and additional inhibitory effects on insect reproduction. It was shown that the ultimate result of AJHAs should be the same as that of surgically removing the corpora allata from early larval instars.[18a,b,c] Since then, the research has progressed to meet the challenge of elucidating the detailed regulation of macromolecular events by examination of the hormone-protein and

receptor-gene interactions on a molecular level.

Accordingly, at the early stages of the research, the application of radiolabeled JH and JH analogs in the biological studies focused mainly on the biosynthesis, distribution, and metabolism of JHs and JHAs, as summarized by Hammock and Quistad[19] and Jennings et al.[20] Most of the labeled compounds covered in these reviews were of low specific radioactivity, that is, less than one ^{3}H or ^{14}C atom-equivalent incorporated into each molecule as a result of the synthetic design and the availability of the source of the radiolabel. Hence, their specific activities were well below the maximum achievable specific radioactivity of 29.1 Ci/mmol for one ^{3}H atom-incorporation calculated based on the half-life time of ^{3}H (12 years).[21] In addition to this, most of these synthetic materials were racemic. Racemic compounds of relatively low specific activity are inadequate for the study of putative low-abundance high affinity JH binding proteins and receptors which are expected and have been shown to have chiral binding sites.[22] Our JH research has recently focused on redesigning the synthesis of labeled JH homologs and JHAs. ^{3}H-labeled chiral JH enantiomers have been synthesized in very high specific activity and enantiomeric purity. Moreover, no-carrier-added, short-half-life radionuclides, such as ^{125}I, have been introduced for the radiolabeling of JH analogs. These newer probes might prove to be more useful in the search for JH receptors.

We will focus our attention on these developments in addition to updating the reviews of Hammock and Quistad[29] and Jennings et al.[20] with the latest reports on the synthesis of labeled JH analogs for studies of their distribution and metabolism. We will also summarize the synthesis of radioiodinated JH derivatives for JH radioimmunoassay. Finally, we will describe some JH analogs designed specifically for the identification and characterization of JH binding proteins and receptor proteins. In particular, an improved synthesis of [10-^{3}H]-10,11-epoxyfarnesyl diazoacetate (EFDA) and its modification into the trifluorodiazopropionate analog (EFTP) will be discussed with experimental details.

RADIOLABELED JUVENILE HORMONES

Until 1984, all the commercially available or readily obtainable JHs, both radiolabeled and radioinert, were racemic mixtures. The [10-^{3}H]-labeled racemates produced by New England Nuclear have maximum specific activities of 10-15 Ci/mmol.[20] Bergot et al. also reported a synthesis of an unnatural JH homolog, [10-^{3}H]-ethyl (2E,6E)-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-dodecadienoate with a specific activity of 6.78 Ci/mmol by treating [10-^{3}H]-JH II with dry ethanol in the presence of NaCN.[23] The labeled homolog was used in the GC-MS determination of JH titers. However, it has been established that the naturally occurring, biologically relevant enantiomers of JHs are those with absolute configuration of (10R,11S) for JH I and JH II, and (10R) for JH III.[24a,b,c] The presence of the unnatural enantiomer could lead to competitive displacement of the natural analog or to excessively high non-specific binding,[25] while the low specific activity causes difficulties in the identification of receptors with dissociation constants smaller than 1 nM. These problems can be overcome by using enantiomerically enriched (10R,11S)-JHs labeled with high specific activity (more than one ^{3}H atom incorporated into one molecule).

Enzymatically synthesized, optically pure ^{3}H-labeled (10R,11S)-JH I with specific radioactivity of 53 Ci/mmol has been reported by using the O-methyltransferase of male Hyalophora cecropia accessory glands to esterify enantioselectively the (10R,11S) antipode of racemic JH I acid with S-[methyl-^{3}H]adenosylmethionine.[26] The acid was obtained in turn from the action of a crude juvenile hormone esterase preparation from Manduca sexta on racemic JH I. Tobe et al.[27a,b,c] observed that the corpora allata of the cockroach Diploptera punctata are efficient scavengers of L-methionine and they are able to utilize even low concentrations of the substrate for JH III biosynthesis at linear high rates for at least 24 hr in vitro. It is possible that the corpora allata of Diplotera punctata can biosynthesize significant quantities (up to 0.6 µg per pair for a 24 hr incubation)[27a] of radiolabeled JH III in vitro, even in the presence of only micromolar quantities of [^{3}H]-L-met which is commercially available with high specific activity (60-80 Ci/mmol). However, these enzymatic procedures have three major drawbacks : (1) the labile methoxyl-^{3}H moiety can cause serious background problems due to nonspecific hydrolysis, (2) frequent repetition of the procedure is necessary in order to produce reasonable amounts of hormones, and (3) labeled, optically active structural analogs could not be obtained in this fashion. Nonetheless, the authenticity of the hormones produced enzymatically should not be ignored.

In 1984, we developed a new strategy by adapting new asymmetric synthetic methodology to traditional stereoselective JH synthesis. Using this strategy, we synthesized both enantiomers (10R,11S) and (10S,11R) of JH I[25] and JH II.[28] Bioassays[25,29] revealed that, in contrast to the JH III enantiomers which differ in relative binding affinity by nearly a factor of 100, the more lipophilic JH I and JH II enantiomers differ by only a factor of less than 10. This implies that the sensitivity to lipophilicity overrides the sensitivity to epoxide absolute configuration for the JH binding proteins used. However, the competitive abilities of the JH I enantiomers appeared equivalent when the unnatural (10S,11R)-JH I is used as the ligand.[25]

The synthesis follows known stereoselective reaction sequences for the elaboration of the trisubstituted (Z)-10 olefin to give (9) and (10), which allows the introduction of the chiral epoxide and highly labeled ethyl moiety late in the synthesis. Thus, asymmetric epoxidations using both enantiomers of diethyl tartrate were performed on (9) and (10) to obtain both enantiomers of the epoxides. The (+)-α-methoxy-α-(trifluoromethyl)phenylacetates of the epoxy alcohols (11) and (12) revealed that the enantiomeric excesses of the epoxidations were better than 95%. The precursors for radiolabeling, (13) and (14), were prepared in two steps from (11) and (12). The labeling was performed in benzene under 740 torr of carrier-free tritium gas with 1.2 equivalent of tritium-presaturated $(PPh_3)_3RhCl$ present. A specific activity of 58 Ci/mmol was achieved in the labeling to get (15) and (16). As we have seen, this is the highest achievable for two ^{3}H atoms incorporated into each molecule.[21]

The value of the intermediates (11) and (12) lies not only in the synthesis of chiral, labeled enantiomers of JH I and JH II, but also in other transformations of the hydroxyl group. Indeed, starting from (11) and (12), we are able to synthesize (i) the ^{125}I labeled JH I analog; (ii) antigenic ligands for use in JH radioimmunoassays; and (iii) chiral and tritium labeled JH III enantiomers.

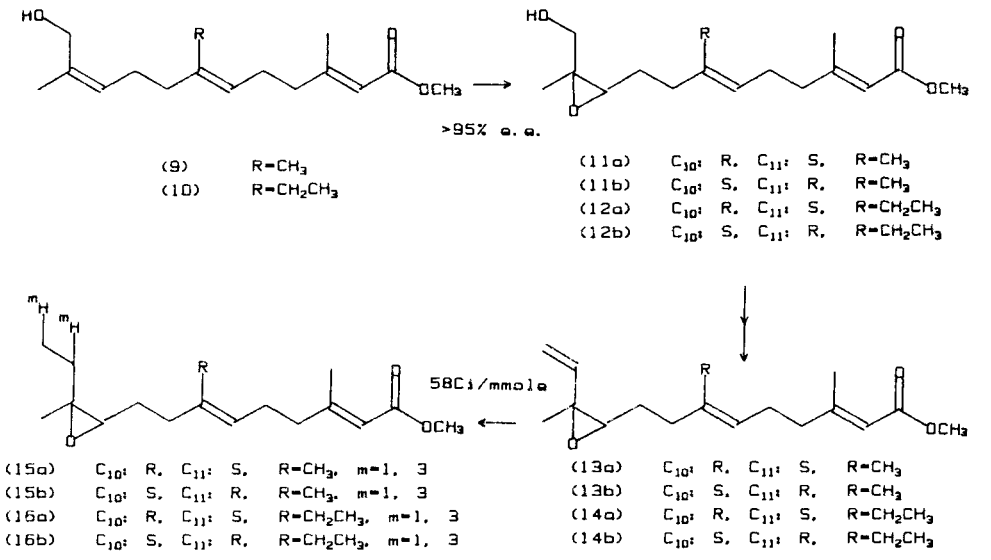


FIG. 3.

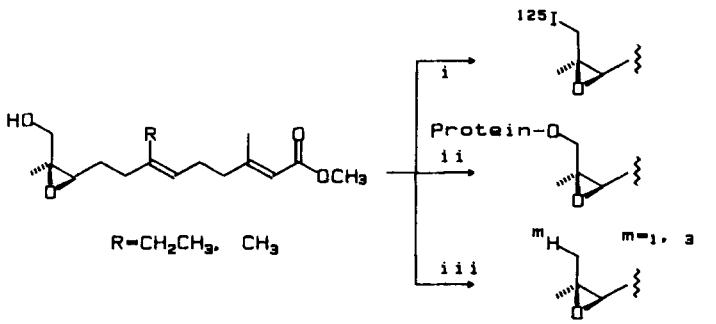


FIG. 4.

RADIOLABELED TRACERS USED IN JH RADIOIMMUNOASSAY

Radioimmunoassays (RIAs), first developed in the late 1950s, have since revolutionized the technology of quantifying submicrogram levels of hormone in endocrinology. The general principles of RIAs are well documented.[30a,b,c]

One of the necessities for an RIA is a radiolabeled tracer, where short half-life γ-emitting radionuclides are favored over β-emitters. This is mainly due to the ease with which the former can be counted and also due to the much higher specific activity the former offers. For instance, the specific activity of ^{131}I is about 16,000 Ci/mmol while that of ^{125}I is 2,200 Ci/mmol, which

is still 75 times higher than ^{3}H and 35,000 times higher than ^{14}C. But, if we take into consideration the potential technical problems caused by the very short half-life time (8 days) and the low commercially available abundance (20%) of ^{131}I, then ^{125}I (half-life 60 days, 100% abundance in no-carrier-added form) would appear to be the preferred isotope for use in RIAs. Finally, the ease of substitution of radioiodine into tyrosine residues of polypeptides allows the convenient application of radioiodine for the preparation of labeled protein tracers. The most widely used methods of iodination, such as in the chloramine-T method, involve the oxidation of $Na^{125}I$ in the presence of a protein. In a pH 7.5 solution, chloramine-T, the sodium salt of the N-monochloro derivative of p-toluenesulfonamide, breaks down slowly forming hypochlorous acid which consequently oxidizes $Na^{125}I$ into cationic iodine, $^{125}I^{+}$. The iodine atom substitutes ortho to the hydroxyl group in tyrosine.[31] Usually, those tyrosine residues on the surface of the protein would get iodinated more readily; histidine and tryptophane residues may also react.

In general, there might be behavior differences between labeled and unlabeled hormones, especially when the label is other than an isotope of an atom in the natural hormone, as in the case of ^{125}I labeling. What is measured in RIAs is, however, immunochemical behavior, which may or may not be identical with biological activity. That is to say, it is possible for a modified hormone to lose one of the biochemical activities, such as the ability to bind to hormone receptor sites, while a second activity, such as the ability to bind to antibodies, is unaffected. For example, RIAs for ecdysone and JH have difficulty in distinguishing hormonally active form from inactive metabolites. Considerable chemical modifications have been employed in the preparation of labeled tracers without regard for the loss of essential binding moieties.

Juvenile hormones, being small molecules, are not independently immunogenic and therefore must be conjugated to a protein which contains either a tyrosine or a histidine residue to elicit the production of antibodies. This is one of the problems encountered in the JH-RIA research, as reviewed by Granger and Goodman[32], which caused the lag of development of JH-RIAs for the critical needs in JH research. We will evaluate several pathways used to synthesize the labeled protein conjugates.

On the skeletons of juvenile hormones, only two moieties can be found to be potential tether points for the required conjugations, each of which defines a completely different radioimmunological system :

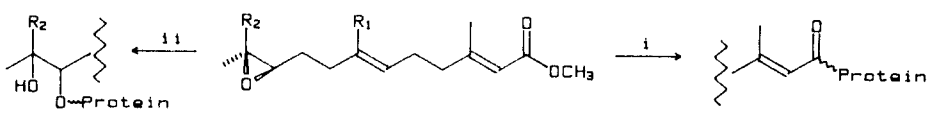


FIG. 5.

i) Carboxy carbon : This conjugation pathway was first explored by Lauer et al.,[33] and was later developed with success by Baehr et al.[34a,b,c] They hydrolyzed the carbomethoxy moiety under mild alkaline conditions and coupled the carboxylic function to N-hydroxysuccinimide (NHS) in the presence of N,N'-dicyclohexylcarbodiimide (DCC). An ε-amino group of lysine in human serum albumin (HSA) then reacted with the activated NHS ester (18) to produce the immunogens (19), in which JH was coupled through an amide linkage. The radioiodinated tracer (21) was made by reaction of histamine with the activated ester. The JH-histamine (20) was then radioiodinated with the chloramine-T technique to achieve a specific activity close to 2000 Ci/mmol.

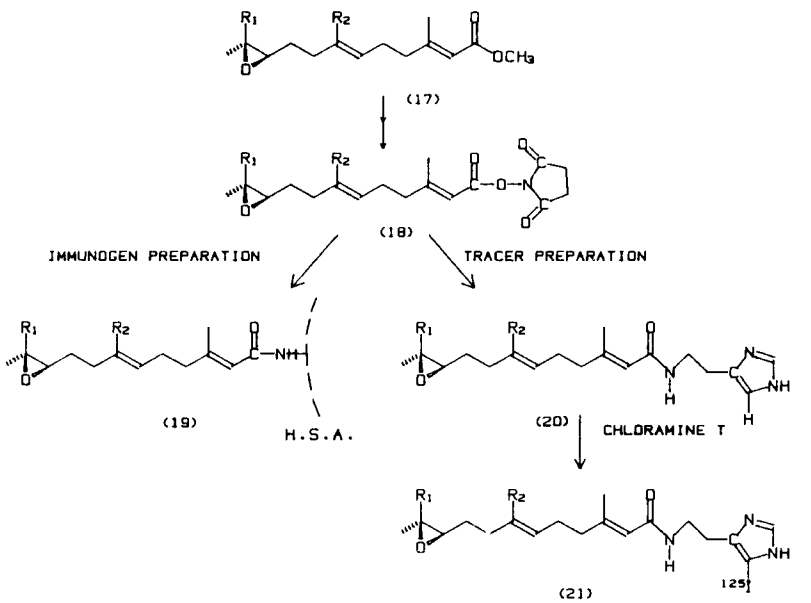
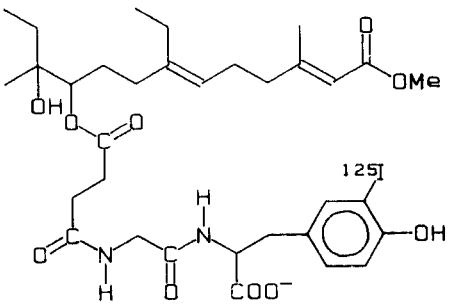


FIG. 6.

High titer antibodies were raised to JH I, JH II, and JH III immunogens, respectively. These were partially specific for their respective haptens so that they can potentially be used to quantify JH I and JH III (JH II cross-reactivity is 37% with JH I antiserum and 0.9% with JH III antiserum). As expected, these antisera cannot distinguish the active JH methyl esters from the inactive JH acids.

A patent by Delaage[35] described the preparation of labeled antigen for JH radioimmunoassay which is composed of an antigen-dipeptide conjugate, the dipeptide being a condensation product of tyrosine and another peptide with the tyrosine group carrying the radiolabel which was achieved using chloramine-T. However, the subsequent assay was not reported.

ii) Epoxy carbon : This alternative pathway was developed by Strambi et al.[36a,b,c] After acidic opening of the epoxide, the glycol obtained was derivatized into the corresponding hemisuccinate to be further coupled to serum albumin. The radioligand (22) was obtained by coupling glycyl-tyrosine to JH I-diol-hemisuccinate, then iodinated (FIG.7). The advantages of this pathway are : (1) the diol is six-fold more immunoreactive than the hormone itself; and (2) the diol has better solubility in water and chromatographic behavior that is adjustable with pH, so that both the diol and the iodinated derivative can be readily separated from liquid contaminants. The sensitivity of the RIA developed through this pathway is comparable with that of physico-chemical analysis.[36c] However, the antibodies thus raised recognized the diols of JH I, JH II and JH III equally with the parent epoxides. Nonetheless, JH acids do not cross-react and homolog specificity of the antisera is acceptable. The RIA utilizing this antiserum can measure total JH titer only after derivatization to the diol forms so that considerable experimental tedium is necessary to obtain titer data.



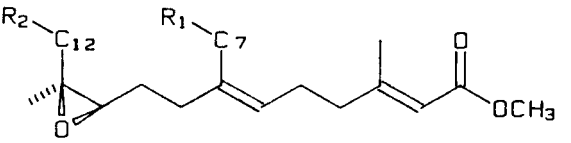
(22)

FIG. 7.

It has long been recognized that antibodies can discriminate between optical and geometrical isomers. Unfortunately antibodies to hydrophobic haptens such as the JHs tend to exhibit greater immunologic cross-reactivity with structurally related haptens than do antibodies to more polar molecules and only commercial racemic JHs were used in both of the pathways mentioned. Therefore, the antibodies thus generated will undoubtedly contain various populations of antibodies which recognize, in part, the configuration around the chiral centers. In addition to this, the functional sites used for the haptenic linkage involved important recognition sites (ester or epoxide), and it was not possible to raise antibodies which can recognize both characteristic functional groups of JHs.[32]

These problems might be solved by using the hydroxyl moiety in the chiral intermediate (11) and (12) mentioned before as the tether point to couple to the carrier macromolecule so that both epoxy and carbomethoxy functionalities

are retained as the recognition sites for the antibodies. This work, as well as the efforts directed toward synthesizing derivatives (23) tethered at the C-7 ethyl group (FIG. 8), are in progress in our laboratories.



(11) R_1= H, R_2= O-protein

(12) R_1= CH_3, R_2= O-protein

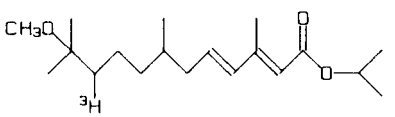
(23a) R_1= O-protein, R_2= H

(23b) R_1= O-protein, R_2= CH_3

FIG. 8.

RADIOLABELED JUVENILE HORMONE ANALOGS

We can classify the labeled JHAs into two groups : (1) those synthesized to study the distribution, metabolism and environmental fates; and (2) those synthesized to assay binding and thus to identify JH binding proteins and receptor proteins. The reviews by Hammock and Quistad[19] and Jennings et al.[20] have covered most of the compounds in the first group, which includes labeled methoprenes (24,25), hydroprene (26), and epoxyfarnesyl propenyl ether (27) [37a,b], as shown in FIG. 9. Here, we will only update the coverage.



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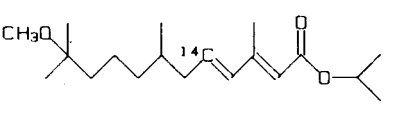

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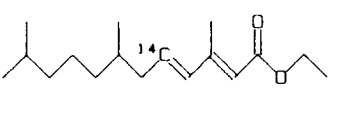

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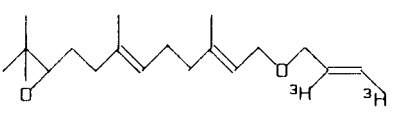
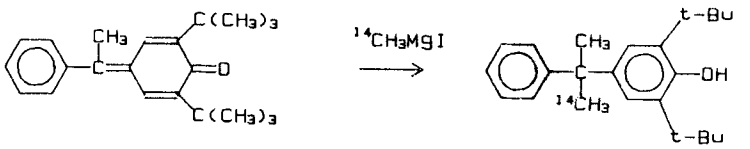


(27)

FIG. 9.

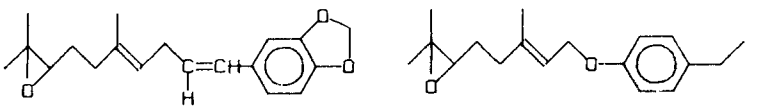
2,6-Di-t-butyl-4-(α,α-dimethylbenzyl)phenol was synthesized and shown to be an effective mosquito larvicide. Its action falls into the category of that of juvenoids. In order to investigate its distribution and decomposition in the environment and in the organisms of insects, the ^{14}C-labeled form of this compound (29) was prepared from (28) (FIG. 10).[38] No specific activity or bioassay results were reported.



(28) (29)

FIG. 10.

In contrast to being insecticides, JH analogs, such as (30) and (31), were shown to prolong the life time of the 5th instar of the silkworm (Bombyx mori), increase the synthesis of silk protein, and thus increase the silk production.[39a,b,c,d]



(30) (31)

FIG. 11.

A wide variety of JHAs have been used in China for this purpose since 1974 and the studies of the mechanism involved have been reported.[40a,b,c,d] Labeled JHAs were synthesized to study the absorption, distribution and excretion of JHA in the silkworm body and the rate of decay and residue of JHA in the fingerling of grass carp (Ctenopharyngodon idellus), since frass and dead pupae of silkworms were sometimes included in the fish food.[41a,b,c]

Thus, 1-[α,α-$^{3}H_2$-p-ethylphenoxy]-7-ethoxy-3,7-dimethyl-2-octene (33)[42] and 1-[α,α-$^{3}H_2$-p-ethylphenoxy]-3,7-dimethyl-6,7-epoxy-2-octene (37)[43] were synthesized by similar pathways in reasonable chemical yields and specific activities : 26.3 Ci/mmol and 21.6 Ci/mmol, respectively. The key labeling reaction was tritium gas reduction of acetylphenol in acetic acid with 10% Pd-C as catalyst. The synthetic schemes are shown in FIG. 12.

Juvenile hormone-protein interactions are involved at all levels of titer regulation, biosynthesis and transport, excretion and metabolism, and cellular mechanisms of action. The interaction of JH with binding proteins controls the

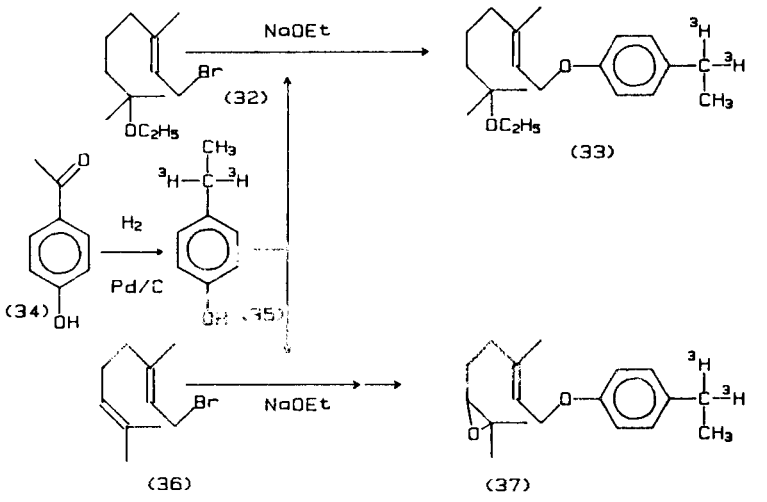
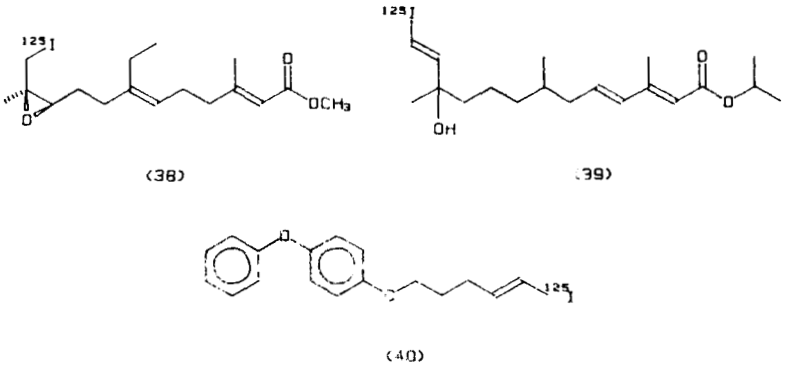


FIG. 12.

JH titer regulation and delivery of the hormone to target sites, while the interaction of JH with receptor proteins is believed to be a key factor in the hormonal regulation of gene expression in insects. The following events have been postulated for the interaction of JH with receptors:[44] (1) binding of JH to a cytoplasmic receptor, (2) translocation of the hormone-protein complex to the nucleus, (3) binding of the complex to an acceptor site on nuclear chromatin; and (4) activation of transcription to give new mRNA. But, the search for the JH receptors has been difficult, largely due to the lack of optically pure probes labeled with very high specific radioactivity. In addition to this, the metabolic degradation of the probe and more importantly the dissociation of the probes from the receptors have caused problems.

An iodine atom can be used as a steric mimic of a methyl group. As we have already discussed, the introduction of the short-half-life γ-emitters can increase the specific activity. Thus, several radioiodinated juvenile homone analogs, (38-40), have been synthesized in our laboratories as experimental JH receptor probes (FIG. 13) (G. Prestwich, W. Eng, M. Boehm and J. McKew, unpublished results). The synthetic details, biological activities and biochemical results will be reported in due course.

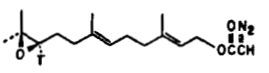
The problems of ligand dissociation from the binding protein were solved by the synthesis of 10,11-epoxyfarnesyl diazoacetates (EFDA, 41),[44] which is a JH photoaffinity label with the diazoacetate mimicking the carbomethoxy group in the parent hormone (FIG.14). The diazoacetate moiety has been shown to be easily incorporated into substrates with minimal stereoelectronic perturbation. The binding affinity of EFDA for the hemolymph JH binding proteins of M. sexta was determined as 40% relative to JH I[45] or as high as 200%[46b], and the ^{3}H-labeled form has been extensively used as a photoaffinity label for JH binding proteins in the hemolymph of Locusta migratoria and Leucophaea maderae as well as



(38) (39) (40)

FIG. 13.

Drosophila Kc cells.[46a,b,c,d,e] Competition assays and photoaffinity labeling involving ^{3}H-EFDA were also performed on the hemolymph JH binding proteins from the budworm H. virescens, the cabbage looper T. ni, and the gypsy moth L. dispar.[29] The results from these bioassays showed that even at concentrations below 10 nM, EFDA was able to bind specifically to the JH binding proteins and modify them covalently upon being irradiated for as short as 5 to 20 sec at 254 nm. Using ^{3}H-EFDA as a tracer, the hemolymph JH binding protein of M. sexta was purified to homogeneity and its N-terminal amino acid sequence was determined up to the 35th residue,[47] thus confirming the sequence of the first eighteen residues reported by Peterson et al.[48] The photomodification of proteins by EFDA is expected to occur via acylcarbene and the corresponding rearranged alkoxyketene, as shown in FIG. 14 :



(41)

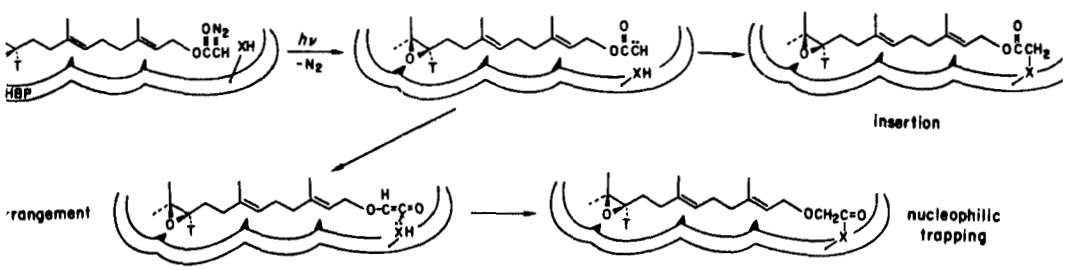
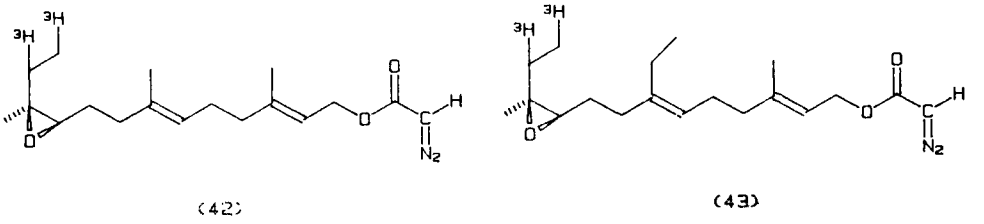


FIG. 14.

The use of [10-^{3}H]-JH III as a natural ligand for photoaffinity labeling of JH-binding proteins was reported by Stupp and Peter.[49] The chromophore used in the study is thought to be dialkylacrylate. The n-π^*-transition of acrylic esters occurs at a rather low wavelength of 240-250 nm, and the reactions of the excited state lead usually to dimerization and polymerization. In the microenvironment where the excited state faces functional groups of the protein in a close geometrical relationship due to high affinity binding, product formation may be different and lead to covalent attachment of the real binding site of the proteins. After prolonged irradiation (15 min) at 254 nm, up to 3.2% of the radioactivity was found bound covalently to the proteins, while 30% of the bound counts were non-specific binding. At the same time, the study also showed that even with only 7 min irradiation at 254 nm, the binding ability of M. sexta JH binding proteins reduced by 44%. Our experiments have concluded that ^{3}H-EFDA is a superior ligand to natural JHs for photoaffinity labeling juvenile hormone binding proteins.[50]

In spite of the success in the binding protein study, several factors have prevented EFDA from being the probe for the search for JH receptor proteins. First, it shows no JH effect both in vitro and in vivo with Manduca sexta or Leucopheae madera. Second, it lacks the desired optical purity and high specific activity, mainly due to the design of synthesis. The third factor is that EFDA's parent hormone, JH III, is not present in the hemolymph of tobacco hornworm M. sexta which is the ideal insect for endocrinological study. Therefore, the synthesis of (42), an JH II photoaffinity label with desired high optical purity and specific radioactivity, has been completed in our laboratories[51] and that of (43), an JH I analog, is also underway.



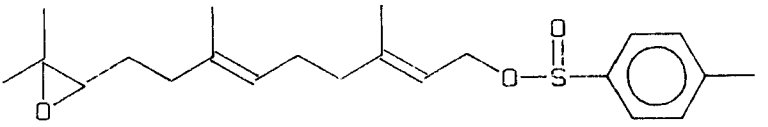
(42) (43)

FIG. 15.

Based on the photolabeling of JH binding proteins by EFDA, (42) and (43) might be able to bind to JH receptor proteins specifically and afford selective, covalent modification upon irradiation. Protein degradation and separation of the labeled fragments followed by amino acid analysis will reveal the active centers of the proteins and thus help us to further explore the hormonal regulation of gene expression in insects.

Radiolabeled EFDA was first developed as the ^{14}C-labeled form with 62% radiochemical yield by using [^{14}C]-glyoxylic acid as the radiolabeled source.[45] The synthesis of [10^3-H]-EFDA was then accomplished in our laboratories with a specific radioactivity of 5.4 Ci/mmol,[44] and later it was improved to 11 Ci/

mmol.[52] The diazoacetylation procedure employed at that time afforded a difficult-to-remove tosyl containing by-product,[44,53] which was suggested later [54a,b] as the corresponding p-toluenesulfinate ester (44).



(44)

FIG. 16.

The amount of this impurity varied depending on the final purification. An improved diazoacetylation procedure was reported[54b], which prompted us to modify our previously published procedure and high purity, specific activity are therefore reported herein for the synthesis of [^{1}H]- and [^{3}H]-EFDA. The major modifications were in the work-up of the NaB^3H_4 reduction of the hydroxy ketone (45), the mesylation of the diol (46), and the diazoacetylation. The EFDA (41) thus obtained was shown to be free from the side product. The bioassays using this batch of ^{3}H-EFDA revealed much less non-specific labeling than those made with our earlier procedure.[55] The key synthetic intermediates and products (EFDA, and ^{3}H-EFDA) are shown in FIG. 17.

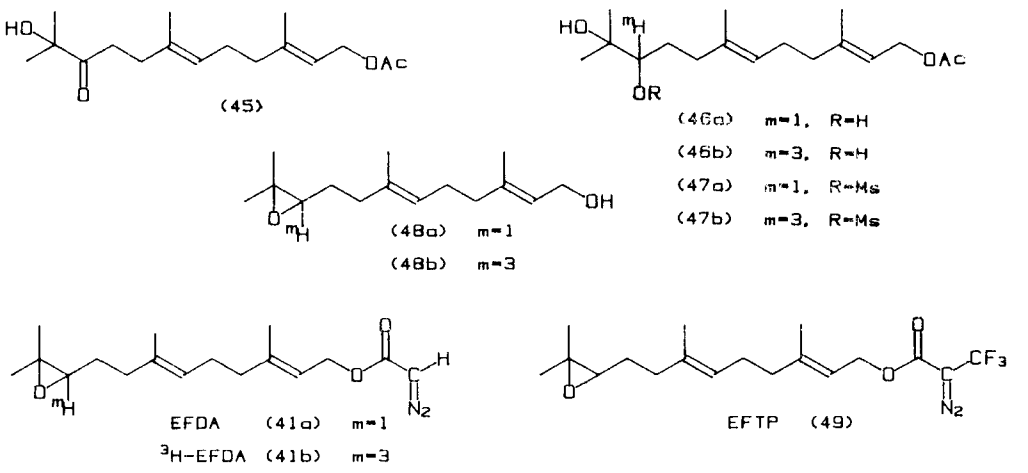


FIG. 17.

Diazotrifluoropropionates have been suggested to be more acid stable and undergo photolysis with substantially less rearrangement than diazoacetates.[56] Thus, epoxyfarnesyl diazotrifluoropropionate (EFTP, 49) might be a better photoaffinity label than EFDA, provided that the increased steric size of the ester does not interfere with binding. The preparation of EFTP (49) is reported herein.

In our study, EFDA and EFTP were photolyzed in methanol and their different rearrangement patterns were determined by NMR studies of the reaction mixture. The NMR integrations of the methanol-trapped products, (50) and (52), revealed 40% rearrangement in the EFDA photolysis. For EFTP, only 10% rearrangement was found, which was indicated by the integrations of (51) and (53), thus confirming Chowdhry's results.[56] We therefore estimated a similar pattern, i.e., EFTP would favor more carbene insertion than EFDA, in their photomodification of the protein binding sites in the bioassays using these probes. This would result in increased labeling since the acylcarbene can react with more residues than the ketene.

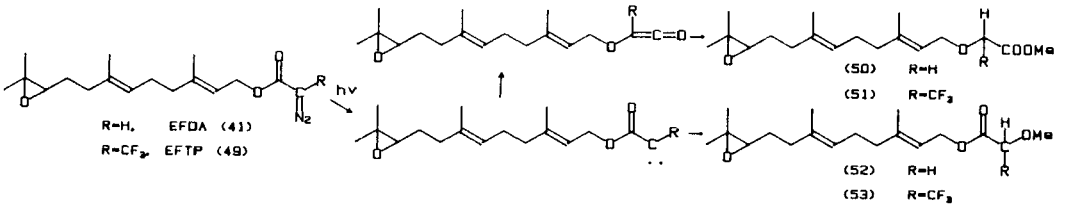


FIG. 18.

However, the surprisingly lower λ_{max} of EFTP (228 nm) was less in favor of EFTP. Furthermore, the competitive displacements of (10R,11S)-JH I from M. sexta JH binding proteins by EFDA and EFTP, respectively, showed that the concentration required for EFTP to displace JH I is about three times higher than that of EFDA. This is obviously due to the steric hindrance caused by the trifluoromethyl group. Therefore, EFTP is not likely to afford more efficient photolabeling of JH binding proteins than EFDA.

EXPERIMENTAL

(2E,6E)-10,11-Dihydroxy-3,7,11-trimethyl-2,6-dodecadienyl acetate (46a)[44,52]

The current batch of starting material, (2E,6E)-10-oxo-11-hydroxy 3,7,11-trimethyl-2,6-dodecadienyl acetate (45), was synthesized by Dr. C. Wawrzeńczyk and Dr. A.K. Singh in our laboratories from (2E,6E)-farnesol (6).[52]

To a stirred solution of (45) (20 mg, 0.06 mmol) in 3 mL absolute ethanol was added $NaBH_4$ (2 mg, 0.06 mmol) at room temperature. After stirring for 1 hr, 0.5 mL of 0.5 M NaH_2PO_4 solution was added, and the reaction mixture was stirred for 15 min. Most of the solvent was removed in vacuo and 50 mL of ether was added. After extraction and washing with 30 mL brine, the organic layer was dried over $MgSO_4$ and concentrated in vacuo to give a quantitative yield of (46a) which was homogeneous by TLC (60% EtOAc(EA)/hexane(H); R_f=0.40). This product was used in the subsequent reaction without further purification.

^{1}H NMR : δ 1.14 (s, 3H), 1.18 (s, 3H), 1.60 (s, 3H), 1.69 (s, 3H), 2.04 (s, 3H), 3.33 (dd, J=10, 3 Hz, 1H), 4.57 (d, J=7.2 Hz, 2H), 5.0-5.5 (m, 2H).

[10-^{3}H]-(2E,6E)-10,11-Dihydroxy-3,7,11-trimethyl-2,6-dodecadienyl acetate (46b) [44,52]

A solution of excess of hydroxyketone (45) (12 mg, 0.039 mmol) in 1.5 mL of absolute ethanol (freshly opened) was added to an ampoule containing a stirring bar and 0.14 mg of [^{3}H]-$NaBH_4$ (250 mCi with specific activity of 68.4 Ci/mmol; 3.65μmol; New England Nuclear) and the mixture was stirred at room temperature for 1.5 hr, 5 drops of 0.5 M NaH_2PO_4 solution were added, and the mixture was stirred for 15 min. Solvent was then blown off with N_2 and the residue was transferred with 10 mL of ether to a test tube containing 1 mL of brine. After washing, the aqueous phase was carefully removed and the organic layer was dried over $MgSO_4$ and concentrated under N_2. Flash chromatography (5 to 10% EA/H) yielded 4.4 mg (0.0148 mmol, quantitative chemical yield) of (46b) with > 92% radiochemical purity determined by TLC. Total radioactivity : 186 mCi (75% radiochemical recovery), specific activity : 12.6 Ci/mmole.

(2E,6E)-11-hydroxy-10-mesyloxy-3,7,11-trimethyl-2,6-dodecadienyl acetate (47a) [44,52]

To a solution of 32 mg (0.107 mmol) of diol (46a) in 1 mL dry CH_2Cl_2 was added Et_3N (50 μL, 0.45 mmol, distilled) in 0.5 mL dry CH_2Cl_2 and MsCl (15 μL, 0.13 mmol distilled) in another 0.5 mL of dry CH_2Cl_2. The mixture was stirred for 48 hr at room temperature before the solvent was removed. TLC (60% EA/H) showed that none of the diol (46a) was left [R_f(46) : 0.40; R_f(47) : 0.42]. The solvent was removed, ether was added and the organic phase was washed (1 M HCl and 10% $NaHCO_3$(aq.), dried ($MgSO_4$) and concentrated to get 40 mg (quantitative yield) of crude product which was used without further purification. An analytical sample was obtained by purification with a silica gel column pretreated with Et_3N.
IR (cm^{-1}) : 3520 (-OH), 1760 (C=O), 1190, 1380 (S=O)
^{1}H NMR : δ 1.26 (s, 6H), 1.64 (s, 3H), 1.70 (s, 3H), 2.05 (s, 3H), 3.12 (s, 3H), 4.10 (m, 1H), 4.60 (d, J=7.4 Hz, 2H), 4.9-5.5 (m, 2H).

(2E,6E)-10,11-Epoxyfarnesol (48a) [44,45,52]

The crude mesylate (47a) 30 mg (0.089 mmol) obtained above was added to 5 mL absolute methanol containing excess K_2CO_3 and stirred for 2 hr at room temperature. After removal of MeOH, ether was added and the organic phase was washed (brine), dried ($MgSO_4$) and concentrated *in vacuo* to give 35 mg of mixture which was purified by flash chromatography (Et_3N pretreated, 5 to 20% EA/H) to yield 19 mg of (48a) (0.080 mmol, 90% yield).
^{1}H NMR : δ 1.25 (s, 3H), 1.29 (s, 3H), 1.61 (s, 3H), 1.66 (s, 3H), 2.69 (t, J=6.1 Hz, 1H), 4.12 (d, J=7.3 Hz, 2H), 5.15 (m, 1H), 5.40 (t, J=6.80 Hz, 1H).

[10-^{3}H]-(2E,6E)-11-Hydroxy-10-mesyloxy-3,7,11-trimethyl-2,6-dodecadienyl acetate (47b) [44,52]

To a reaction vessel containing 0.5 ml of dry CH_2Cl_2 solution of 4.4 mg of diol (46b) (0.0148 mmol) was added 4 mg of Et_3N (0.04 mmol) in 0.5 mL of CH_2Cl_2, and 0.5 mL CH_2Cl_2 solution of 4 mg MsCl (0.035 mmol) and the mixture was stirred at room temperature for 23 hr before an additional 1 L of MsCl was added and the stirring continued for eight more hours. The mixture was then diluted with 6 mL of ether, washed (1 M HCl, $NaHCO_3$(aq), and brine), and dried ($MgSO_4$). The solution was then filtered through $MgSO_4$ and the solvent was removed to get the mesylate as the major product, identical to (47a) on TLC.

[10-^{3}H]-(2E,6E)-10,11-Epoxyfarnesol (48b) [44,52]

The crude mesylate (47b) of the above reaction was dissolved into 2 mL of absolute MeOH in a reaction vessel containing excess K_2CO_3 and stirred for 3 hr at room temperature. After removal of the solvent by blowing with N_2, 5 mL of ether and 1 mL brine were added. The organic phase was washed and dried ($MgSO_4$), and the solvents blown off under N_2 to give (48b) as the crude product. This material was identical to (48a) on TLC, and was used without further purification.

(2E,6E)-10,11-Epoxyfarnesyl diazoacetate (EFDA) (41a)[44,45,52]

To a 5 ml dry CH_2Cl_2 solution of epoxyfarnesol (48a) (12 mg, 0.05 mmol) and 17 mg of glyoxylic acid chloride p-toluenesulfonylhydrazone (0.065 mmol) was added 9 µL (8 mg, 0.065 mmol) of N,N-dimethylaniline at 0°C and the greenish resulting solution was stirred at 0°C for 30 min while TLC monitored the appearance of the ester. Then 22 µL of Et_3N (20 mg, 0.20 mmol) were added into the solution which was stirred for another 1 hr at 0°C before brought to room temperature at which it was further stirred for 1 hr. Solvents (20% EA/H, 50 mL) were added followed by 3 mL of H_2O. The organic phase was separated, washed with 10 mL brine and the combined aqueous phases were extracted with an additional 20 ml of 20% EA/H. The organic portions were combined and dried over $MgSO_4$. The removal of the solvent followed by purification with flash chromatography (5% EA/H) yielded 8 mg of (41a) (0.026 mmol, 51% yield).
IR (cm^{-1}) : 3120 (CHN_2), 2100 (C=N=N), 1690 (C=O), 1230, 1190 (C-O)
^1H NMR : δ 1.25 (s, 3H), 1.29 (s, 3H), 1.61 (s, 3H), 1.70 (s, 3H), 1.9-2.2 (br m, 8H), 2.69 (t, J=6.3 Hz, 1H), 4.70 (d, J=7.0 Hz, 2H), 4.75 (s, 1H), 5.13 (t, J=6.7 Hz, 1H), 5.33 (t, J=6.9 Hz, 1H)
UV λ_{max} (hexane) : 244 nm, $\varepsilon=1.5\times10^4$ with g/mL as the unit for concentration.

[10-^{3}H]-(2E,6E)-10,11-Epoxyfarnesyl diazoacetate (^{3}H-EFDA) (41b)[44,52]

To a dry CH_2Cl_2 solution (0.5 mL) of crude epoxyfarnesol (48b) in a 10 ml round bottom flask was added glyoxylic acid chloride p-toluenesulfonylhydrazone (8 mg, 0.031 mmol) in 0.5 mL CH_2Cl_2 followed by 4 mg (0.033 mol) of N,N-dimethylaniline in 0.5 mL CH_2Cl_2 at 0°C. The mixture was stirred at 0°C for 45 min before Et_3N (15 mg, 0.15 mmol) in 0.5 mL CH_2Cl_2 was added. The mixture was further stirred for 1 hr at 0°C, then 1 hr at room temperature, diluted with 5 mL EA/H (20%) and washed with 1 mL brine. After washing, the organic phase was dried over $MgSO_4$, filtered through $MgSO_4$ and the solvent was removed with N_2. Flash chromatography (1 to 5% EA/H) afforded 1.9 ± 0.2 mg of (41b) (0.0062 mmol, 42% overall yield from (45) with > 90% radiochemical purity determined by TLC. Radioactivity : 75 mCi; specific activity : 12.1 Ci/mmol (total radiochemical recovery 30% from NaB^3H_4). The product was stored in sealed ampoules as 5:1 hexane:toluene solution.

(2E,6E)-10,11-Epoxyfarnesyl 2-diazo-3,3,3-trifluoropropionate (EFTP) (49)

To a 3 ml dry CH_2Cl_2 solution of 19 mg epoxyfarnesol (48a) (19 mg, 0.080 mmol), was added pyridine (15 mg, 0.16 mmol) in 1 mL of CH_2Cl_2 followed by 15 µL of 2-diazo-3,3,3-trifluoropropionic acid chloride (ca. 0.12 mmol). The reaction mixture was stirred at room temperature overnight in an aluminium-foil-wrapped flask. Then, the mixture was diluted with 50 mL ether, washed (brine), and dried ($MgSO_4$). The solvent was removed in vacuo and the residue was purified by flash chromatography (5 to 20% EA/H) in a dark room equipped with a red light to give 16 mg of (49) (0.042 mmol, 53% yield).
IR (cm^{-1}) : 2130 (C=N=N), 1720 (C=O)
^1H NMR : δ 1.25 (s, 3H), 1.29 (s, 3H), 1.61 (s, 3H), 1.72 (s, 3H), 1.9-2.2 (brm, 8H), 2.69 (t, J=6.2 Hz, 1H), 4.75 (d, J=7.1 Hz, 2H), 5.12 (t, J=6.7 Hz, 1H), 5.33 (t, J=7.2 Hz, 1H)
UV λ_{max} (hexane) : 226 nm, $\varepsilon = 1\times10^4$, with g/mL as the concentration unit.

Photolysis of EFDA and EFTP in methanol

1) In 3 mL of MeOH was dissolved 0.2 mg of EFDA (41a) and the solution was subjected to irradiationat 254 nm in a Rayonet photochemical reactor. Absorption readings were taken at λ_{max} (249 nm) : A=1.50 (0 sec), 0.40 (20 sec), 0.06 (60 sec), 0.06 (120 sec).

2) A 3 mL MeOH solution of 0.5 mg of EFDA (41a) was irradiated at 254 nm for 12 min. ^1H NMR and IR spectra were taken before and after the irradiation:
^1H NMR (after) : 3.45 ppm (s, 3H, 80 units) [OCH_3 in (52), insertion]
3.77 ppm (s, 3H, 40 units) [OCH_3 in (50), rearrangement].
IR (after) : no absorption at 2100 cm^{-1} (C=N=N).

3) 0.08 mg of EFTP (49) was used to perform an analogous experiment described in 1). The absorption readings were taken at λ_{max} (228 nm) : A=0.44 (0 sec), 0.20 (60 sec), 0.14 (120 sec).

4) 0.5 mg of EFTP (49) was used to perform an analogous experiment as described in 2). The ^1H NMR and IR spectra were taken before and after the irradiation.

IR (after) : no absorption at 2100 cm^{-1} (C=N=N)
^1H NMR (after) : 3.58 ppm (s, 3H, 82 units) [OCH_3 in (53), insertion], 3.85 ppm (s, 3H, 9 units) [OCH_3 in (51), rearrangement]. 4.0-4.1 ppm [two quartets, J_{CF}=7.2 Hz, 2H, $OC(O)C\underline{H}(OCH_3)CF_3$, and $OC\underline{H}(CF_3)COOCH_3$]

Competition Binding assay.[29,52]

Manduca sexta JH binding proteins were obtained as described by Prestwich and Wawrzeńczyk.[24,29] A solution (50 µL) of competitors, EFDA or EFTP (1 x 10^{-9} to 1 x 10^{-5} M), in TK buffer containing 0.5% ethanol was added to a 6 x 50 mm PEG-coated assay tube, followed by 50 µL of 1:4 diluted hemolymph JHBP containing 2 x 10^{-5} M MOTFP (3-[3,7-dimethyl-7-methoxyoctylthiol]-1,1,1-trifluoro-2-propanone)[57] as a JH esterase inhibitor. After vortexing and incubation at 4°C for 2 hr, 50 µL of a 5 x 10^{-9} M (10R,11S)-^{3}H-JH I solution in TK buffer was added, and the tubes were vortexed and incubated 3 hr at 4°C. Then, 100 µL of a 1.1% DCC solution was added, and the tubes were vortexed, incubated 15 min at 4°C, and centrifuged (12,000 x g, 5 min). Two 100 µL aliquots were withdrawn for scintillation counting. "No protein" blanks were subtracted and a "no charcoal" sample gave total counts present. Relative percent dpm bound was then plotted against log competitor concentration and the value for 50% displacement was obtained graphically. It was determined that a 50% displacement of JH I by EFDA was achieved at a concentration of 3.0 x 10^{-6} M, while that for EFTP occured at a concentration of 8.6 x 10^{-6} M.

ACKNOWLEDGEMENTS

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