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The use of methoprene in the preparation of an immunogen from non-immunogenic insect growth regulators with juvenile hormone activity : development of a model system.

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The Use of Methoprene In the Preparation of an Immunogen from Non-Immunogenic Insect Growth Regulators with Juvenile Hormone Activity: Development of a Model System

A Thesis Presented

by

Joanne V. Mei

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of the requirements for the degree of

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May, 1988

Entomology

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Approved as to style and Content by:

Chih-Ming Yin

Chih-Ming Yin, Chairperson of Committee

J. Marshall Clark, Member

Louis A. Carpino, Member

John D. Edman, Department Head
Department of Entomology

This thesis is dedicated to my parents, Rita and Enrico, for their patience and support ("when are you going to be done with school?"), to C.J., my cat, without his morning stompings, I would have never made it out of bed, and to David, whose support, critical advice, and love helped me through it all and made it worthwhile, not to mention tons of fun.

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Abstract

The Use of Methoprene In the Preparation of an Immunogen from Non-immunogenic Insect Growth Regulators with Juvenile Hormone Activity: Development of a Model System

May, 1988

Joanne V. Mei, B.S., University of Massachusetts

M.S., University of Massachusetts

Directed by: Professor Chih-Ming Yin

The insect growth regulator , methoprene, is a small, non-immunogenic molecule which must be conjugated to a large carrier molecule to generate an immunological response in mammals. An immunogen has been developed which retains the features of the methoprene molecule, namely a methoxy and an ester function. The immunogen consists of methoprene acid linked as an ester to a spacer group, which is in turn conjugated to a carrier protein. The spacer group, 4-hydroxybutanoic acid, was protected to prevent its cyclyzation prior to coupling to methoprene acid. The protecting groups used were removed with specific reagents under mild conditions.

Two methods of conjugating methoprene to a protein via activated esters were studied. The first method used an N-hydroxysuccinimde ester derivative and conjugated 53 molecules of methoprene per molecule of protein. A second method used a water soluble, 1-hydroxy-2-nitro-4-

benzenesulfonate ester derivative of methoprene and conjugated 30 molecules of the methoprene per molecule of protein.

The immunogen was used to immunize eight BALB/c mice. The indirect enzyme-linked immunosorbent assay was used to analyze the polyclonal antibodies collected from blood serum. All eight mice produced antibodies which showed specificity for the methoprene immunogen.

Table of Contents

List of Tables

List of Figures

Page

Chapter 1.

Introduction

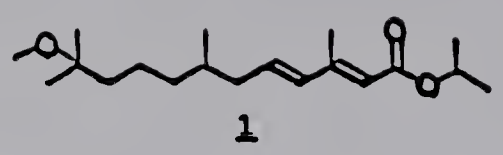
1.1. Uses of an Immunoassay for Methoprene

The need for a quantitative assay to quantify methoprene, an insect growth regulator with juvenile hormone activity, is apparent when one considers the many uses of methoprene in the field. Field residue detection is becoming increasingly important as government regulation and public scrutiny place more and more restrictions on pesticide residue levels (Brosten, 1987). immunological assays for pesticides would allow sensitive and economical detection of residues. These assays have been only recently developed for detecting pesticides. To my knowledge, an immunoassay for methoprene is not available, despite the fact that methoprene has been used to control several insect pests.

Methoprene is also widely used as a research tool to investigate juvenile hormone-like activities in insects (Staal, 1975). Recent studies on receptor proteins using radiolabeled juvenile hormone and methoprene analogs, found them binding to different receptor sites (Prestwich, 1987). An immunoassay for methoprene could be used to investigate the roles of methoprene *in vivo*.

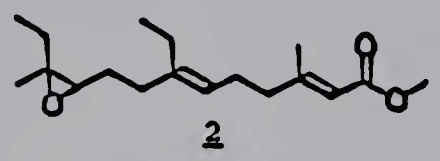
This thesis has developed immunological methods which will facilitate the detection and quantification of

Figure 1. The chemical structure of methoprene, the five naturally occuring juvenile hormones, and juvenile hormone diol.



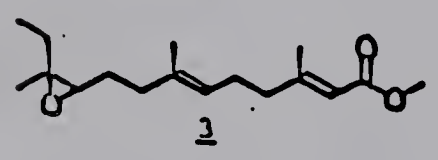
1

Methoprene



2

Juvenile Hormone I



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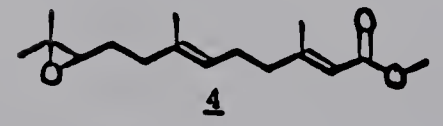
Juvenile Hormone II



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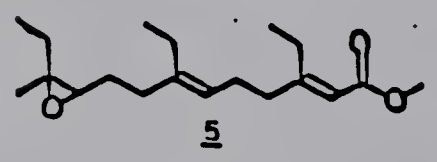
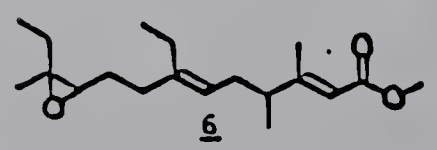
Juvenile Hormone III



5

Juvenile Hormone 0



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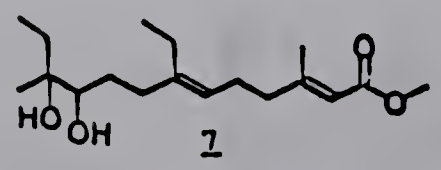
4-Methyl Juvenile Hormone I



7

Juvenile Hormone I Diol

methoprene (1) (Figure 1). It is hoped that similar protocols can then be applied to generate antibodies for the structurally related juvenile hormones, and other non-immunogenic materials, facilitating their detection and quantification. Existing immunological techniques for the juvenile hormones are laborious, expensive, not readily available, and at times unreproducible or their results are in conflict with those obtained by physical-chemical methods.

The main goal of this work was to covalently link methoprene to a protein and to use the resulting conjugate to produce specific polyclonal antibodies against methoprene. The antibodies produced would be used to develop immunological assays in the form of enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). ELISA and RIA have been developed for numerous substances, including chemicals with insecticidal activity, steroids, peptides, proteins, and viruses. These assays are fast, specific, and sensitive in their detection of minute quantities of materials of agricultural, environmental, industrial, and medical importance. Protocols and procedures of the immunological techniques developed for methoprene (1), will, hopefully, provide insight on the development of similar techniques specific for the juvenile hormones (structures 2, 3, 4, 5 and 6, Figure 1), and, thus, would greatly facilitate research on juvenile hormone and juvenile hormone related compounds.

Since methoprene is a small molecule, which does not arouse an immunological response, it must be covalently bound to a large molecule, such as a protein, to form an immunogen which can then elicit immune responses. The protein acts as a carrier for the small molecule (hapten), which together challenge the immune system to produce antibodies against the hapten.

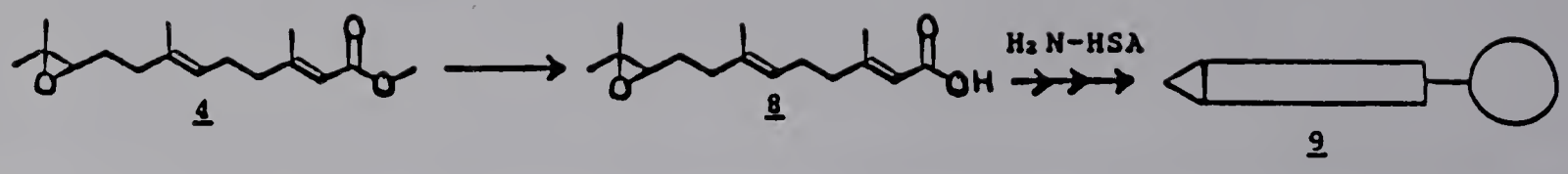
1.2. Immunogens Prepared for the Juvenile Hormones

The first attempts to conjugate juvenile hormone III (4), which is structurally related to methoprene (1), to a protein relied on converting juvenile hormone to its acid form, derivatizing the acid to an active ester with N-hydroxysuccinimde and coupling the ester to human serum albumin (HSA) through an amide bond (Figure 2a) (Lauer et al., 1974a; Baehr et al., 1976). The resulting immunogen, illustrated by the model 9, where the triangle represents the epoxy function, the rectangle is the parent molecule, the hexagon is the ester function, and the circle represents the bulky protein molecule, has lost the ester function of the hormone. Antisera raised to this juvenile hormone immunogen recognized both juvenile hormone III (4) and juvenile hormone III acid (8).

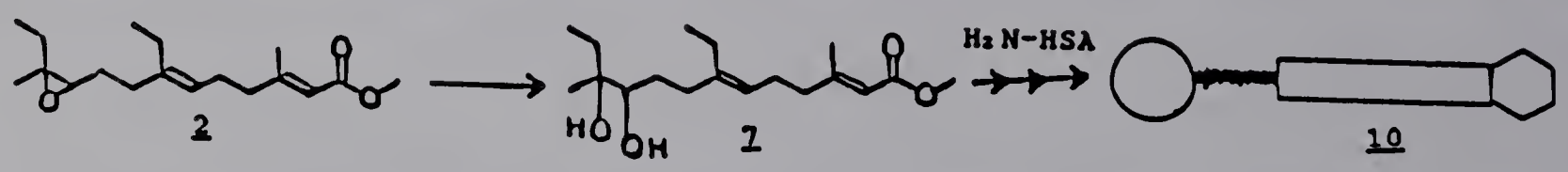
Another effort conjugated juvenile hormone I (2) to a protein through its diol derivative (7), which was formed by opening the epoxide ring at carbons 10 and 11,

Figure 2. The various juvenile hormone immunogens have been prepared by different intermediates and can be visualized by different models. a: The juvenile hormone iii immunogen (9) was made with juvenile hormone iii acid (4) and retains the epoxide function (Lauer et al, 1974a; Baehr et al, 1976). b: The immunogen (10) prepared from juvneile hormone i diol (7) retains the ester function of 2, while incorporating a four carbon spacer group between the diol and the protein (HSA) (Strambi et al., 1981). c: The proposed immunogens for juvenile hormone i (12 and 14) (Eng and Prestwich, 1986) would be prepared from the intermediates 11 and 13 and would retain both the epoxide and ester functions of juvenile hormone i (2).

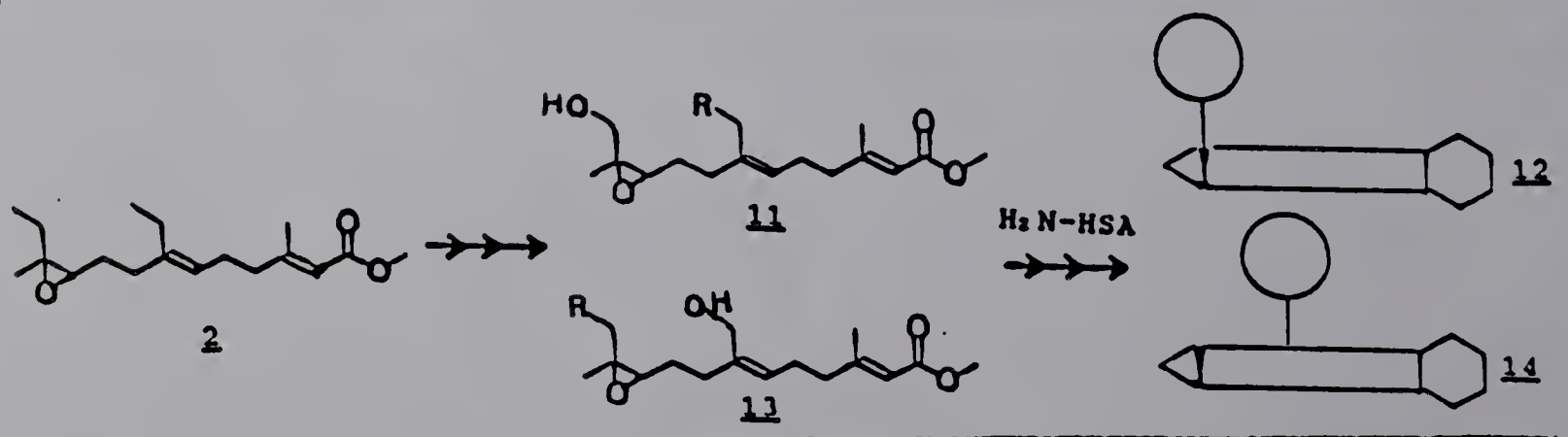
a

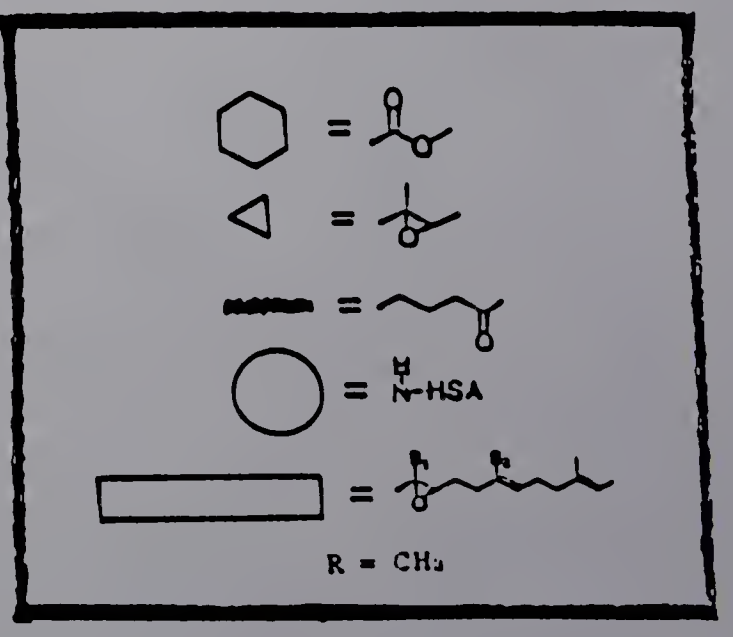

4
8
OH
H₂N-HSA
9

b


2
HO
OH
7
H₂N-HSA
10

c


HO
R
11
R
OH
13
2
H₂N-HSA
12
14


=
=
=
=
N-HSA
R = CH₃

(Figure 2b) (Strambi et al., 1981). This immunogen (10) contains the ester function of the hormone, but not the epoxide. A spacer group, represented by the thick line in the model, was incorporated into the structure of the immunogen. The antisera raised against the immunogen (10) recognized only diol derivatives of the juvenile hormones. No antiserum reported solely recognizes juvenile hormone. Eng and Prestwhich (1986) have proposed immunogens for juvenile hormone (12, 14) where the hormone would be linked to a protein via a hydroxyl group as depicted in Figure 2c (compounds 12 or 13), thus maintaining both functional ends of the hormone.

1.3. Design of an Immunogen for Methoprene

Based on the results from juvenile hormone radioimmunoassays, it was apparent that a useful immunoassay for methoprene would have to be: a) specific for the methoprene molecule in its native form; b) sensitive to quantities of methoprene in the nanogram to picogram range; c) able to be used with a minimum of sample purification and prepartion; d) reproducible, such that different laboratories could compare results; and e) in agreement with the results obtained from physical-chemical methods for similar samples.

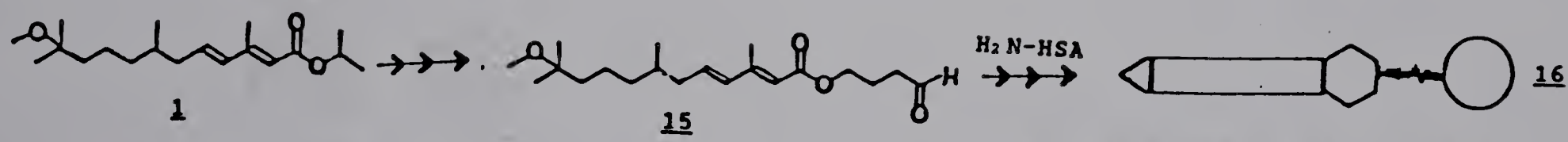
The design of the methoprene immunogen was proposed to meet the above criteria. The production of specific antibodies against methoprene is dependent on the ability of an animal's immune system to "see" methoprene in its

"native" form. To allow better recognition of the methoprene derivative on the surface of its protein carrier, a spacer chain was incorporated between methoprene and the protein. The spacer serves as a flexible linker between methoprene and the protein, thus reducing steric hindrance between the two. By choosing the proper spacer incorporation and protein conjugation scheme, the immunogen retained both the ester and methoxy moieties of the methoprene molecule. The structure of the prepared immunogen (16) is illustrated in Figure 3.

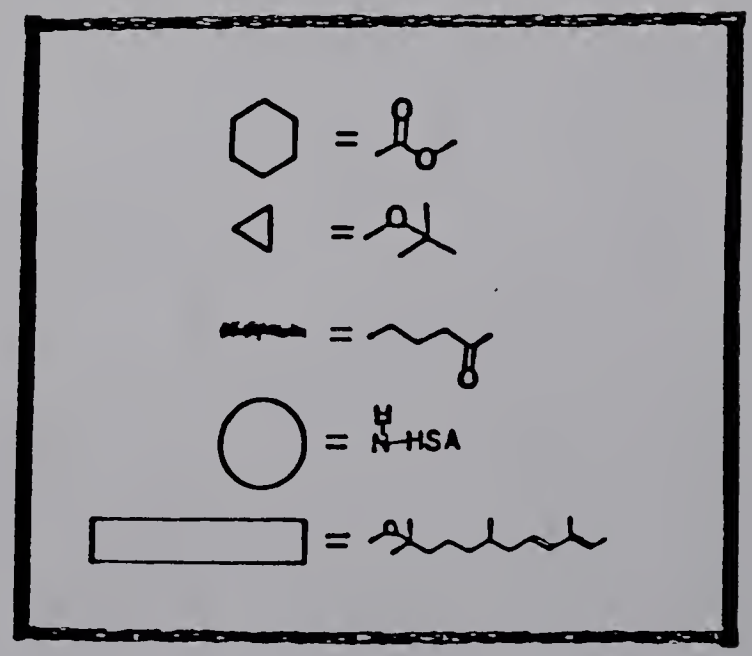
To encourage the detection of methoprene in its native form in the animal, its ester function was retained in the derivatized methoprene molecule by coupling it to the spacer group as an ester. This served two purposes: a) an ester function, which is native to methoprene, is in the same postion; and b) the ester allowed for the incorporation of a spacer between methoprene and the protein carrier. The spacer group is depicted by the thick line in the immunogen model 16 (p. 11).

Previous attempts at preparing immunogens for juvenile hormone have always destroyed one of the functional groups of the juvenile hormone molecule. The immunogen prepared from juvenile hormone acid (9) (Lauer et al., 1974a; Baehr et al., 1976) lacked the ester function, but contained the epoxide. The immunogen

Figure 3. The methoprene immunogen (16) was prepared from methoprene (1), which had been modified to incorporate a four carbon spacer group with a carboxylic acid function (15). The resulting immunogen retains both the methoxy and ester functions of methoprene.



1 15 16



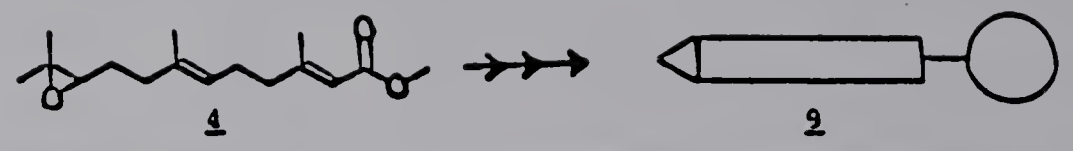
prepared from juvenile hormone diol (10) lacked the epoxide molety, but contained the ester (Strambi et al., 1981). Two additional immunogens for juvenile hormone (12 and 14) were proposed (but not prepared) by Eng and Prestwich (1986), in which the functionalities of both ends of methoprene will be retained. The immunogen prepared for methoprene (16) also contains the functionality of both ends of the molecule. By our design, a bridge (spacer) between methoprene and the bulky protein molecule allows for the maintenance of both the ester and the epoxy functionalities (Figure 4).

The incorporation of the spacer group required a number of protection-deprotection steps (Figure 5) and insure that reactions occured mostly between selected functional groups as intended. Such precautions reduced the possibility of unwanted side reactions. Specific reagents and conditions were employed to remove the protecting groups so that functional groups (i.e. esters, double bonds, and methoxides) were not affected.

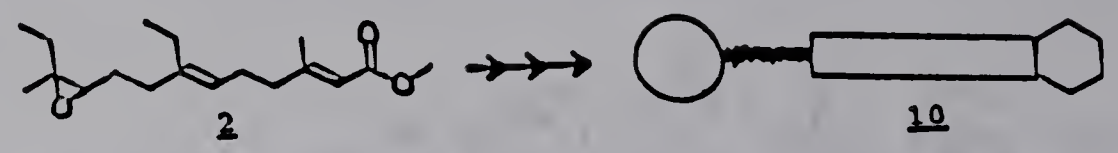
Two other problems might be anticipated: a) differential solubility of hapten (lipid) and carrier (protein) in the reaction mixture, and b) the monitoring of the extent of the coupling reaction. Methoprene, which has a long hydrocarbon chain, is extremely insoluble in aqueous solutions (1.39 ppm, Farm Chemicals Handbook, 1987). Its solubility is greater in organic or polar organic/aqueous solutions. Proteins, however, are

Figure 4. The immunogens for juvenile hormone and methoprene have various characteristics in common. Only the methoprene immunogen (16) has all the functionalities of the native molecule (1), in addition to having a spacer group between methoprene and its protein carrier. See figures 2 and 3 for explanation of symbols.

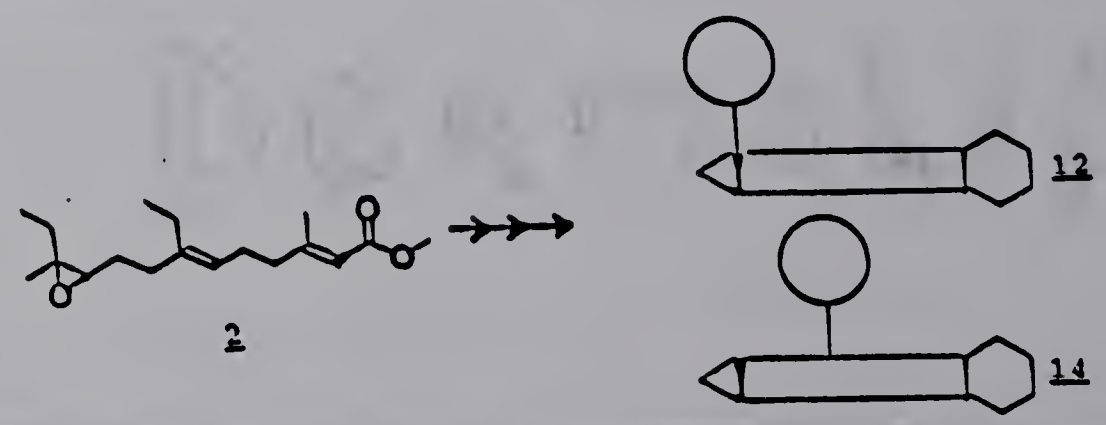
a



b



c



d

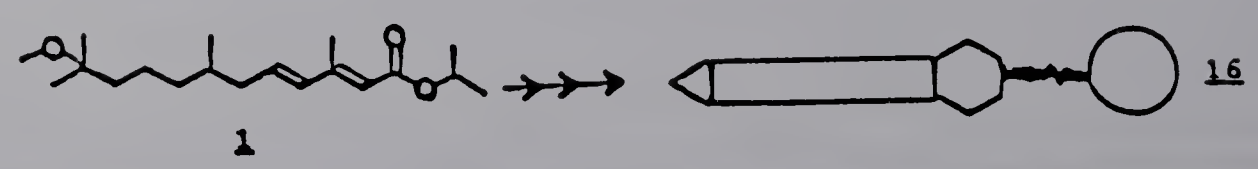
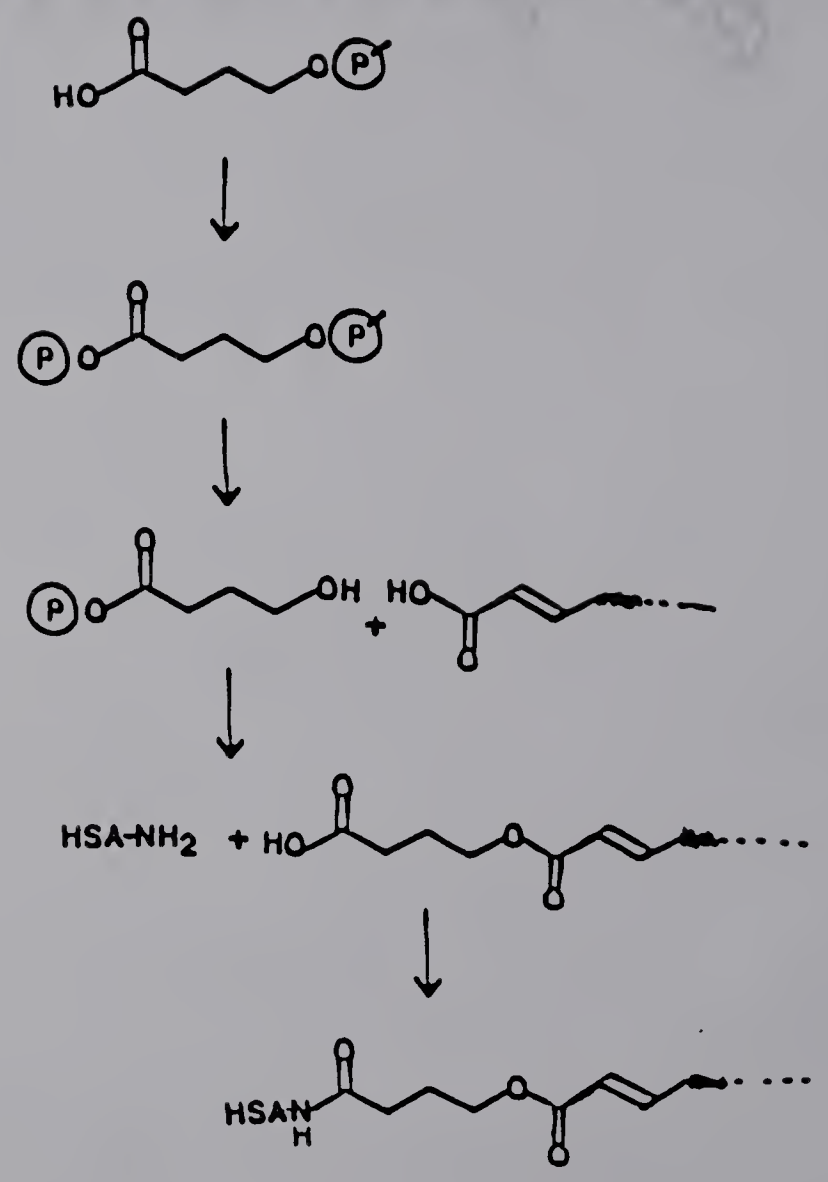


Figure 5. The four carbon spacer group was incorporated between methoprene and a carrier protein by a series of protection, deprotection, and coupling reactions. This synthetic route insured that the desired products were formed, minimizing unwanted side reactions.



P, P = Protecting Group

very water soluble, but their solubility decreases greatly in organic/aqueous solutions. Difficulties arise when one attempts to solubilize methoprene and a protein in a single solvent.

To monitor the extent of the methoprene-protein coupling reaction, it is necessary to either use a radiolabeled tracer or a spectrophotometric method to quantify the number of molecules of methoprene coupled per molecule of protein. A number of methods were used in this study (see Results section 4.4 and 4.5).

It is hoped that this work will develop a new technique for methoprene quantifition, and will shed new light on the improvement of present immunological techniques available for juvenile hormone quantification.

Chapter 2.

Literature Review

2.1 Role of Juvenile Hormone in Insects

The physiological and developmental events in insects are controlled by a hormonal system responding to internal and external cues. There are five major hormones responsible for integrating growth and development: juvenile hormone; prothoracicotropic hormone; ecdysone, eclosion hormone; and bursicon. This integration is illustrated in Figure 6. Briefly, juvenile hormone, a sesquiterpene epoxide, has a variety of roles in the life processes of insects. Prothoracicotropic hormone, a peptide hormone, is secreted by the brain and is responsible for stimulating the prothoracic or ecdysial glands to produce a sterold, ecdysone. Ecdysone, the molting pre-hormone, is converted to 20-hydroxyecdysone by the epidermis, causing the secretion of new cuticle by immature insects. Eclosion hormone, a neurosecretory product, initiates preeclosion and eclosion behaviors which free the insect from its old cuticle. Bursicon, another peptide neurosecretion, is responsible for the tanning and strengthening of the new cuticle. These five hormones maintain the delicate internal environment necessary for successful growth and development in insects.

Juvenile hormone is, thus, of major importance to

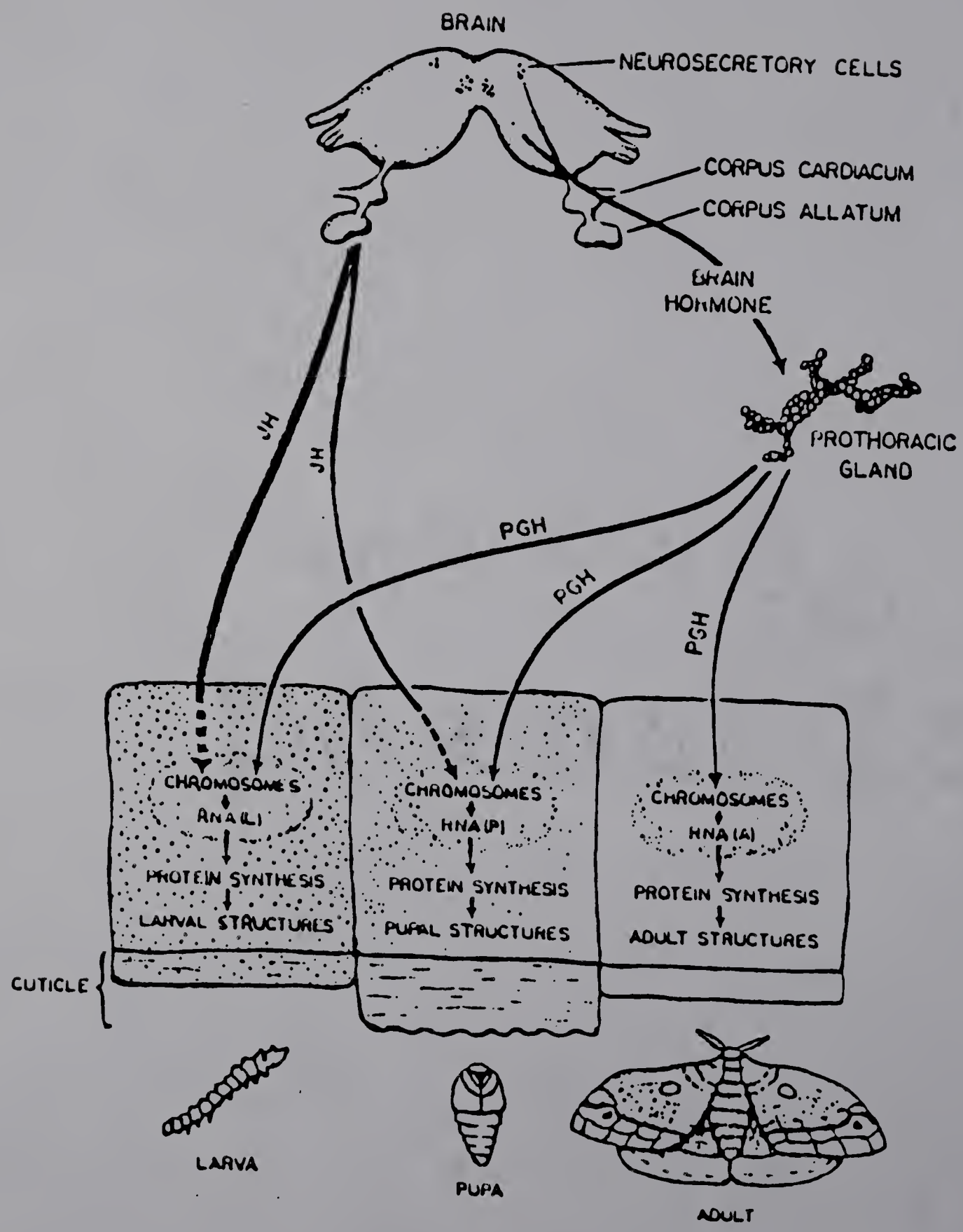
the growth and development of insects. There are five homologous forms of juvenile hormone in insects. They are all in the form of methyl esters of sesquiterpene epoxides (Figure 1). Much data has been gathered concerning the variety of physiological processes controlled by juvenile hormone. A few of the processes are metamorphosis, reproduction, behavior, diapause, and caste determination.

2.1.1. Control of metamorphosis

In order for an insect to grow, it must periodically shed its old cuticle, forming in the process, a larger cuticle into which it can expand. Throughout an insect's life, different types of cuticle are produced. In species which undergo complete metamorphosis, larval cuticle is made during larval stages; pupal cuticle is made at the end of larval development; and adult cuticle is formed as a result of metamorphosis in the pupal stage. The molting hormone, 20-hydroxyecdysone, controls the synthesis of the cuticle. The presence of juvenile hormone at the critical period before each molt has the role of directing the nature of newly formed cuticle, or the nature of the molt: larval to larval; larval to pupal, or pupal to adult (Figure 6).

Juvenile hormone is present at precise times during

Figure 6. The classical scheme of juvenile hormone and ecdysone action. Eclosion hormone initiates behaviors necessary for old cuticle removal at each molt. Bursicon aids in the tanning of new cuticle. (Redrawn from Gilbert and Schniederman, 1961).


BRAIN
NEUROSECRETORY CELLS
CORPUS CARDIACUM
CORPUS ALLATUM
BRAIN HORMONE
PROTHORACIC GLAND
JH
JH
PGH
PGH
PGH
CHROMOSOMES
RNA (L)
PROTEIN SYNTHESIS
LARVAL STRUCTURES
CHROMOSOMES
RNA (P)
PROTEIN SYNTHESIS
PUPAL STRUCTURES
CHROMOSOMES
RNA (A)
PROTEIN SYNTHESIS
ADULT STRUCTURES
CUTICLE
LARVA
PUPA
ADULT

larval life. A considerable level of juvenile hormone in the hemolymph is maintained at the critical period with each successive larval instar, until it is undetectablein the last larval instar. This decline allows the insect to undergo metamorphosis (Wigglesworth, 1960; Van der Kloot, 1961). Declining juvenile hormone titer was also found to stimulate the synthesis of major hemolymph proteins by the fat body in the last larval and pupal stages in the silk moth, Bombyx mori (Plantevin et al., 1987). During pupal development, the juvenile hormone titer declines and remains at a low level for the rest of metamorphosis. It rises again in the adult where it is involved in the regulation of reproduction (Gilbert and Schneiderman, 1961).

2.1.2. Reproduction Regulation

The role of juvenile hormone as a modulator of developmental processes is further expanded in adult female insects. Juvenile hormone has been shown to control the synthesis and/or uptake of egg precursor yolk proteins in many species of insects. The precursor yolk proteins, known as vitellogenins, are synthesized by the insect fat body (with some exceptions), released into the hemolymph and taken up by developing oocytes.

Juvenile hormone controls the synthesis of vitellogenins by the fat body. Vitellogenin synthesis is prevented in locusts whose corpora allata, the primary source of juvenile hormone, have been removed soon after

adult emergence. Vitellogenin synthesis may be induced in allectomized females by topical application of juvenile hormone I or methoprene (Chen et al., 1976). Fat body removed from allectomized females will produce mRNA's specific for the vitellogenin proteins after treatment with juvenile hormone I or methoprene. These *in vitro* studies by Chen et al. (1976) demonstrate the action of juvenile hormone at the cellular level, where specific proteins are made by cells responding to an endocrine signal. An analogous system has been found in cockroaches (Englemann, 1974). The control of vitellogenin synthesis by juvenile hormone in the fat body of adult female insects provides a system for biochemical and modular study of hormonal control of gene expression (for reviews see Englemann, 1983; Koeppe et al., 1985; Riddiford, 1985).

The control of yolk protein synthesis in the mosquito demonstrates a different role for juvenile hormone in reproduction. In the mosquito, *Aedes aegypti*, an increase in juvenile hormone titer after adult emergence causes growth of the oocytes before vitellogenin uptake (Shapiro, 1986). The increase in juvenile hormone levels prepares the mosquito for blood meals which are required for egg development. As juvenile hormone levels drop off after a blood meal, ecdysone levels increase, stimulating the synthesis of vitellogenin by the fat body (Hagedorn et al., 1979).

2.1.3. Control of Insect Behavior

The endocrine system in insects plays an important role in the control of behavior. It provides an economic means of obtaining a rich behavioral repertoire within the limited capacity of the insect central nervous system (Riddiford and Truman, 1974). Juvenile hormone has the effect of coordinating many behaviors in insects, including reproductive behavior, migratory behavior, and foraging behavior. Reproductive behavior must coordinate a series of activities which are designed to ensure the fertilization of the gametes. The presence of juvenile hormone has been shown to regulate the sexual behavior of the female cricket, Acheta domesticus (Koudele et al., 1987). The phonotactic responsiveness and copulatory readiness of females were greatly reduced after allatectomy. Topical application of juvenile hormone III fully restored these sexual behaviors. Juvenile hormone also controls sexual receptiveness of the female grasshopper, Gomphocercus rufus (Chapman, 1982). Allatectomy of the male grasshopper, Schistocerca gregaria, resulted in reduced protein synthesis in the male accessory gland, inhibition of yellow coloration, and cessation of mating activity (Pener, 1965).

Migratory behavior is regulated, in part, by juvenile hormone. Most insect species which migrate do so by flying as adults, before the onset of reproduction. In the milkweed bug, monarch butterfly, and Colorado potato beetle, flight behavior is stimulated by

intermediate titers of juvenile hormone (for reviews see Rankin, 1978; and Pener, 1985). Short days and poor food quality induce intermediate juvenile hormone levels in these insects. Such conditions mimic the autumn season, where migratory flights are observed and most of these insects are forced to forage farther for resources.

Foraging behavior in honey bees may be induced by juvenile hormone. Worker honey bees perform a number of behaviors during adult life, including cell cleaning, brood nest care, food handling, and foraging. These behaviors are spacially segregated, where the youngest workers perform cell cleaning in the center of the nest; and the oldest workers engage in forging away from the nest. Topical application of methoprene or synthetic juvenile hormone I (Robinson, 1985) caused young worker bees to engage in foraging flights much sooner than untreated bees. Africanized honey bees forage at a significantly younger age than European bees (Winston and Katz, 1982); however, juvenile hormone hemolymph concentration did not significantly differ from European bees (Robinson, et al., 1987). Environmental conditions may modulate juvenile hormone concentration, which may enable a honey bee colony to regulate its division of labor in preparation for unfavorable conditions.

2.1.4. Control of Diapause

Environmental conditions rarely allow insects to sustain continuous development throughout the year. Many

insect species enter a period of diapause, where low metabolic rates enable them to stretch their food reserves to bridge the unfavorable period. In species which diapause in the larval stage, there is clear evidence that the brain regulates diapause induction. The brain integrates environmental information with the synthesis and release of hormonal products from the prothoracic gland, ecdysone, and the corpora allata, juvenile hormone.

In response to short days and cool temperatures, diapause is initiated in the last larval instar of the southwestern corn borer, Diatraea grandiosella. A drop in the hemolymph ecdysteroid titer and an increase in juvenile hormone titer is required for diapause (Chippendale and Yin, 1976). The diapause state is maintained by high levels of juvenile hormone. Removal of the corpora allata causes most larvae to terminate diapause and pupate within 30 days. Application of juvenile hormone to allatectomized larvae prolongs diapause (Yin and Chippendale, 1979). The presence of an intermediate level of juvenile hormone in these diapausing larvae appears to inhibit production of prothoracicotropic hormone by the brain (Sehnal et al., 1981). Production of ecdysone by prothoracic glands and declining juvenile hormone titers can terminate diapause (for review see Denlinger, 1985).

2.1.5. Caste Determination

The term polymorphism is used to describe individuals in a species with distinct but dissimilar external characteristics. Many different forms may be found within an insect species. They may consist of larvae, pupae, and adults, and adult males and females may differ. In some species adults of the same sex may be differentiated by division of labor. A hierarchy of tasks divides the social insects such as termites, bees, and ants into morphologically different individuals within a colony of the same species. Caste formation is under the control of juvenile hormone. In lower termites, high juvenile hormone levels topically applied to nymphs are required for the the production of soldiers, while very low juvenile hormone titers in nymphs allow them to develop into supplementary reproductives (wingless reproductive adults) (Hardee and Lees, 1985). In higher termites an egg laying queen produces large amounts of juvenile hormone, some of which is passed on to developing nymphs through anal secretions. High juvenile hormone titers prevent the production of reproductives, in favor of the neuter caste. If the reproducing male and female, the royal pair, are removed from the colony, new reproductives are formed. For a complete review of the role of juvenile hormone in caste determination see Hardie and Lees (1985).

2.2. Quantification of Juvenile Hormone

The importance of juvenile hormone during the life span of an insect is apparent from the above discussion. Juvenile hormone titers change frequently throughout an insect's life, thus allowing for an integrated control of the insect's physiological needs. To understand the endocrinology involved, accurate and precise methods of measuring juvenile hormone titers at different stages in the insect life cycle are necessary. Juvenile hormones may be extracted from insect tissues and fluids; however, the physiological concentration of the hormone is quite small, in the range of 10^{-9} to 10^{-12} M (Mauchamp et al., 1984). The small amount of juvenile hormones present along with interfering endogenous organic materials make the quantification of juvenile hormones difficult.

Early methods of juvenile hormone quantification relied on indirect chemical and biological assays which provided information on the relative activity of the corpora allata, but were qualitative at best. Biological assays have, for the most part been replaced by more direct assays, which include radiochemical assays, physical-chemical methods and radioimmunoassays.

2.2.1. Bioassays for Juvenile Hormone Detection

The detection of juvenile hormone by bioassay relies on the response of a whole or part of another insect body. Juvenile hormone or samples which may contain

juvenile hormone are applied to the test animal or tissue. Juvenile hormone activity is determined as the degree of inhibition of metamorphosis of the test animal.

One of the most widely used bioassays, the Galleria wax moth test (see Yin and Chippendale, 1976), uses newly molted Galleria mellonella pupae. Newly molted pupal cuticle is extremely responsive to juvenile hormone. Juvenile hormone presence in a test sample is determined by inhibition of pupal-adult metamorphosis of the treated tissue. Verification of this method by electron capture-gas chromatography showed that juvenile hormone titers in whole body extracts of the southwestern corn borer could not be estimated with the Galleria bioassay due to interfering substances (Bergot, et al., 1976). However, both the bioassay and the chromatographic method could be used to estimate juvenile hormone titer in the hemolymph of the southwestern corn borer (Bergot at al., 1976). The Galleria wax test has also been used to demonstrate juvenile hormone activity in larvae (Blight and Wenham, 1976) and adult male of Schistocerca gregaria and Locusta migratoria (Johnson and Hill, 1973).

Other bioassays for juvenile hormone include tissue culture, larval cuticle pigmentation, and metamorphosis inhibition of whole insects. Cultured integumental tissue of the rice stem borer, Chilo suppressalis (Kitahara, 1983), or the tobacco hornworm, Manduca sexta (Riddiford, 1975), failed to undergo larval-pupal

metamorphosis in the presence of juvenile hormone. Estimation of juvenile hormone titers in the hemolymph of Manduca sexta was determined by a black mutant larval pigmentation assay where juvenile hormone prevented melanization of the black mutant larval cuticle. (Fain and Riddiford, 1975). Bioassays for insect growth regulators with juvenile hormone activity have been developed using inhibition of metamorphosis of a number of economically important insects including: the house fly, pea aphid, tobacco budworm, greater wax moth, yellow meal worm, and the yellow fever mosquito (Henrick et al., 1973; Henrick et al., 1975). The effectivness of the insect growth regulator was measured as a function of the percentage of insects unable to molt to their next developmental stage.

Although the above assays indicate the presence of juvenile hormone and some of them may be very sensitive (detecting picogram levels of the hormone), they fail to distinguish between the different juvenile hormone homologs. They also lack prescision in that the exact quantity of juvenile hormone in a sample is difficult to assess, particularly when interfering substances may give false results. If a quantitative measure of juvenile hormone is to be obtained, the bioassay must be coupled with a physical-chemical analysis.

2.2.2. Radiochemical Assay for Juvenile Hormone Biosynthesis

Since bioassays were found to be, at best, qualitative, some researchers have developed assays which measure the *in vitro* biosynthesis of juvenile hormone. The realization that the corpora allata produce and secrete juvenile hormone in insects has led to techniques which allow the hormones to be extracted from media in which active glands are placed. Early work by Judy et al. (1973) enabled the *in vitro* incubation of the corpora allata in a commercial medium containg no methionine. Radiolabeled L-[methyl-^{14}C] methionine was added to the medium to act as a labeled donor for the incorproation of the methyl ester moiety of juvenile hormone. The incubated gland synthesized ^{14}C-juvenile hormone into the surrounding medium. The medium was then extracted and an estimate was made of the amount of juvenile hormone biosynthesized by the glands. Subsequent use of high pressure liquid chromatography (HPLC) and gas chromatography-mass spectrometry further identified the nature of the juvenile hormones produced *in vitro*. The radiochemical assay shows a sensitivity of 0.1 pmol when ^{14}C-methyl-methionine is used as the methyl donor in juvenile hormone biosynthesis. A number of reviews and reports document the data obtained by the *in vitro* radiochemical assay for juvenile hormone biosynthesis

(Tobe and Feyereisen, 1982; Pratt, 1984; Tobe et al., 1984; Tobe and Stay, 1985).

2.2.3. Physical-chemical Methods for Juvenile Hormone Quantification

One of the most rapid, sensitive, and specific methods for identification and quantification of juvenile hormones from insect tissues is the use of physical-chemical methods, such as gas chromatography-mass spectrometry (GC-MS) or high-performance liquid chromatography-mass spectrometry (HPLC-MS). To use such techniques, a few requirements are needed. First, access to such equipment and expertise is necessary. Second, insect tissues must first be purified chromatographically to remove interfering substances, and third the juvenile hormones must be derivatized before they can be analyzed by gas chromatography. Physical-chemical techniques offer verification and validation of the various assays used for juvenile hormone quantification.

To prepare the juvenile hormones for GC analysis, micro-derivatization reactions are used to convert the epoxide ring of the juvenile hormones to from their respective methoxy hydrins, which are converted to their 11-methoxy-10-pentafluorophenoxy acetate esters. These esters may then be subjected to gas chromatography. Early work used gas chromatography coupled with electron capture detection to quantitatively measure the juvenile

hormone biosynthesis from *in vitro* incubation of the corpora allata (Schooley et al., 1975) or juvenile hormone titer on hemolymph or whole body extracts (Bergot et al.,1976). More recently, the addition of mass spectrometry to gas chromatographic analysis has simplified and shortened of derivatization procedures; enabled the use of small sample sizes, and produced a level of sensitivity of less than 0.01 ng/ml. These methods have provided precise determination of juvenile hormone titers in a number of insects species (Bergot et al., 1981a; 1981b; Rembold, 1981; de Kort et al., 1982; Mauchamp et al., 1984; Schooley et al., 1984; Shapiro, 1986), and identification of juvenile hormone analogs from embryonic stages of *Manduca sexta*: juvenile hormone 0 and 4-methyl juvenile hormone I (Figure 1) (Bergot et al., 1980; Bergot et al., 1981c).

High-performance liquid chromatography (HPLC) has also been used extensively to purify the juvenile hormones from juvenile hormone metabolites in biological samples prior to analysis by radioimmunoassay (Granger et al., 1979). However, since juvenile hormone concentrations in biological samples are too low to be detected by direct UV analysis, a system was developed which coupled HPLC to mass spectrometry (HPLC-MS) (Mauchamp et al., 1981). This system allowed direct quantification of biologigal samples. This method required two prepurification steps of the samples to be

analyzed. Elutuion on a glass packed alumina column is first required, followed by filtration through a C_{18} Sep-pak^R cartridge (Waters Associates). The eluent is then analyzed with the HPLC-MS system.

The data collected by physical-chemical methods coupled to mass spectrometry are clearly more accurate, reproducible, and may be the best method for juvenile hormone quantification. The major advantages of these methods are their high sensitivity and the reduction of time required for analysis. The expense and availability for such analyses, however, may be problematic for many laboratories.

2.2.4. Radioimmunoassays for Juvenile Hormone Quantification

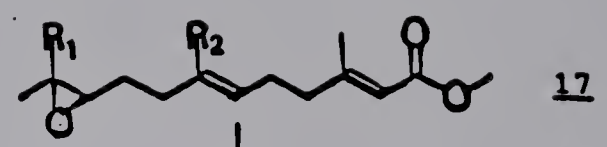
Radioimmunoassays have been successfully used to quantify hormones in biological samples from both vertebrate and invertebrate systems. They are generally sensitive, with detection limits in the nanogram to picogram range, reproducible, and simple to use with a minimum of sample preparation. Radioimmunoassays for juvenile hormone have been reported, however, their effectiveness has been limited due to the physical nature of the juvenile hormones, the presence of juvenile hormone homologs, degradation products, and interfering substances in the samples to be analyzed.

Juvenile hormone is a small, hydrophobic molecule, with poor solubility in aqueous solutions (Giese et al., 1977). In order for juvenile hormone, (or methoprene) to become immunogenic, it must be conjugated to a large carrier molecule such as a protein. To do this, juvenile hormone must be modified to make functional groups available for reaction with the protein. Two methods of juvenile hormone modification have been reported thus far: a) the hydrolysis of the methyl ester to form a carboxylic acid (Figure 2a); and b) the opening of the epoxide ring to form the diol (Figure 2b). A third method of conjugating juvenile hormone has been proposed, but no results have been reported (Eng and Prestwich, 1986). This last method would retain both the epoxy and carboxy moieties (Figure 2c). Antibodies have been raised against immunogens prepared by methods (a) and (b) and have been used in the quantification of juvenile hormone in biological samples.

2.2.4.1. Conjugation of Juvenile Hormone via the Carboxylic Acid

Lauer et al. (1974a) described the preparation of a juvenile hormone immunogen by conjugating the hormone to the protein, human serum albumin (HSA). This method was further expanded by Baehr et al. (1976). In both methods, mild alkaline conditions were used to hydrolyze the methyl ester (17) to the acid (18) (Figure 7a), which

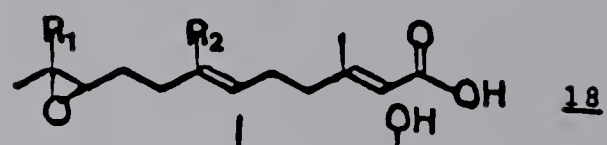
Figure 7. Synthetic scheme for the preparation of an immunogen and an iodinated tracer from juvenile hormone acids.



17

a

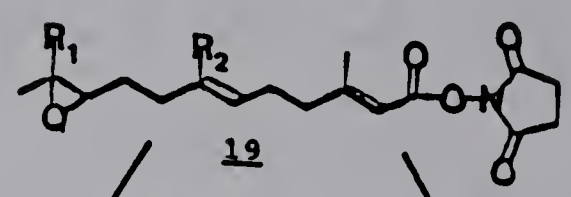
mild base



18

b

DCC,

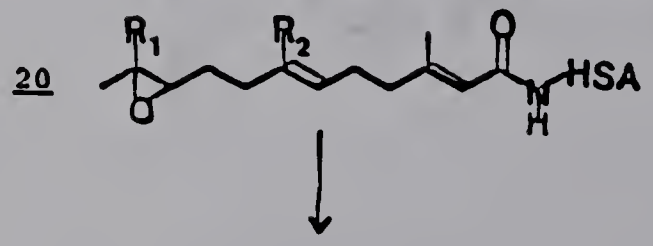


19

C Immunogen Preparation

H_2N-HSA

Tracer Preparation d



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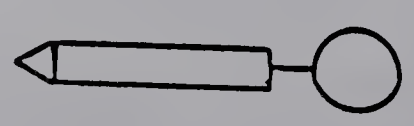

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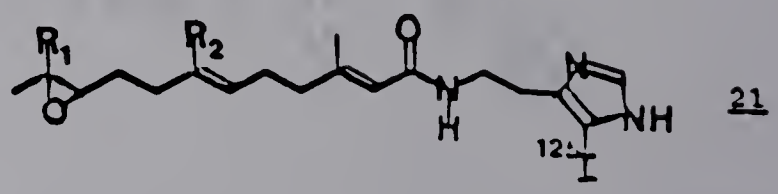

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was then coupled to N-hydroxysuccinimide (NHS) to make the activated ester (19) using the reagent 1,3-dicyclohexylcarbodiimide (DCC) (Figure 7b). Free lysine groups on the protein were reacted with the activated NHS-ester (19), conjugating juvenile hormone to the protein through an amide bond (20) (Figure 7c). Spectrophotometric estimation of the number of juvenile hormone residues per molecule of protein gave 20 (Lauer et al., 1974a) and 26 (Baehr, 1976). A radiolabeled tracer (21) was also prepared for subsequent characterization of the antibodies raised against the immunogen (20). The activated NHS-ester (19) was reacted with histamine and then radioiodinated with chloramine T to give a specific activity of 2000 ci/mmol (Figure 7d).

Immunogens were prepared in this way for the juvenile hormone homologs, juvenile hormone I, II, III, and were used to raise high titer polyclonal antibodies against each of them. These antibodies reacted 100% with their respective juvenile hormone acids, and cross reacted with other analogs to a lesser extent (Table 1). The first antibody produced (Lauer et al., 1974a) showed low binding affinity for juvenile hormone III (33 ug/ml assay for 50% displacement). The antibody generated by Baehr et al. (1976) had improved sensitivity (1.5 ng/ml for 50% displacement). Using antisera from the same source, Granger et al. (1979; 1982) reported affinities

Table 1. Reactivities and cross reactivities of antisera raised against juvenile hormone immunogens I, II, and III.

Hormone/analog	Reactivity and Cross-reactivity (%)					
	JH I			JH II	JH III	
	A	B	C	D	E	F
JH I	100	100	16.7	26	0.6	--
JH II	37	12.6	--	100	0.9	50
JH III	0.4	1.7	--	12	100	100
JH O	--	37	--	--	0.6	--
JH I acid	50	100	--	--	--	--
JH III acid	--	--	--	--	77.1	--
JH I diol	14	0.5	100	--	--	--
JH II diol	--	--	100	--	--	--
JH III diol	--	--	100	--	12.0	--

A: Baehr et al. (1976).
B: Granger et al. (1979).
C: Strambi et al. (1981).
D: Baehr et al., (1981).
E: Granger et al. (1982).
F: Lauer et al. (1974a).

for juvenile hormone I and III of 1.2 and 1.0 ng/ml, respectively, for 50% displacement. The antisera raised against juvenile hormone II by this method (Baehr et al., 1981) had high cross reactivity against both juvenile hormone I and III (Table 1), and has not been used to develop radioimmunoassays.

The antibodies raised against juvenile hormone I were used to develop a radioimmunoassay to study the biosynthesis of both juvenile hormones I and II in day 5 last instar larvae of Manduca sexta (Granger et al., 1979). A biosynthesis ratio of juvenile hormones I:II (1:4) was calculated from HPLC purified extracts of medium in which corpora allata were incubated.

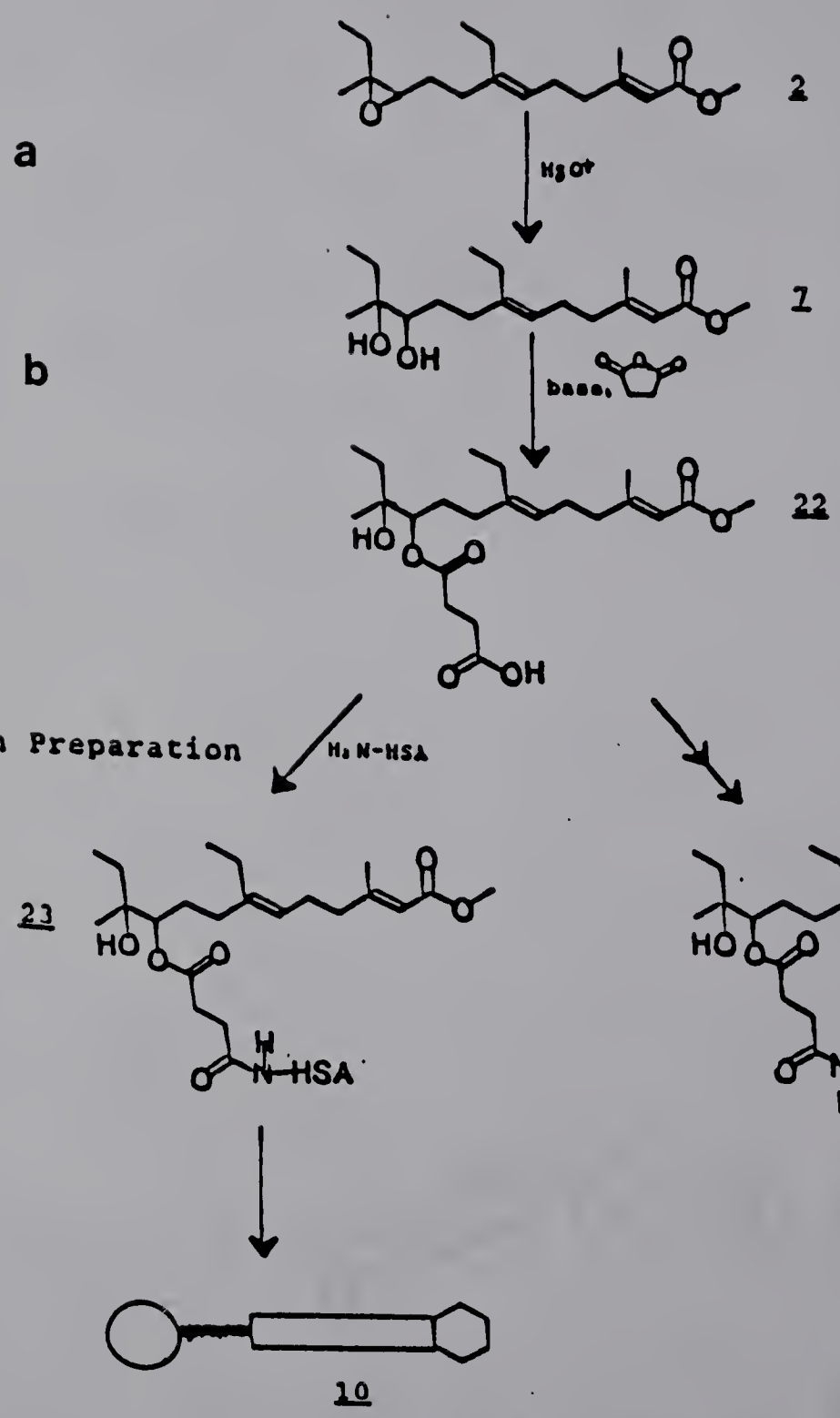
Granger et al. (1982) developed a radioimmunoassay for juvenile hormone III, and quantified juvenile hormone biosynthesis in the corpora allata of day 5 Manduca sexta larvae. Radioimmunoassay of HPLC-purified extracts in which corpora allata were incubated gave a biosynthesis ratio of juvenile hormones I:II:III (1:3.6:2.2). Granger et al. (1979) claimed that the 1:4 ratio of biosynthesis of juvenile hormone I:II is consistant with radiochemical-gas chromatographic results reported for day 5 Manduca sexta larvae by Dahm et al. (1976). However, Dahm et al. (1976), detected no juvenile hormone in larvae of this stage, and no reported values for juvenile hormone biosynthsis have the ratio of 1:4 for any Manduca sexta stage tested.

Additional problems have arisen when the radioimmunoassay for juvenile hormone I was used to quantify the juvenile hormone content in the hemolymph of fourth and fifth instar Locusta migratoria (Baehr et al., 1979). High levels of juvenile hormone I were found in both the fourth and fifth instar stage. When gas chromatography-mass spectrometry was used to identify the nature of juvenile hormone in the L. migratoria , at the same developmental stages, only juvenile hormone III was found (Bergot et al., 1981). Verification of the amount and nature of juvenile hormone (in biological samples) by physical-chemical methods has not been reported by groups using the antisera prepared against juvenile hormone acid immunogens (Granger et al., 1982; 1987; Bollenbacher et al., 1987).

2.2.4.2. Conjugation of Juvenile Hormone via the Epoxide

The second reported method for a juvenile hormone radioimmunoassay utilizes the epoxy moiety for the site of conjugation to a protein (Strambi et al., 1981). The epoxide ring of a mixture of juvenile hormone I (2) and ^{3}H-juvenile hormone I was opened to the diol (7) by treatment with acid (Figure 8a). The diol (7) was then treated with succinic anhydride in the presence of base to form the hemisuccinate (22) (Figure 8b), which was coupled to human serum albumin with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) (Figure 8c). On

Figure 8. Synthetic scheme for the preparation of an immunogen and an iodinated tracer from juvenile hormone diol.


a
2
H_3O^+
b
HO
OH
base,
7
HO
OH
22
C Immunogen Preparation
H_2N-HSA
d Tracer Preparation
23
HO
H
N-HSA
24
HO
125I
OH
H
N
H
N
H
O
COO^-
10

the basis of its radioactivity, the conjugate (23) was found to contain 17 molecules of succinyl juvenile hormone (22) per molecule of protein. An iodinated tracer (24) was also prepared by reaction of the juvenile hormone hemisuccinate (22) with glycyltyrosine, followed by iodination with ^{125}I-iodide (Figure 8d). This tracer had a radioactivity of 2×10^5 counts min^{-1} ml^{-1} when diluted with a citrate-hemoglobin buffer. Antibodies raised against the diol immunogen (23) had high specificity for the diol (7).

The diol radioimmunoassay has three advantages (Strambi et al., 1981): a) the diol is more immunoreactive than juvenile hormone; b) the diol is more water soluble and less absorbed onto surfaces; and c) the diol is easier to separate from lipids which are the main source of non-specific interference in juvenile hormone radioimmunoassays. Two major disadvantages of this radioimmunoassay are: a) the antibodies raised cannot distinguish between juvenile hormone diol homologs (Table 1); and b) all biological samples containing juvenile hormones must first be converted to their respective diol derivatives. This process entails lengthly extraction and purification procedures. Strambi et al. (1981) caution that the user should pay scrupulous attention to each step of the diol conversion so that the hormones are carefully purified and products are handled properly.

Despite the need for care in sample handling and purification, the radioimmunoassay developed for juvenile hormone diols was used to quantify the juvenile hormone titers in the hemolymph of the Colorado potato beetle (Strambi et al., 1984). The method was validated by physical-chemical analysis (de Kort et al., 1985). These results have encouraged the use of this assay for the determination of juvenile hormone titers in Bombyx mori (Plantevin et al., 1987) and Apis mellifera (Robinson et al., 1987).

2.3. Methoprene as an Insect Control Agent

Juvenile hormone analogs are chemicals structurally related to juvenile hormone. A juvenoid is any chemical capable of showing the same physiological activity in an insect species as the native juvenile hormone, and may be capable of restoring normal juvenile hormone-mediated functions in an allatectomized insect (Henrick, 1982). The juvenile hormone analog and juvenoid, methoprene, is the most commercially advanced example of a class of compounds known as the insect growth regulators (IGR's). Insect growth regulators interfere with the developmental processes of insects and pose few undesirable effects on man, wildlife, and the environment. The development-interfering activity of methoprene has proven to be effective against some species of insect pests with low

toxicity to non-target species and rapid degradation in the environment.

Methoprene has been shown to have low toxicity to protozoa, annelids, molluscs, crustacea, fish, amphibians, birds, and some non-pest insects such as damselflies, mayflies, and water beetles (Miura and Takahashi, 1973). Mammalian toxicity to methoprene was very low, where no toxicity was found in rats given 10,500 mg/kg (Siddall and Slade, 1974). In cattle [5-^{14}C]-methoprene was metabolized to acetate and other labeled intermediates (Quistad et al., 1975a). A large amount of the ^{14}C-label was lost by respiration ($^{14}CO_2$) in the cow and guinea pig (Chamberlain et al., 1975). The non-toxic effects of methoprene on mammals and some non-pest insects is a welcome advantage compared with traditional insecticides.

The life of methoprene in the environment is limited. Methoprene has been shown to be very susceptible to photolytic decomposition under enviromental conditions (Quistad et al., 1975b). It is also metabolized by aquatic microorganisms (Schooley et al., 1975). The half life of ^{3}H-methoprene in pond water containing unknown microorganisms was 30 h at a concentration of 0.001 ppm.

The Environmental Protection Agency has recognized the value of methoprene in pest control. Methoprene and another juvenoid, kinoprene, are registered by the Agency

for insect control (Henrick, 1982). Products in use include a mosquito larvicide (Altosid SR-10, Altosid Briquet), a horn fly larvicide (Altosid 10-f and Bolus), a mushroom fly control agent (Apex), and a flea control agent (Precor). Methoprene was the first successful commercial application of a juvenoid on a stored product, tobacco (Menn, 1981). Henrick (1982) lists many of the insect species which are susceptible to methoprene.

2.4. Mode of Action of Methoprene

Although juvenoids have juvenile hormone activity, their mode of action differs from that of juvenile hormone itself. The physiological activities induced by both juvenile hormone and juvenoids are similar. Studies at the molecular level, however, show that juvenoids have different binding sites than those of juvenile hormone.

High affinity juvenile hormone I binding sites have been isolated from Drosophila larval integument (Klages et al., 1980). Methoprene and other juvenile hormone analogs were found to be poor competitors for these binding sites in the presence of juvenile hormone I. An ^{125}I-labeled derivative of methoprene (Boehm and Prestwich, 1986) was also a poor competitor for juvenile hormone receptor sites in Manduca sexta integumental tissue, indicating for the first time that there may be different receptor sites for both juvenile hormone homologs and juvenile hormone analogs (Prestwich, 1987).

The molecular basis of action of dodecadienoates with juvenile hormone activity is still unclear, but it may be postulated that once lipid molecules like the juvenile hormone homologs or analogs, bind to specific cellular receptors, the releasing mechanism for physiological action may be the same. The binding affinites of cellular receptors may differ greatly. More study is needed on the mode of action of juvenile hormone homologs and analogs.

2.5. The Importance of Methoprene in the Development of Immunological Assays

Though problems with juvenile hormone radioimmunoassays exist, the merits of developing such a technique do not preclude its use as a means of juvenile hormone detection and determination. The value of juvenile hormone radioimmunoassays has been demonstrated (Granger and Goodman, 1983; Gilbert, 1984; Tobe and Stay, 1985; Eng and Prestwich, 1986). Before data generated from juvenile hormone radioimmunoassays can be accepted, more work needs to be done on antisera generation and characterization, and assay validation. Care must be used when preparing samples and separating juvenile hormone homologs from whole insect bodies or incubation media. The routine use of internal standards should be employed to monitor juvenile hormone recovery from any quantification method. The final test for a successful juvenile hormone radioimmunoassay will be its comparison

with data obtained from physical-chemical methods. Unless the assay has been proven unequivocally, it is no longer enough to claim the presence and quantity of a juvenile hormone homolog based on radioimmunoassay data alone.

To overcome the problems encountered with existing assays, the use of methoprene is proposed for the development of immunological assays. Each step in the preparation of the immunogen for methoprene has been documented with spectral data and radioisotope labeling where appropriate. The merits of methoprene as an insect control agent and a research tool are great, warranting its use as a model for the development of immunological assays. The information and experience gathered here may help the development of other immunological assays for other juvenoids.

Chapter 3

Methoprene Immunogen Preparation

3.1. Materials and Methods

Methoprene, isopropyl 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, and 5-^{14}C-methoprene were gifts from Zoecon Corporation. 1,3-Dicyclohexylcarbodiimide (DCC), 4-(N,N-dimethyl)amino pyridine (DMAP), sodium 4-hydroxybenzene sulfonate dihydrate, 2-(trimethylsilyl)ethanol, benzyl alcohol, 4-butyrolactone, N-hydroxysuccinimide (NHS), magnesium sulfate ($MgSO_4$), silver nitrate ($AgNO_3$), potassium phosphate monobasic (KH_2PO_4), and farnesol, 3,7,11-trimethyl-2,6,10-dodecatrienol, were obtained from Aldrich (Milwaukee, WI). Sodium phosphate monobasic (NaH_2PO_4) and sodium acetate were obtained from Baker (Phillipsburg, NJ). Tetraethylammonium fluoride (Et_4F) was obtained from Kodak (Rochester, NY). Pyridinium chlorochromate (PCC) was prepared by the method of Corey and Suggs (1975).

Solvents used were Fisher reagent grade. Tetrahydrofuran (THF) and acetonitrile (CH_3CN) were distilled from calcium hydride. The mixture of hexanes used as a solvent was the petroleum distillate Skelly B (common name for a mixture of hexanes). Hydrochloric acid (HCl), acetic acid (HOAc), sulfuric acid (H_2SO_4), nitric acid (HNO_3), sodium bicarbonate ($NaHCO_3$), sodium

hydroxide (NaOH), potassium hydroxide (KOH), and hydrogen peroxide (H_2O_2) were also obtained from Fisher. Flash chromatography was carried out according to Still et al. (1978) using technical grade solvents. Silica gel, Merck 230-400 mesh, 60 Å, and silica gel coated glass plates, 60 Å, were obtained from Aldrich.

Bovine serum albumin (BSA), fraction V, and Freund's complete and incomplete adjuvants were obtained from Gibco (Grand Island, NY). Human serum albumin (HSA, fraction V, fatty acid free), thimersal (sodium ethylmercurithiosalicyate), 2,2'-azino(3-ethylbenzthiazolin sulfonate)-diammonium salt (ABTS), Tween 20 (polyoxyethylenesorbitan monolaurate), and goat antimouse horseradish peroxidase conjugated antibody were purchased from Sigma (St. Louis, MO). Tris (tri(hydroxymethyl) aminoethane) was obtained from Bio-Rad (Richmond, CA). Dialysis tubing, molecular weight cut off 12,000-14,000, was obtained from Fisher. Turberculin syringes, 1 and 5 ml, were obtained from Becton-Dickinson (Rutherford, NJ). Immulon 1, poly(vinyl chloride) (PVC) wells were obtained from Dynatech Labs (Alexandria, VA).

Liquid scintillation counting was done on a Packard Tri-carb spectrometer with scintillation counting medium (Aquasol II) from New England Nuclear (Cambridge, MA). Values are reported in counts min^{-1} (cpm). Ultraviolet and visible spectrophotometric analysis were carried out in a LKB Ultraspec 4050. Absorbances from enzyme linked

Immunosorbant assays (ELISA) were read on a MR 600 Microtitre Plate Instrument from Dynatech Instruments (Torrance, CA), and reported in optical density (O.D.). Infrared (IR) spectra were recorded on a Perkin Elmer 1420 ratio recording spectrophotometer. The chart was calibrated against polystyrene (1601 cm^{-1}). Proton nuclear magnetic resonance (^1H NMR) was performed on either a 60 MHz Perkin Elmer R12A or a 200 MHz Varian spectrometer. Chemical shifts (*k*) are reported in parts per million (ppm), down field from the internal standard, tetramethylsilane (*k* 0.0). All nmr and ir spectra may be found in Appendix A, page 138.

Eight female, BALB/c mice were obtained from Charles River Breeding Labs (Cambridge, MA). The mice were kept at 27°C on a diet of rat chow.

3.2. Experimental Section

The following sections describe the synthesis of all intermediates necessary for the preparation of an immunogen for methoprene.

4-Benzyloxybutanoic acid (28)

A solution of benzyl alcohol (25) (27.12 g, 0.25 mol) in toluene (150 ml) was added slowly to sodium (5.75 g, 0.25 mol) in xylene (50 ml). Upon completion of the addition, the mixture was heated to reflux for 4 h. 4-Butyrolactone (27) (21.51 g, 0.25 mol) was added and the solvent was removed by heating the mixture slowly at

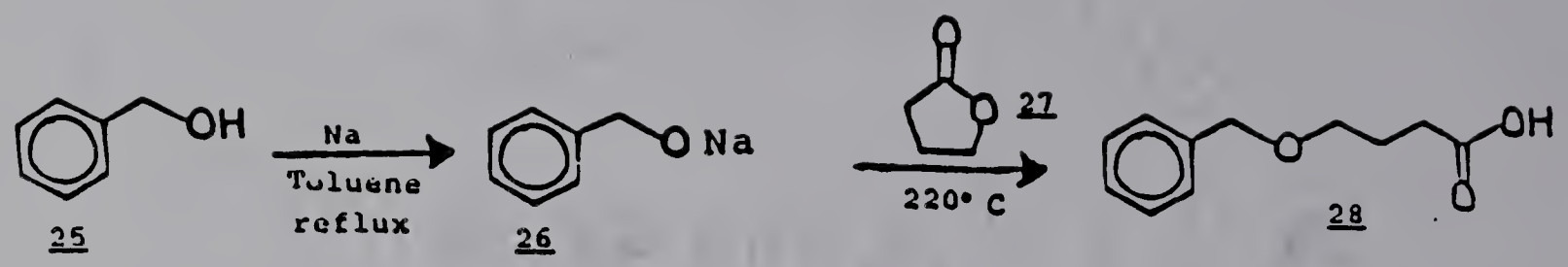
220°C. The temperature was maintained at 220°C for 2 h. The mixture was cooled and the yellow solid was dissolved in water (300 ml), washed with diethyl ether (Et_2O) (2 x 100 ml) and acidified with glacial acetic acid. The mixture was extracted with Et_2O (3 x 200 ml), the combined extracts were dried ($MgSO_4$) and the solvent was removed. The residue was distilled to give 9.52 g (21%) of (28) (Figure 9a): bp 140°C/0.8 mmHg (150°C/2.5 mmHg, Sudo et al., 1967). ^1H NMR ($CDCl_3$, 200 MHz): *k* 1.8-2.1 (m, 2H, 3-CH_2), 2.50 (t, J = 7 Hz, 2H, 2-CH_2), 3.55 (t, J = 6 Hz, 2H, 4-CH_2), 4.03 (s, 2H, benzylic-CH_2), 7.25 (s, 5H, phenyl). IR (neat): cm^{-1} 3600-2400 (w, COOH), 1710 (s, C=O), 700, 750 (m, Ph).

2-(Trimethylsilyl)ethyl 4-benzyloxybutanoate (30)

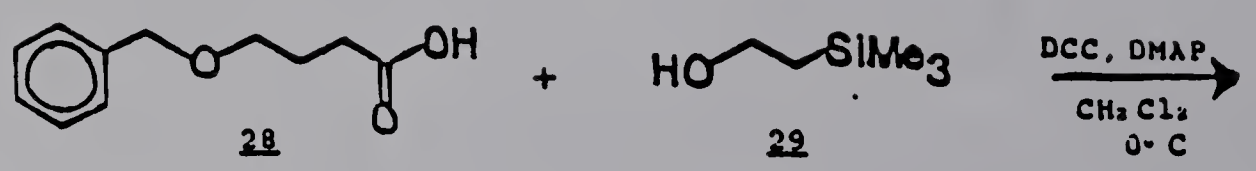
4-(N,N-Dimethyl)amino pyridine (DMAP) (0.73 g, 5.97 mmol) and 1,3-dicyclohexylcarbodiimide (DCC) (5.92 g, 28.69 mmol) were added to a solution of 4-benzyloxybutanoic acid (28) (4.44 g, 22.85 mmol) and 2-(trimethylsilyl)ethanol (29) (4.07 g, 34.41 mmol) in dichloromethane (CH_2Cl_2) (50 ml) at 0°C (Sieber et al., 1977). The mixture was allowed to stand at 0°C for 48 h. Dicyclohexylurea (DCU) was filtered from the mixture and the filtrate was washed with 10% aq $NaHCO_3$ (3 x 50 ml), water (3 x 50 ml), 10% aq acetic acid (3 x 50 ml), water (3 x 50 ml), saturated NaCl (1 x 50 ml), and dried

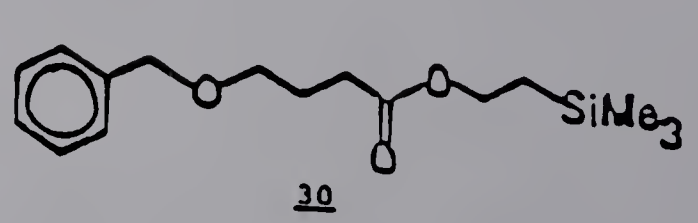
Figure 9. Synthetic routes leading to the preparation of 2-(trimethylsilyl)ethyl-4-hydroxybutanoate (31).

a

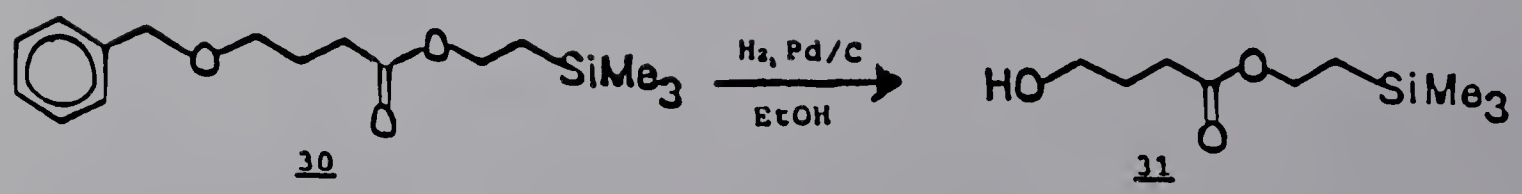

OH
Na
Toluene
reflux
O Na
27
220° C
OH
25
26
28

b


OH
28
+
HO
SiMe3
29
DCC, DMAP
CH2Cl2
0° C


SiMe3
30

c


SiMe3
30
H2, Pd/C
EtOH
HO
SiMe3
31

($MgSO_4$). The solvent was removed to give a yellow liquid which was purified by flash chromatography (ethyl acetate:Skelly B, 10:90), to afford 30 as a pale yellow liquid (6.26 g, 93%) (Figure 9b). ^1H NMR ($CDCl_3$, 60 MHz): *k* 0.14 (s, 9H, $SiMe_3$), 0.83 (t, J = 7 Hz, 2H, 2'-CH_2), 1.4-2.0 (m, 2H, 3-CH_2), 2.23 (t, J = 6 Hz, 2H, 2-CH_2), 3.32 (t, J = 6 Hz, 2H, 4-CH_2), 4.01(t, J = 7 Hz, 2H, 1'-CH_2), 4.32 (s, 2H, benzylic-CH_2), 7.16 (s, 5H, Ph). IR (neat): cm^{-1} 3020 (w, Ph), 1730 (s, COOR) 1250 (m, Si-C) 700,750 (m, Ph).

2-(Trimethylsilyl)ethyl-4-hydroxybutanoate (31)

Palladium on carbon (5%, 300 mg, Aesar, Type II unreduced) was added to a cold solution of 30 (3.10 g, 10.52 mmol) in absolute ethanol (100 ml) in a 500 ml hydrogenation flask (Greene, 1981a). The mixture was shaken under 2 atm hydrogen for 1.5 h. The catalyst was removed by filtration and the solvent was evaporated to give 2.11 g (98%) of 31 as a clear liquid (Figure 9c). ^1H NMR ($CDCl_3$, 60 MHz): *k* 0.14 (s, 9H, $SiMe_3$), 0.90 (t, J = 7 Hz, 2H, 2'-CH_2), 1.5-2.0 (m, 2H, 3-CH_2), 2.25 (t, J = 6 Hz, 2H, 2-CH_2), 3.14 (br s, 1H, OH), 3.50 (t, J = 6 Hz, 4-CH_2), 4.06 (t, J = 7 Hz, 1'-CH_2). IR (neat): cm-1 3400 (br, m, OH), 1730 (s, COOR), 1250 (m, C-Si).

3,7,11-Trimethyl-2,6,10-dodecatrienal (33)

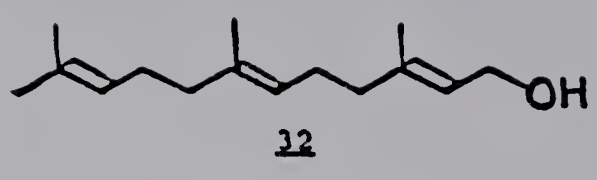
Farnesol (31) (5.62 g, 25.27 mmol) in CH_2Cl_2 (10 ml) was added to a stirred solution of pyridinium

chlorochromate (PCC) (9.41 g, 43.65 mmol) in CH_2Cl_2 (50 ml) (Corey and Suggs, 1975). The solution was stirred at room temperature for 4 h. Diethyl ether (100 ml) was added and the supernatant was decanted from the black gum. The insoluble residue was washed with Et_2O (4 x 25 ml) and the combined washings were passed through a pad (3") of Florisil (100-200 mesh), the solvent was removed and the residue was purified by flash chromatography (ethyl acetate:Skelly B 10:90), to give 33 as a yellow oil (4.49 g, 79%) (Figure 10a). ^1H NMR ($CDCl_3$, 60 MHz): δ 1.60 (s, 3H, 12-Me), 1.68 (s, 3H, 11-Me), 1.0-2.6 (m, 14H, allylic H, 3,7-Me), 4.7-5.2 (m, 2H, vinylic 6,10-H), 5.5-5.7 (m, 1H, vinylic 2-H) 9.7-10.0 (m, 1H, CHO). IR (neat): cm^{-1} 2700 (w, aldehyde C-H), 1675 (s, C=O), 1630 (w, C=C). The aldehyde was identified by comparison of its IR spectrum with that of an authentic sample (Sadtler No. 1050K).

3,7,11-Trimethyl-2,6,10-dodecatrienoic acid (34)

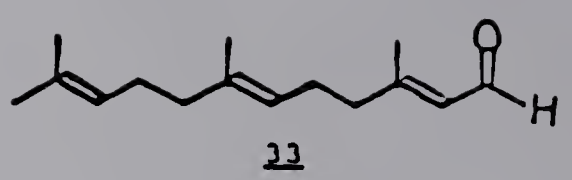
A saturated aq solution of silver nitrate (6.21 g, 36.55 mmol) was added with cooling (0-10°C) to a solution of farnesal (33) (4.06 g, 18.42 mmol) in ethanol (95%, 30 ml) (Caliezi and Schinz, 1949). Potassium hydroxide (4.80 g, 85.54 mmol) in water (200 ml) was added and the reaction was stirred at room temperature for 24 h. Silver and silver oxide were filtered from the solution, which was acidified with 50% H_2SO_4. The mixture was extracted

Figure 10. Oxidation and hydrolysis of juvenile hormone analogs. a and b: Two-step oxidation of farnesol (32) to form its carboxylic acid (34). c: Hydrolysis of methoprene (1) using base to form the acid (35).

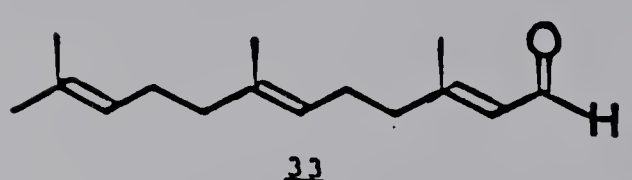
a 

32

PCC
CH_2Cl_2

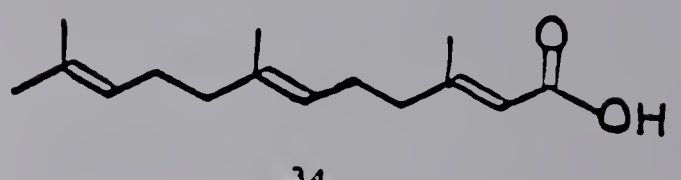


33

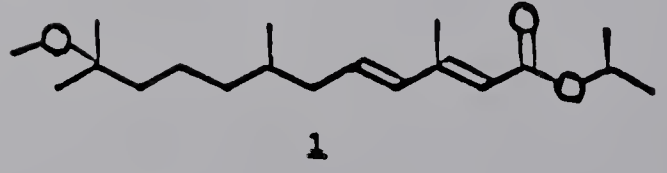
b 

33

1. $AgNO_3$
2 KOH
aq EtOH

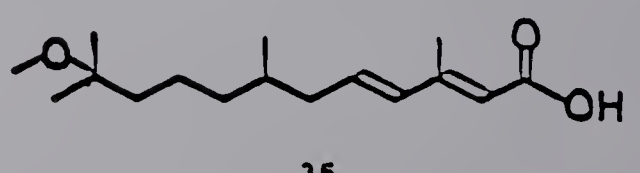


34

c 

1

KOH
EtOH



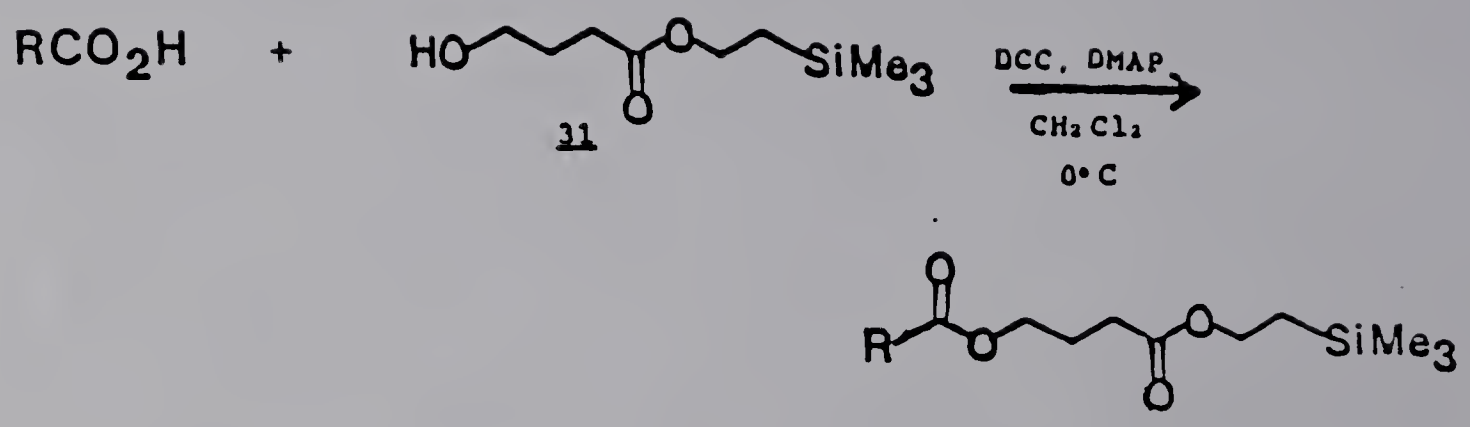
35

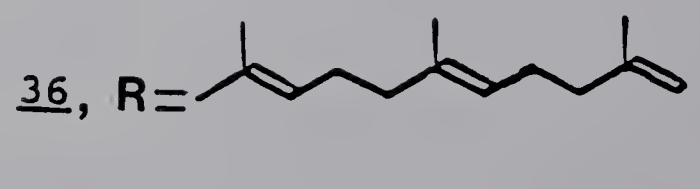
into Et_2O (4 x 100 ml). The combined extracts were dried ($MgSO_4$), the solvent was removed, and the remaining oil was purified by flash chromatography (ethyl acetate:Skelly B, 25:75) to give **34** as a yellow oil (3.13 g, 81%) (Figure 10b). ^1H NMR ($CDCl_3$, 60 MHz): *k* 1.60 (s, 3H, 12-Me), 1.68 (s, 3H, 11-Me), 1.0-2.6 (m, 14H, allylic H, 3,7-Me), 4.8-5.2 (m, 2H, vinylic 6,10-H), 5.5-5.7 (m, 1H, vinylic 2-H), 11.2 (br s, COOH). IR (neat): cm^{-1} 3500-2500 (m br, COOH), 1690 (s, C=O), 1640 (m, C=C), 1250 (m, C-O). The acid was identified by comparison of its ^1H NMR spectrum with that of an authentic sample (Sadtler No. 14666).

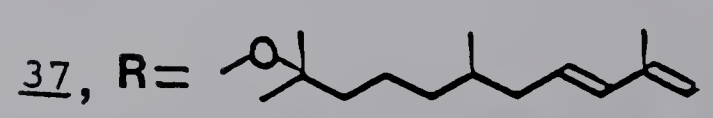
2-(Trimethylsilyl)ethyl 4-(3,7,11-trimethyl-2,6,10-dodecatrienoyloxy)butanoate (36)

DMAP (0.039 g, 0.32 mmol) and DCC (0.26 g, 1.26 mmol) were added to a chilled solution of **34** (0.17 g, 0.76 mmol) and (3) in CH_2Cl_2 (10 ml). The mixture was stirred at 0^oC for 5 min and allowed to stand at 0^oC for 24 h. DCU was filtered from the reaction mixture and the filtrate was washed with aq $NaHCO_3$ (3 x 30 ml), water (3 x 30 ml), 10% aq acetic acid (3 x 30 ml), dried ($MgSO_4$), and the solvent was removed. The oily residue was purified by flash chromatography (ethyl acetate:Skelly B, 10:90), leaving **36** as a pale yellow oil, (0.20 g, 63%) (Figure 11). ^1H NMR ($CDCl_3$, 60 MHz): *k* 0.14 (s, 9H, Me), 1.54 (s, 3H, 11-Me), 1.0-2.6 (m, 16H, allylic H,

Figure 11. Coupling reaction used to synthesize the trimethylsilyl-esters **36** and **37**.


RCO2H
+
HO
SiMe3
31
DCC, DMAP
CH2Cl2
0°C
R
SiMe3


36, R=


37, R=

$SIMe_3$), 0.84 (t, J = 7 Hz, 2H, 2'-CH_2), 1.45 (s, 3H, 12-3,7-Me), 3.92 (t, J = 6 Hz, 2H), 4.03, (t, J = 7 Hz, 2H, 1'-CH_2), 4.7-5.1 (m, 2H, vinylic 6,10-H), 5.45 (m, 1H, vinlylc 2-H). IR (neat): cm^{-1} 1720-1750 (s, C=O), 1650 (m, C=C), 1250 (m. Si-C).

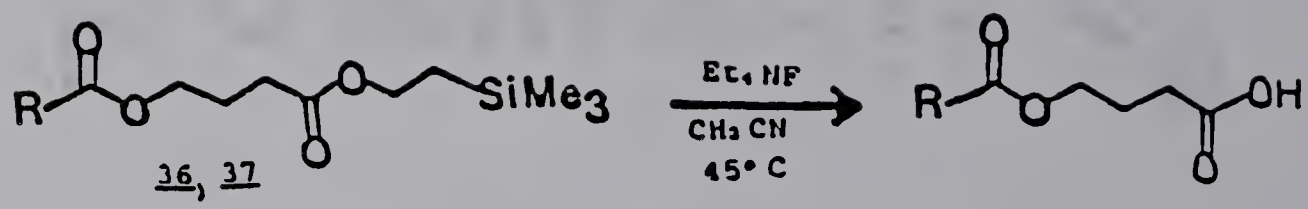
4-(3,7,11-trimethyl-2,6,10-dodecatrienoyloxy)butanoic acid (38)

The 2-trimethylsilyl ester (36) (0.16 g, 0.38 mmol) was slowly added to a solution of tetraethylammonium fluoride (Et_4F) (0.57 g, 3.78 mmol) in dry acetonitrile (CH_3CN) (25 ml) and the reaction mixture was heated to 45°C for 6 h (Carpino and Tsao, 1978). The mixture was concentrated and water (25 ml) was added. The aq solution was extracted with hexane (1 x 50 ml), acidified with H_2SO_4, and extracted with Et_2O (4 x 25 ml), dried ($MgSO_4$), and the solvent was removed. Flash chromatography (ethyl acetate:Skelly B:acetic acid, 50:50:0.1) gave 38 as an orange-yellow oil (0.10 g, 80%) (Figure 12). ^1H HMR ($CDCl_3$, 60 MHz): *k* 1.56 (s, 3H, 12-Me), 1.63 (s, 3H, 11-Me), 1.0-2.4 (m, 18H, 3,7-Me), 4.08 (t, J = 7 Hz, 2H, 4-CH_2), 4.8-5.2 (m, 2H, vinylic 6,10-H), 5.5-5.6 (m, 1H, vinylic 2-H). IR (neat): cm^{-1} 3600-2500 (br m, COOH), 1720 (s, C=O), 1650 (m, C=C).

(2E,4E)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid (35)

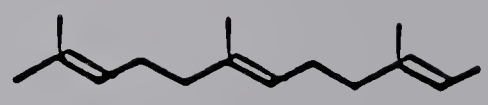
Methoprene (1) (Isopropyl (2E,4E)-11-methoxy-3,7,11-

Figure 12. Deprotection reaction used to remove the trimethylsilyl protecting residue.

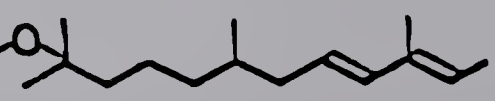


36, 37

38, R =



39, R =



trimethyl-2,4-dodecadienoate) (1.09 g, 3.51 mmol) was added to 25 ml of a 2.5 M solution of KOH in ethanol (Henrick, et al., 1973). The reaction mixture was stirred for 18 h, and the solution was concentrated under reduced pressure. Water (10 ml) was added, and the aqueous solution was extracted with Et_2O (2 x 25 ml). The aqueous solution was acidified with 50% H_2SO_4, extracted with Et_2O (3 x 25 ml), and dried ($MgSO_4$). The solvent was removed leaving an orange residue which was purified by flash chromatography (ethyl acetate:Skelly B, 20:80), to give **35** as a yellow oil (1.04 g, 95%) (Figure 10c). ^1H NMR ($CDCl_3$, 60 MHz): *k* 0.93 (d, J = 6 Hz, 3H), 1.11 (s, 6H, 11-Me), 1.2-1.5 (m, 7H, allylic H), 2.0-2.2 (m, 2H, 6-CH_2), 2.29 (s, 3H, 3-Me), 3.13 (s, 3H, OMe), 5.6-5.7 (m, 1H, vinylic 2-H), 6.2-6.3 (m, 2H, vinylic 4,5-H), 11.2 (br s, 1H, acidic H). IR (neat): cm^{-1} 3500-2500 (s br, COOH), 1690 (s, C=O), 1610 (s, C=C), 1260 (s, C-O). ^1H NMR ($CDCl_3$, 60 MHz) (Henrick et al., 1973): *k* 0.88 (d, J = 6 Hz, 3H, 7-Me), 1.13 (s, 6H, 11-Me), 3.18 (s, 3H, OMe), 5.72 (br, s, 1H, vinylic H-2), 6.13 (m, 2H, vinylic 4,5-H).

2-(Trimethylsilyl)ethyl 4-(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy)butanoate (37)

DMAP (13.4 mg, 0.11 mmol) and DCC (142 mg, 0.69 mmol) were added to a cold (0°C) solution of **9** and **3** in CH_2Cl_2. The solution was stirred for 5 min and allowed to stand at 0°C for 24 h. DCU was filtered from the

solution, which was then washed with 10% aq $NaHCO_3$ (3 x 25ml), water (3 x 25 ml), 10% aq acetic acid (3 x 25 ml), water (3 x 25 ml), and saturated aq NaCl (2 x 25 ml), and dried ($MgSO_4$). The solvent was removed to give a yellow residue, which was purified by flash chromatography (ethyl acetate:Skelly B 10:90), to give 37 as a pale yellow oil (95.5 mg, 66%) (Figure 11). ^1H NMR ($CDCl_3$, 60 MHz): *k* 0.11 (s, 9H, $SiMe_3$), 0.7-1.0 (m, 5H, 7-Me; 1'-CH_2), 1.12 (s, 6H, 11-Me), 1.2-1.5 (m, 9H, allylic H), 1.7-2.6 (m, 7H, 3-Me, allylic H), 3.15 (s, OMe), 3.9-4.4 (m, 4H, O-CH_2), 5.6-5.7 (m, 2H, vinylic H), 6.0-6.2 (m, 1H, vinylic H). IR (neat): cm^{-1} 1730 (s, C=O), 1710 (s, C=O), 1610 (m C=C), 1250 (m, Si-C). Elemental anaylsis: calculated for $C_{25}H_{46}O_5Si$: C, 66.04; H, 10.20. Found: C, 65.98; H, 9.97.

4-(2E,4E)-11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy) butanoic acid (39)

The 2-(trimethylsilyl)ethyl ester, 37 (0.16 g, 0.33 mmol), was added to a solution of Et_4F (0.61 g, 3.30 mmol) in dry CH_3CN (25 ml), and heated to 45°C for 6 h. The reaction mixture was concentrated under reduced pressure and water (25 ml) was added. The aqueous solution was washed with hexane (2 x 25 ml) and acidified (50% H_2SO_4). The solution was extracted with Et_2O (3 x 25 ml), dried ($MgSO_4$) and the solvent was removed, leaving an orange residue. The residue was purified by flash chromatography (ethyl acetate:hexane:acetic acid,

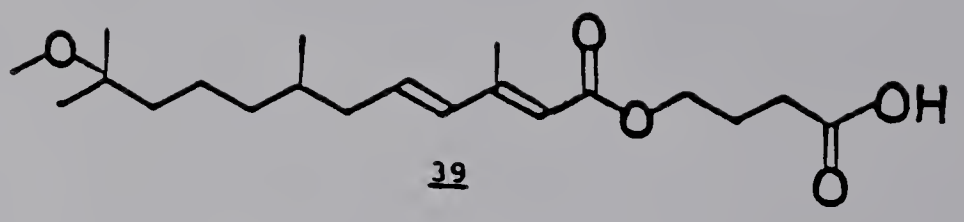
50:50:0.1) to give 39 as a yellow oil (79.4 mg, 68%) (Figure 12). ^{1}H NMR ($CDCl_3$, 60 MHz): *k* 0.8 (d, J = 6 Hz, 3H, 7-Me), 1.12 (s, 6H, 11-Me), 1.3-1.5 (m, 9H, allylic H), 1.9-2.1 (m, 4H, 6',3-CH_2), 2.2-2.5 (m, 5H, 3'-Me, 1-CH_2), 3.15 (s, 3H, OMe), 4.15 (t, J = 6 Hz, 2H, 4-CH_2), 5.6-5.7 (m, 1H, vinylic H), 6.0-6.1 (m, 2H, vinlylc H). IR (neat): cm^{-1} 3500-2500 (m br, COOH), 1710 (s, C=O), 1610 (m, C=C), 1240 (s, C-O), 1150 (s, C-O). Elemental analysis: calculated for $C_{20}H_{34}O_5$: C, 67.76; H, 9.67. Found: C, 67.60; H, 9.65.

N-hydroxysuccinimidyl 4-(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy)butanoate (41)

DCC (156 mg, 0.75 mmol) was added to a cold (0°C) solution of N-hydroxysuccinimide (40) (54.7 mg, 0.47 mmol) and 10 (135 mg, 0.38 mmol) in THF (5 ml) (Lauer et al., 1974a; Baehr et al., 1976). The mixture was stirred for 5 min and allowed to stand at 0°C for 24 h. DCU was filtered from the solution and DCC was added (15 ml). The organic solution was washed with 10% aq $NaHCO_3$ (3 x 10 ml), water (3 x 10 ml), 10% aq acetic acid (3 x 10 ml), water (3 x 10 ml), dried ($MgSO_4$) and the solvent was removed. The yellow residue was taken up in chloroform (2 ml), filtered from DCU, and the solvent was removed to give impure 41 an oily solid (134 mg, 78%) (Figure 13a). ^{1}H NMR ($CDCl_3$, 60 MHz): *k* 0.8 (d, J = 6 Hz, 3H, 7-Me),

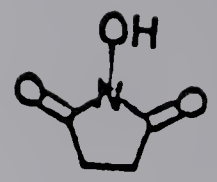
Figure 13. Formation of activated esters of methoprene>spacer (39). a: N-hydroxysuccinimide (NHS) ester (41). b: 1-Hydroxy-2-nitro-4-benzenesulfonate (HNSA) ester (44).

a



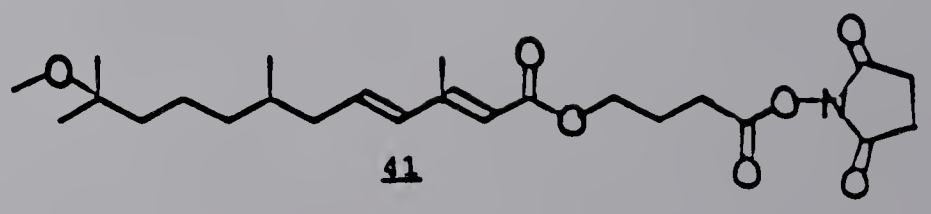
39

+



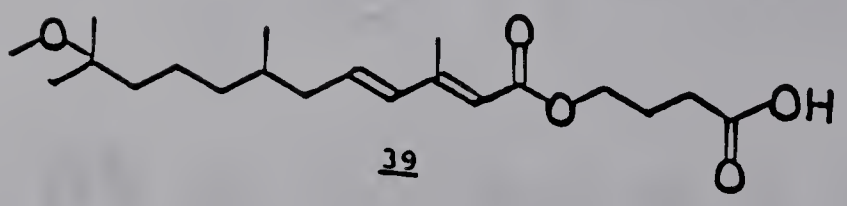
40

DCC
THF
0° C



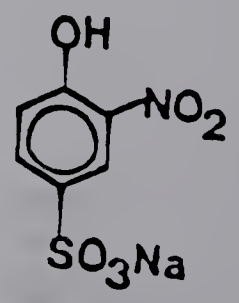
41

b



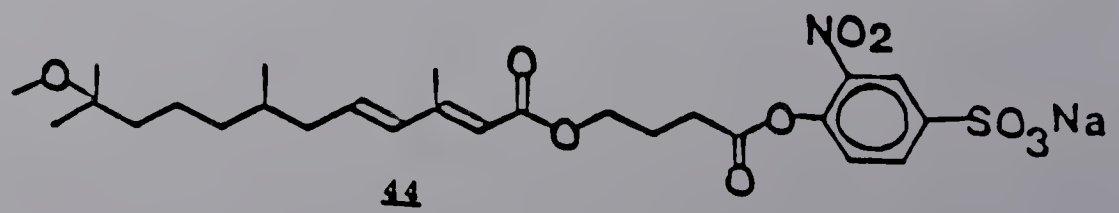
39

+



43

DCC
DMF
RT



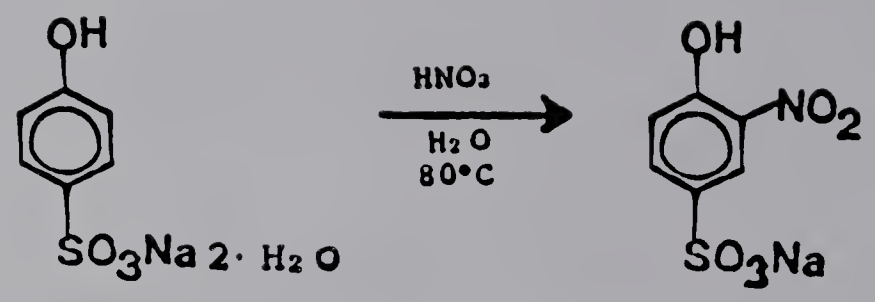
44

1.2 (s, 6H, 11-Me), 2.8 (s, 4H, succinimidyl CH_2), 3.15 (s, 3H, OMe), 4.15 (t, J = 6 Hz, 2H, 4-CH_2), 5.6-5.7 (m, 1H, vinylic H), 6.0-6.1 (m, 2H, vinylic H). IR (neat): cm^{-1} 3320 (m, NH, DCU), 1820, 1790, 1740, 1710 (m, m, s, s, C=O), 1610 (m, C=C), 1250, 1210, 1160 (m, C=C).

Sodium 1-Hydroxy-2-nitro-4-benzenesulfonate (43)

Sodium 4-hydroxybenzenesulfonate dihydrate (42) (20 g, 86.14 mmol) was added to water (50 ml) at 60°C. Nitric acid (12.0 ml, 276.24 mmol) was added dropwise over 30 min (King, 1921). The temperature rose to 80°C and was maintained for 15 min after addition of the nitric acid. The reaction mixture was cooled and orange crystals were filtered from the solution. The material was recrystallized from ethanol:water (1:2), to give 43 as yellow crystals (11.79 g, 57%) (Figure 14). ^1H NMR (D_2O, 200 MHz): δ 6.62 (d, J = 10 Hz, 1H, C6-H), 7.31 (d of d, J = 10 Hz, 3Hz, 1H, C5-H), 7.80 (d, J = 3 Hz, 1H, C3-H). IR (KBr): cm^{-1} 3460 (s br, OH), 1625 (s, C=C, Ar), 1525, 1330 (s, NO_2), 1200 (s, C-O), 1160, 1050 (s, SO_3), 730-850 (m, 1,2,4-substituted Ar ring). Elemental anaylsis: calculated for $C_6H_4NNaO_6S$: C, 29.88; H, 1.87; N, 5.81; Na, 9.53. Found: C, 29.71; H, 1.58; N, 5.70; Na, 9.57. According to King(1921), the salt was obtained as the trihydrate.

Figure 14. Preparation of sodium 1-hydroxy-2-nitro-4-benzenesulfonate.



42 43

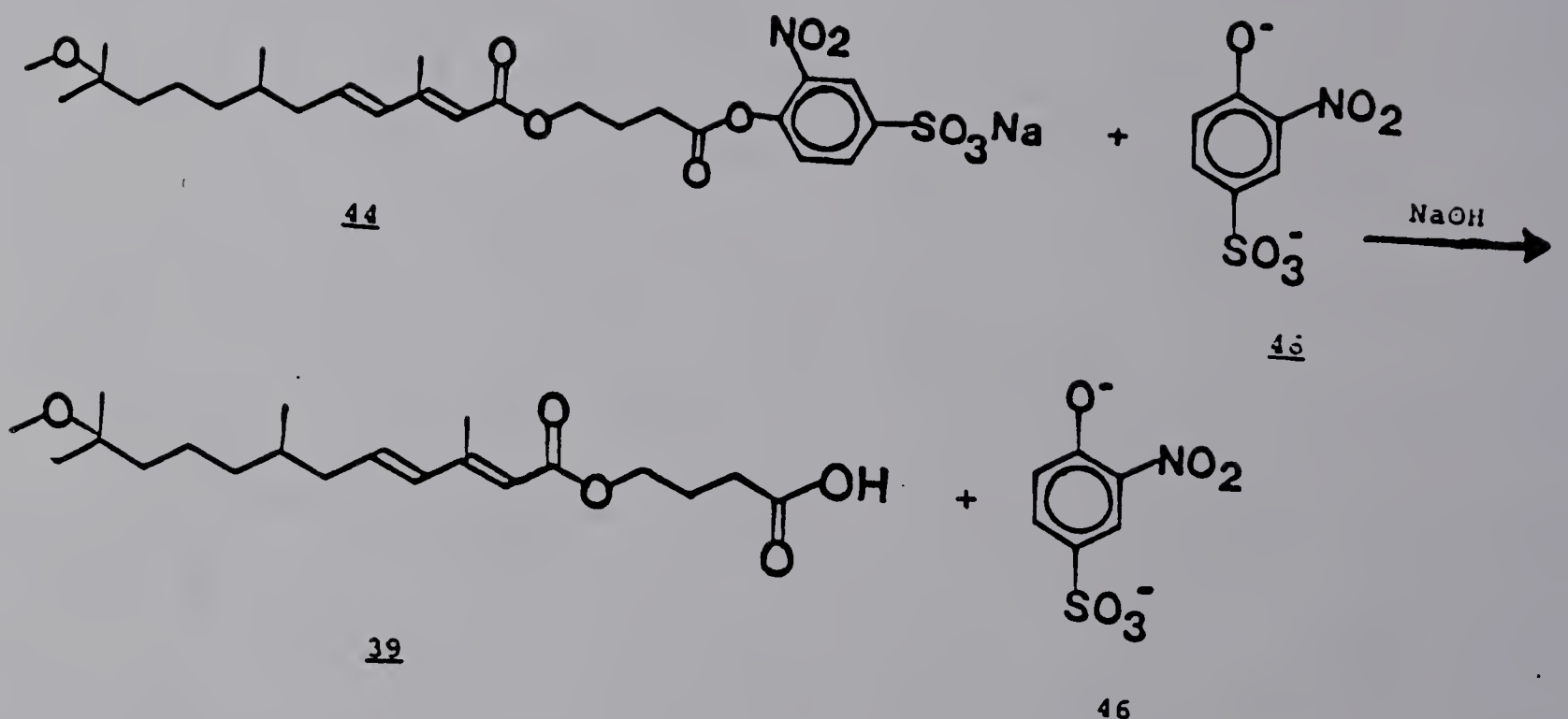
Sodium 1-(4-(2E,4E-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy)butanoyloxy)-2-nitro-4-benzenesulfonate (44)

Methoprene>spacer (39) (60 mg, 0.17 mmol) was added to a solution of 43 in N,N-dimethylformamide (DMF) (2 ml). DCC (60 mg, 0.29 mmol) was added and the solution was stirred for 24 h at room temperature (Aldwin and Nitecki, 1987) . DCU was filtered from the solution and the filtrate was washed with Et_2O (5 ml). The solvent was removed under reduced pressure with gentle heating. Et_2O was added to the orange residue until it solidified. Et_2O was removed, the solid was taken up in water (3 ml) and lyophilized, leaving 44 as a fluffy yellow solid (76 mg, 77%) (Figure 13b).

Analysis of Esters of 1-Hydroxy-2-Nitro-4-Benzenesulfonic Acid (HNSA)

The amount of material in the form of an ester of 1-hydroxy-2-nitro-benzenesulfonic acid (HNSA) was determined by base hydrolysis of a mixture of the HNSA ester and the free dianion (46) (Figure 15). Methoprene>Spacer-HNSA ester (44) was dissolved in 0.01M phosphate buffer (pH 7.0, 1 ml). The free dianion (46) was determined by reading the absorbance at 406 nm. A second reading was taken after complete hydrolysis of the ester with 5N NaOH (50 uL). The difference between the two absorbances represents the total amount of HNSA-ester

Figure 15. Base hydrolysis of the HNSA ester (44) to form methoprene>spacer (39) and the yellow dianion (46).



44 46

39 46

(Aldwin and Nitecki, 1987). This can be represented as

$$\frac{A_{406}(NaOH) - A_{406}}{A_{406}(NaOH)} \times 100 \qquad \text{Equation 1.}$$

Three sets of absorbance readings were obtained for Methoprene>spacer-HNSA ester, giving 29.9% as the total amount of ester present in the crude product (Table 2).

3.3. Protein Coupling Reactions

Two methods of coupling the derivatized methoprene molecule to human serum albumin were investigated.

3.3.1. Coupling of Methoprene>Spacer-N-Hydroxysuccinimide Ester (41) to Human Serum Albumin

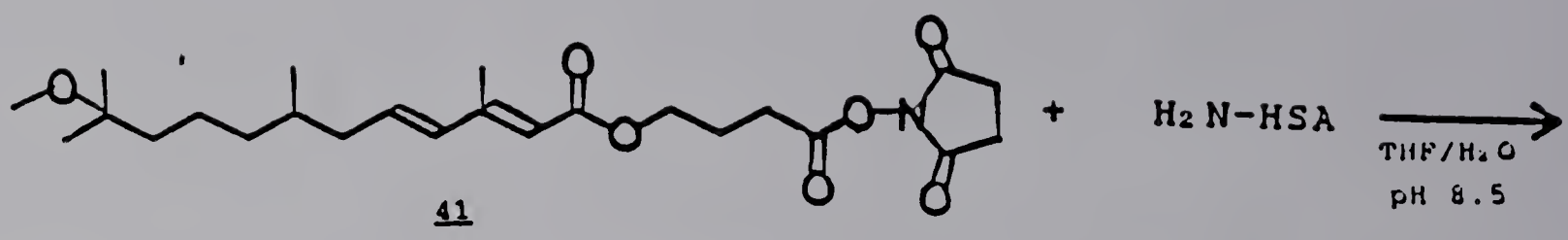
^{14}C-5-Methoprene>spacer-N-hydroxysuccinimide ester (41) (4.17 mg, 9.2 umol, 2.03×10^4 cpm mg^{-1}) was added to a solution of human serum albumin (HSA) (13.15 mg, 0.19 umol) in THF/H_2O (56:44, pH 8.5, 500 uL), and stirred for 24 h (Figure 16) (Baehr et al., 1976). The solution was transferred to a 4" dialysis tube and dialyzed against phosphate buffered saline (0.05M $PO_4^=$, 0.05M NaCl, pH 7.4, 1.5 L), and water (1.5 L). Aliquots (1 ml) of the dialysis washings were removed, liquid scintillation counting medium (8 ml) was added, and the aliquots were counted. Dialysis was ended when no more radioactivity was detected in the washings. The contents of the dialysis tube were lyophilized (24 h), giving a white fluffy powder (45) (12.87 mg, 4344 cpm mg^{-1}). Based on its radioactivity, the protein product contained

Table 2. Percentage of Methoprene>spacer-HNSA ester as determined by spectrophotometry at 406 nm.

Amount HNSA-ester (ug)	A_{406}	A_{406}(NaOH)	% ester
140	0.500	0.714	29.97
50	0.217	0.306	29.08
53	0.221	0.319	30.72
		Ave.=	29.92

HNSA: 1-Hydroxy-2-nitro-4-benzenesulfonate.

Figure 16. Formation of the methoprene immunogen (45) from the activated NHS-ester (41) and human serum albumin (H_2N-HSA).



41

45

16

21 molecules of methoprene>spacer per molecule of protein (see sample calculations, Appendix 2). The reaction was repeated twice under similar conditions, but the reaction time was increased to 48 h, giving 53 and 48 molecules ofmethoprene>spacer per molecule of protein (Table 3).

3.3.2. Coupling of Methoprene>Spacer-1-Hydroxy-2-Nitro-4-Benzenesulfonate (44) to Human Serum Albumin

^{14}C-5-Methoprene>spacer-1-hydroxy-2-nitobenzenesulfonate (44) (10.52 mg, 18.3 umol, 1.5×10^{-4} cpm mg^{-1}) was added to a solution of human serum albumin (5.0 mg, 0.072 umol) in phosphate buffer (0.1M $PO_4^{=}$, pH 7.0, 500 uL) (Aldwin and Nitecki, 1987). The reaction was stirred for 15 min (Figure 17). An aliquot of the reaction mixture (10 uL) was added to phosphate buffer (1 ml). The absorption of this test solution was measured at 406 nm to assay the amount of liberated HNSA-ester (see section discussion for method). Spectrophotometry predicted that 34 residues on human serum albumin had reacted with the activated ester to form amide bonds (Table 3).

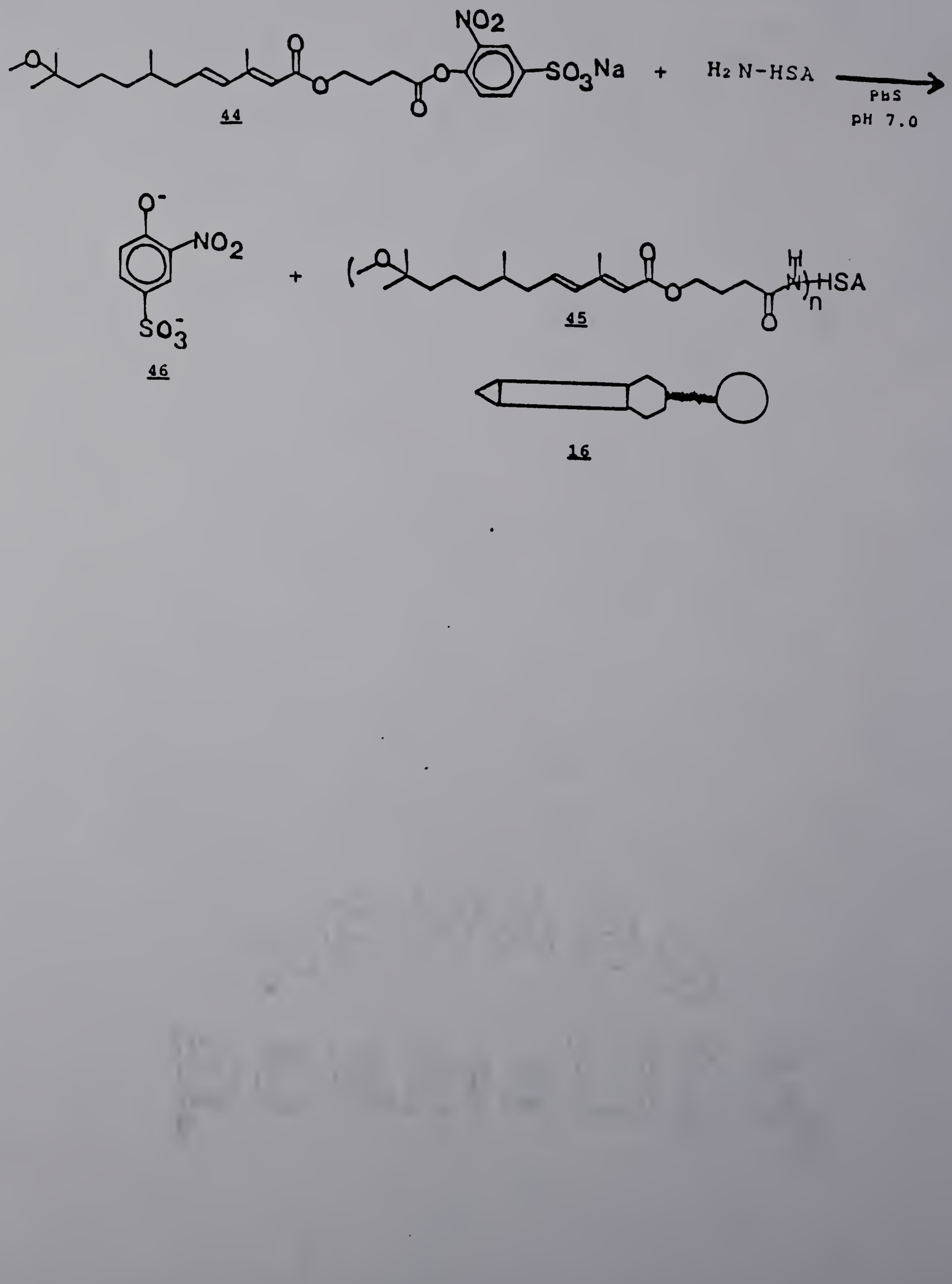
The reaction mixture was transferred to a 10.5 cm dialysis tube and was dialized against phosphate buffered saline (1.5 L) and water (1.5 L), until aliquots removed (1 ml) contained no radioactivity. The contents of the dialysis tube were lyophilized (24 h) to give a white fluffy solid (45) (4.43 mg). Based on its radioactivity,

Table 3. Comparison of the use of two activated esters for the protein coupling reactions used to form the methoprene immunogen, and the quantification of residues reacted.

Ester	Trial	Reaction Time	Residues Reacted ^{14}C[a]	A_{406}[b]
NHS	1	24 h	21	NA
	2	48 h	53	NA
	3	48 h	48	NA
HNSA	1	15 min	31	34
	2	30 min	28	32
	3	180 min	28	27

[a]Number of residues reacted was calculated using 5-^{14}C-labeled-methoprene.
[b]Number of residues reacted was calculated using the spectrophotometric method, where the absorbance of the free dianion was measured at A_{406}.
NHS: N-Hydroxysuccinimide.
HNSA: 1-Hydroxy-2-nitro-4-benzenesulfonate.

Figure 17. Formation of the methoprene immunogen (45) from the activated HNSA ester (44) and human serum albumin. The yellow dianion (46) is liberated by the reaction of free lysine groups on the protein with the activated ester.


NO2
SO3Na
H2N-HSA
PbS
pH 7.0
44
O-
NO2
SO3-
46
45
HSA
n
16

the protein was found to contain 31 molecules of methoprene>spacer per of molecule protein. This reaction was repeated two additional times, with reaction times of 30 min and 3 h. Based on their radioactivity, the protein products contained 28 and 27 molecules of methoprene>spacer per molecule of protein, respectively (Table 3).

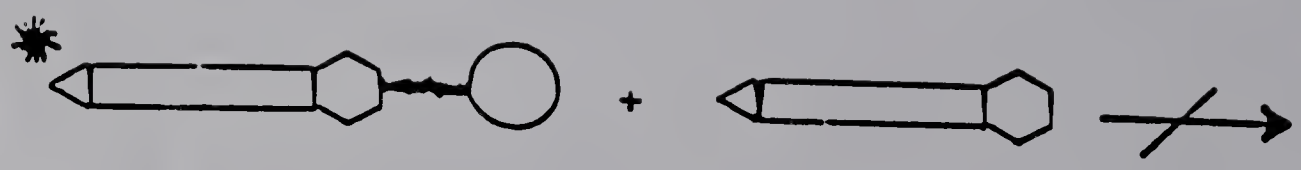
3.3.3. Competitive Binding Assay of the Methoprene Immunogen

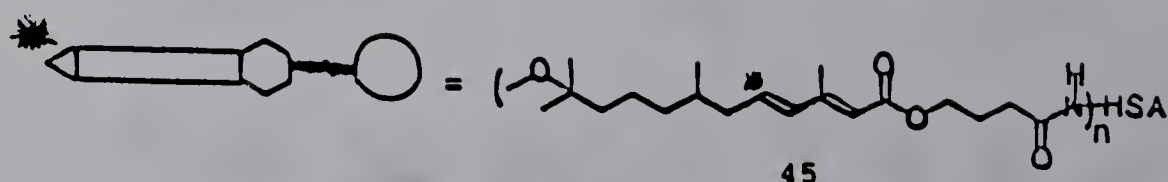
Methoprene>spacer acid (39) (185 ug, 5.2 umol) was added to a solution of the methoprene immunogen (45) (390 ug, 4.9 nmol, 1500 counts min^{-1}) in phosphate buffered saline (500 uL), and stirred for 24 h (Figure 18). The solution was transferred to a 10.5 cm dialysis tube and was dialyzed against phosphate buffered saline for 24 h. Aliquots (1 ml) of the dialysis washing were removed and counted. The contents of the dialysis tube were also counted. The radioactivity of the dialysis washing was not significantly above background. All radioactivity was recovered from the contents of the dialysis tube. When the same procedure was carried out in the absence of the competitor (39) all radioactivity was associated with the methoprene immunogen.

3.4. Antibody Production

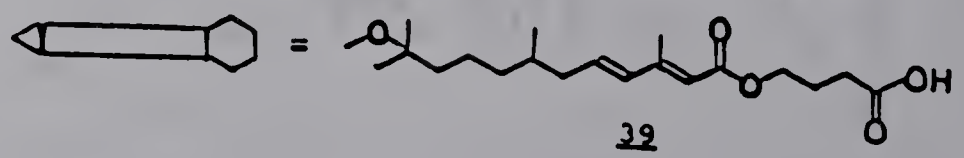
Eight 8-week-old female BALB/c mice were each injected interperitoneally with an emulsion of 25-30 ug

Figure 18. Competition study of the nature of the radiolabel on the methoprene immunogen. If the radiolabel was in the form of nonspecific binding to the protein, it would have been displaced by the excess competitor (39). This was not the case. All radiolabel was retained on the immunogen.





45



39

of the methoprene immunogen (45) (53 molecules methoprene>spacer per molecule protein) in 50 uL of sterilized phosphate buffered saline (0.05M $PO_4^=$, 0.05M NaCl, pH 7.4) and 50 uL of Freund's incomplete adjuvant. Booster shots of the immunogen (25-30 ug per mouse) in Freund's complete adjuvant (50 uL) and buffer (50 uL) were given one month after the initial injection, and again two weeks after the first booster shot.

Serum from each mouse was collected two weeks after the second booster shot. A small length of the mouse tail tip (1 mm) from each mouse was cut off and blood (30 uL) was collected by gentle massaging of the tail. The blood was allowed to stand at room temperature for 1 h, refrigerated (4° C) for 24 h, and centrifuged (10 min, 5000 rpm). The serum (supernatant, ca 25 uL) was diluted twenty times with antibody buffer (TBS-Tween 20: 20 mM Tris, 500 mM NaCl, 147 uM BSA, 0.1% thimerosal, 0.05% Tween 20, pH 7.5), and stored under refrigeration.

The presence of antibodies against the methoprene immunogen (45) was determined by an indirect enzyme linked immunosorbant assay (ELISA). Ten Immulon 1 PVC wells were set up. All wells but well 1 (no antigen control) were coated with 10 ug of the methoprene immunogen in carbonate buffer (100 uL, 0.05 M $CO_3^=$, 0.1% thimerosal, pH 9.6). The wells were incubated (27°C, 24 h), and washed (3 x, TBS-Tween 20). All wells were incubated (27°C, 24 h) with BSA blocker solution (200 uL,

1% BSA-TBS, without Tween 20), and washed (3 x, TBS-Tween 20). All wells but well 2 (no antibody control) were incubated (27°C, 24 h) with the mouse antisera (100 uL, diluted 1:20 with antibody buffer). Individual wells 3 through 10 corresponded to serum collected from individual mice numbered 1 through 8. The wells were washed (3 x, TBS, without Tween 20). Goat antimouse horseradish peroxidase conjugated antibody (100 uL, diluted 1:300 with antibody buffer) was added to all wells and incubated (27°C, 5h). The wells were washed (3x, TBS-Tween 20) and substrate solution (100 uL, 0.16 M sodium acetate, 0.05 M NaH_2PO_4, 0.12 M H_2O_2, 20 mM ABTS, 0.01% thimerosal) was added to each well. The wells were incubated (27°C, 15 min) and their absorbances (O.D.) were recorded at 410 nm.

Chapter 4.

Results

4.1. Synthesis of the Four Carbon Spacer Group

The synthesis of the protected four carbon spacer group (31) began with the reaction between benzyl alcohol (25) and sodium to form sodium benzyloxide (26). The addition of 4-butyrolactone (27), with heating, formed 4-benzyloxybutanoic acid (28) (Figure 9a) (Sudo et al., 1967). The benzylether of 28 served as a protecting group in the four-position. The overall yield of the reaction was poor (21%). No other byproducts from the reaction were isolated.

The next step required the protection of the carboxylic acid group of 28 (see discussion section 5.3.2., p. 114). This was accomplished by the reaction of 28 with 2-(trimethylsilyl)ethanol (29) using the coupling reagent, 1,3-dicyclohexylcarbodiimide (DCC), and the amine, 4-(N,N-dimethyl)amino pryidine (DMAP) (Sieber et al., 1977) (Figure 9b). The formation of the trimethylsilyl ester (30) resulted, along with the byproduct, 1,3-dicyclohexylurea. The urea precipitated from the reaction solution and the majority of it was removed by filtration. Traces of the urea, however, were always present in the product. The most efficient way of removing the urea was by flash column chromatography (Still et al., 1978).

The protected ester (30) underwent its first deprotection step by catalytic hydrogenation (Greene, 1981a). The benzylether (30) was cleaved exclusively between the benzyl group and the oxygen to form the alcohol 31 (Figure 9c). Unreduced, Aesar type II, palladium catalyst gave the best yield (98%) over the shortest period of time (1.5 h) with 2 atm H_2. 2-(Trimethylsilyl)ethyl-4-hydroxybutanoate (31) was then coupled to the carboxylic acids of two juvenile hormone analogs, farnesol (32) and methoprene (1), forming diesters.

4.2. Formation of Carboxylic Acids of Two Juvenile Hormone Analogs

The juvenile hormone analog, farnesol (32), had to be oxidized to its carboxylic acid in a two step procedure. The alcohol (32) was treated with pyridinium chlorochromate (Corey and Suggs, 1975) to form the aldehyde (33) (Figure 10a). This reagent has been used as a general oxidizer of primary and secondary alcohols to their respective aldehydes. Oxidation of 33 with silver nitrate and potassium hydroxide in aqueous ethanol (Callezi and Schinz, 1949) gave the carboxylic acid (34) (Figure 10b), along with metallic silver which was filtered from solution. Both the aldehyde (33) and the acid (34) were in the form of yellow oils which were purified by flash column chromatography.

Methoprene (1) was hydrolysed to its acid (35) by treatment with potassium hydroxide in ethanol (Henrick et al., 1973) (Figure 10c). It, too, was in the form of a yellow oil and was purified by column chromatography.

4.3. Coupling of the Four Carbon Spacer Group to Juvenile Hormone Analogs

2-(Trimethylsilyl)ethyl-4-hydroxy butanoate (31) was coupled to the carboxylic acids 34 and 35 to form the protected diesters 36 and 37, respectively (Figure 11). The coupling reagent used was DCC. Purification of these esters was by flash chromatography.

The protected trimethylsilyl esters 36 and 37 were deprotected using tetraethylammonium fluoride (Et_4F) (Sieber et al.,1977; Carpino et al., 1978) (Figure 12). This reagent selectively deprotected the esters to form the carboxylic acids 38 and 39, without attacking other functional groups.

4.4. Preparation of Activated Esters of Methoprene>Spacer (39)

The carboxylic acid function generated by the above deprotection step enabled the further reaction of the methoprene homolog (39, methoprene>spacer) with alcohols to form activated esters. It should be noted that 5-^{14}C-methoprene was added to unlabeled methoprene prior to its hydrolysis and subsequent derivatization to 39. The ^{14}C-

label acted as a tracer during the preparation of the methoprene immunogen (45). Two methods of preparing activated esters of methoprene>spacer (39) were employed (Figure 13).

The first method followed the procedures reported by Lauer et al. (1974a) and Baehr et al. (1976) for the preparation of an activated N-hydroxysuccinimide ester of juvenile hormone (19, Figure 7b). ^{14}C-5-Methoprene>spacer was coupled to N-hydroxysuccinimide with DCC Figure 13a). The resulting activated ester 41 was isolated in an impure form, with DCU as the contaminant (IR 3320, m, NH). The ester was used, without further purification, in protein coupling reactions.

A water soluble activated ester of 39 was also prepared by the method of Alwin and Nitecki (1987) (Figure 13b). The reagent, 1-hydroxy-2-nitro-4-benzenesulfonic acid (43) was formed by the method of King (1921). Sodium 4-hydroxybenzenesulfonate, dihydrate (42) was nitrated in water with an excess of nitric acid to form 43 (Figure 14). The elemental anaylsis reported by King for the trihydrate, $C_6H_4NNaO_6S \cdot 3H_2O$, found H_2O: 18.7, Na:9.55 per cent. When the reaction was repeated and the product was thoroughly dried, the analysis gave, for the unhydrated acid (43), $C_6H_4NNaO_6S$: required C: 29.88, H: 1.67, N: 5.81, Na: 9.53; found C: 29.71, H: 1.58, N: 5.70, Na:9.57. Spectral data (Appendix 1,

Figures A-23 and A-24) confirmed the structure of the material as unhydrated, 1-hydroxy-2-nitro-4-benzenesulfonic acid. It is unclear as to why there is a discrepancy between the literature analysis and the analysis reported here. It is clear, however, that the theoretical content of sodium for the trihydrate should be 7.79 per cent, not 9.55 per cent, which corresponds to the sodium content of unhydrated material (43).

Once the structure of 43 was confirmed, it was reacted with ^{14}C-5-methoprene>spacer (39) using DCC in DMF at room temperature (Figure 13b) (previous DCC couplings were carried out at 0^{o}C). The activated ester (44) was also isolated in an impure form; however, it was very water soluble. This is the first report of a water soluble derivative of methoprene (1).

4.5. Immunogen Preparation

As previously stated, methoprene must be conjugated to a large molecule to elicit an immune response in mammals. The formation of two activated esters of methoprene enabled the comparison of two protein conjugation reactions.

4.5.1. The N-Hydroxysuccinimide Ester Method

The formation of an immunogen for juvenile hormone using the activated N-hydroxysuccinimide ester of the hormone (Lauer et al., 1974a; Baehr et al., 1976) (Figure 7) served as a model for the coupling of methoprene>spacer-N-hydroxysuccinimide ester (41) to the protein, human serum albumin. The protein was first dissolved in a THF/H_2O solution (50:50, pH 8.5). Methoprene>spacer-N-hydroxysuccinimide ester (41) was added and the final THF/H_2O concentration was adjusted by the addition of small amounts of THF (Figure 15). This is in contrast to previously reported juvenile hormone protein coupling reactions, where the pH was 9.5 and the solution favors the solubility of the protein (Baehr et al., 1976). Hydrolysis of activated N-hydroxysuccinimide esters is encouraged at higher pH's (unreported results). To reduce hydrolysis of the methoprene>spacer-N-hydroxysuccinimide ester, the pH of the reaction was lowered.

A reaction time of 48 hours seemed to allow for the reaction of many of the available amine residues on the protein (Table 3). Human serum albumin has 99 basic amino acids, 59 of those are lysine; 24 are arginine; and 16 are histidine (Meloun et al., 1975; Walker, 1976; Dayhoff, 1978). Of the uncharged and nonpolar amino acids on human serum albumin, 18 are tyrosine, and one is tryptophan. All of these amino acids may contribute to the conjugation of methoprene>spacer to the protein,

however, it has been shown that lysine is involved in conjugation reactions more often than the other amino acids (Jacobsen et al., 1972). Because no attempt was made to determine which amine residues reacted with the activated esters, the number of potential residues which could react was assumed to be 99.

The use of ^{14}C-5-methoprene enabled the quantitative measurement of the number of amine residues reacted by direct liquid scintillation counting of the protein product. A maximum of 53 amine residues on one molecule of human serum albumin reacted with 41 to form the methoprene immunogen (45). The number of residues taken up in this reaction is greater than for previous reports for juvenile hormone immunogen preparation (Table 4). The radiolabeled methoprene>spacer hapten (39) was not competitively removed from the immunogen (45) by an excess of unlabeled methoprene>spacer (39), signifying the hapten was covalently bound to the protein (Figure 18).

4.5.2. The 1-Hydroxy-2-Nitro-4-Benzenesulfonate Ester Method

The second method of conjugating methoprene>spacer (39) to a protein used the water soluble activated HNSA-

Table 4. Comparison of the number of haptens per molecule of protein for juvenile hormone and methoprene immunogen preparations.

Hapten	#Haptens/Protein	Method of Immunogen Preparation
JH I	26[a]	NHS Activated Ester
JH I Diol	17[b]	Carbodiimide Coupling
JH III	20[c]	NHS Activated Ester
JH III Diol	20[d]	NHS Activated Ester
Methoprene	53[e]	NHS Activated Ester
Methoprene	31[e]	HNSA Activated Ester

[a]Baehr et al., 1976.
[b]Strambi et al., 1981.
[c]Lauer et al., 1974a.
[d]Baehr et al., 1987.
[e]Mei, 1988, this report.
NHS: N-hydroxysuccinimide.
HNSA: 1-Hydroxy-2-nitro-4-benzenesufonate.

ester (44). This ester was extremely water soluble despite the hydrophobic nature of methoprene. This was advantageous since the concern over solubilizing the reactants was eliminated (Aldwin and Nitecki, 1987).

Both the activated ester (44) and human serum albumin were soluble in phosphate buffer at pH 7.0 (Figure 17). The reaction time for this method was much faster than that of the N-hydroxysuccinimide ester method (30 min vs. 48 h). Again, the use of ^{14}C-methoprene allowed for direct quantification of the number of amine residues on the protein which had reacted with 44. In this case, 28 residues on human serum albumin had reacted with the activated ester after 30 min (Tables 3 and 5).

4.5.3. Assay of Methoprene>Spacer-HNSA Ester Protein Coupling Reaction

The 1-hydroxy-2-nitro-4-benzenesulfonate ester method also has the advantage of being monitorable by spectrophotometry. A yellow dianion (46) is formed when the activated ester 44 reacts with nucleophiles (base or free amine groups on a protein) (Figures 15 and 17). When the methoprene>spacer-HNSA ester (44) and human serum albumin were allowed to react, the free HNSA dianion (46) was liberated. The dianion absorbs light in the visible region at 406 nm, and was detected by spectrophotometry. As the reaction proceeded, the absorption of the dianion increased. When the spectrophotometric readings at A_{406} were corrected for

the free dianion, 1-hydroxy-2-nitro-4-benzenesulfonate, the number of amine residues reacted were directly related to the amount of liberated dianion (Table 5).

When values obtained from the spectrophotometric method were compared with those obtained from the covalently bound radiolabeled methoprene>spacer (39), the two methods agreed with the number of methoprene molecules bound per molecule of protein (Tables 3 and 5). Aldwin and Nitecki (1987) monitored the reaction of their HNSA ester with a protein by spectrophotometry alone, and did not offer a comparison with a radiolabeled tracer.

4.6. Preliminary Production of Antibodies Against the Methoprene Immunogen

The immunization schedule employed showed the presence of antibodies in mice eight weeks after the first injection of the methoprene immunogen (45). The introduction of the immunogen into the mice caused each mouse to mount an immune response. Antibodies against the immunogen were secreted into the blood stream. Serum, rich in antibodies, was collected from each mouse by removal of the blood cells by centrifugation (Figure 19).

Using the indirect ELISA method for antibody detection (Figure 20), antibodies against the methoprene immunogen were seen in mouse sera which had been diluted

Table 5. The spectrophotometric method of calculating the number of methoprene molecules conjugated per molecule of protein. A comparison of the two methods shows that they are in agreement.

Reaction Time (min)	Crude Ester (mg)	Dianion Start (umol)	A_0	A_t	Residues Reacted (Method) A_{406}[a]	^{14}C[b]
15	10.52	21.86	0.750	0.837	34	31
30	8.11	16.86	0.579	0.640	32	28
180	8.50	17.66	0.607	0.656	27	28

[a] The spectrophotometric method required two readings at 406 nm; A_0 determined the amount of free dianion in the sample at time = 0 min; A_t determined the amount of dianion liberated by the reaction of the HNSA-ester with protein at the end of a reaction time.
[b] ^{14}C-methoprene was used to determine the number of methoprene molecules conjugated per molecule protein.

Figure 19. Injection of the methoprene immunogen into mice caused an immune response to be mounted. Antibodies specific to the immunogen were collected in the serum portion of the blood.

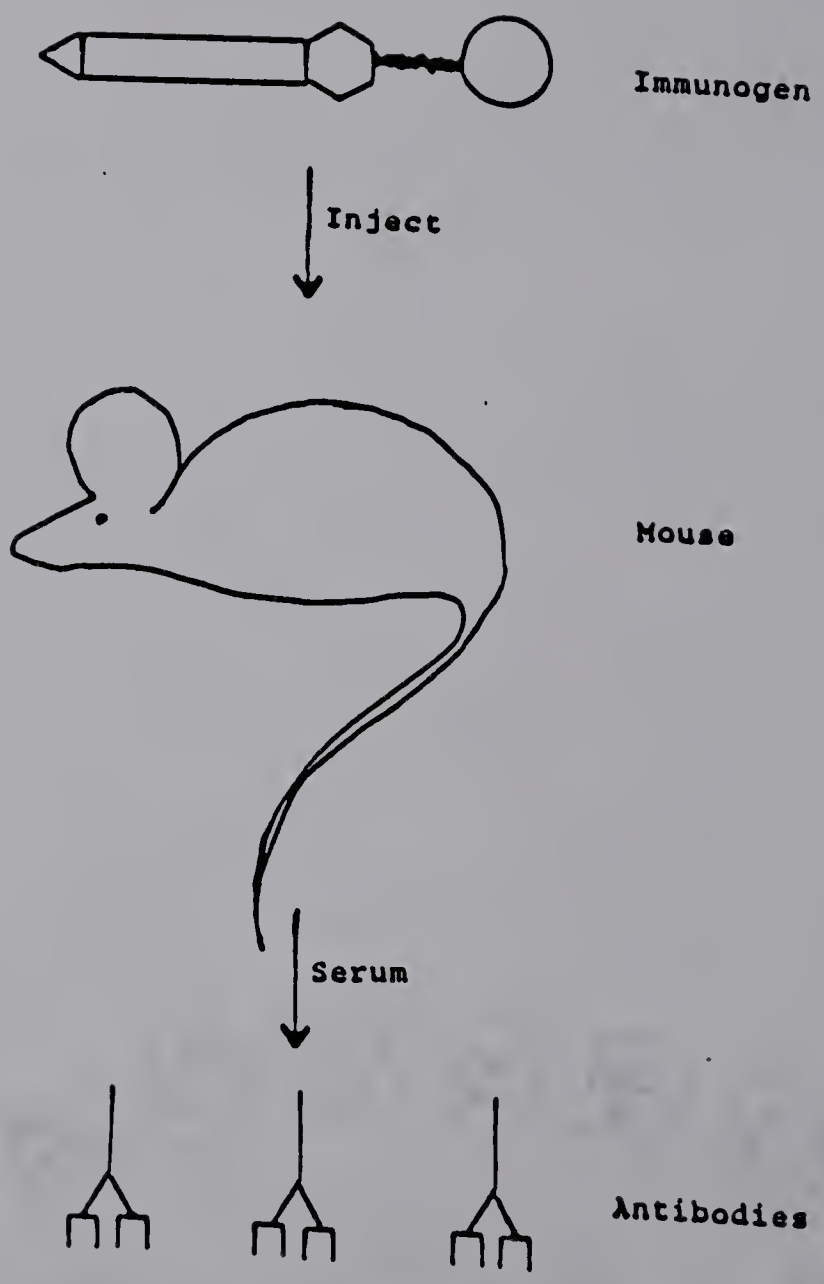
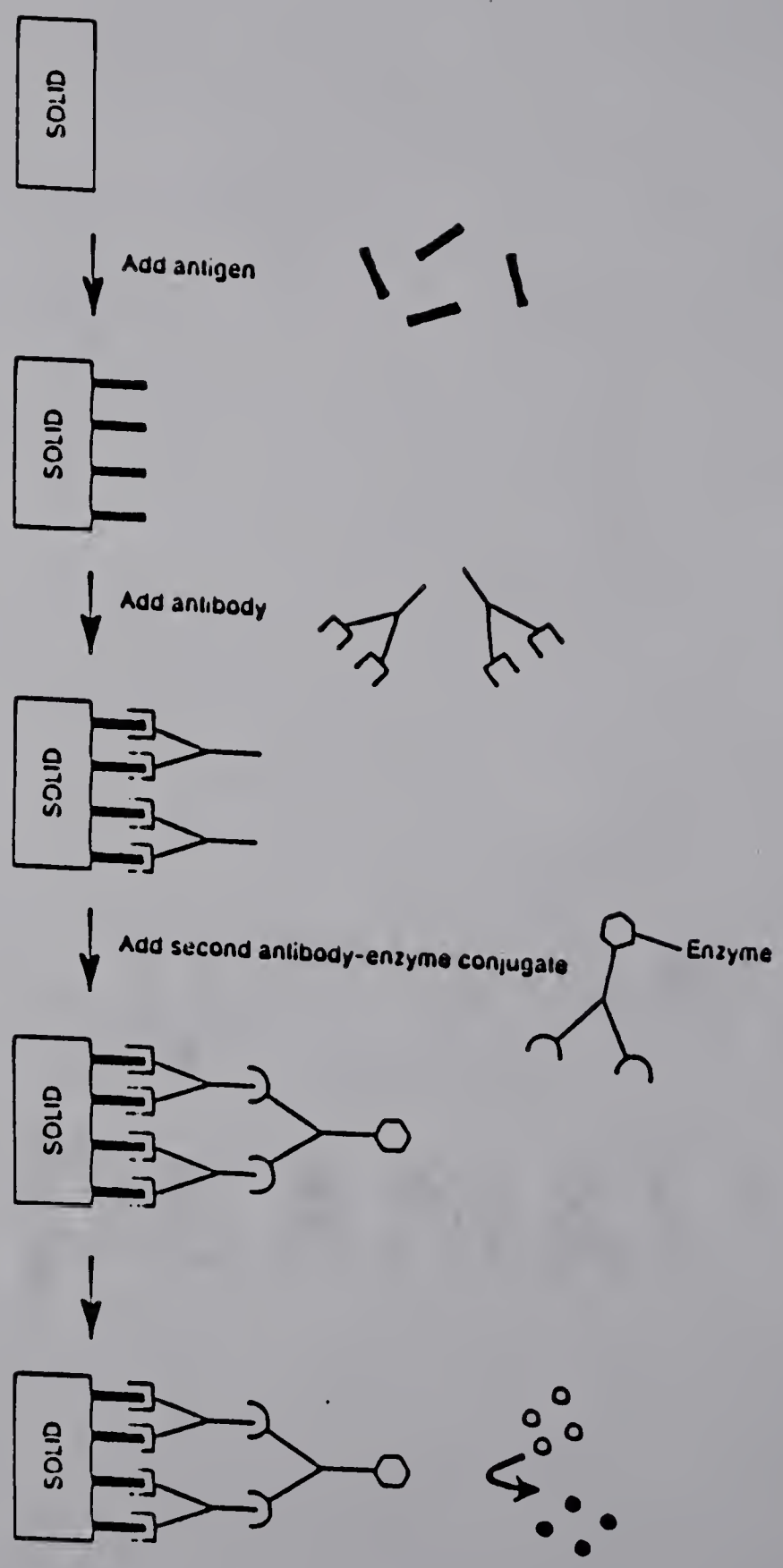

Immunogen
Inject
Mouse
Serum
Antibodies

Figure 20. The indirect enzyme linked immunosorbent assay (ELISA). (From Detoma and MacDonald, 1987).


SOLID
Add antigen
SOLID
Add antibody
SOLID
Add second antibody-enzyme conjugate
Enzyme
SOLID
SOLID

1:20. Based on the optical density readings after only 15 min of incubation of the goat antimouse horseradish peroxidase conjugated antibody with substrate, it seemed that the titer and specificity of the mouse antibodies raised against the methoprene immunogen was high. All eight mice showed the presence of antibodies, with three mice giving a very positive response (Table 6, # 6, 7, 8).

Table 6. Optical Density ($O.D._{410}$) of mouse serum produced against the methoprene immunogen after 15 min incubation using the ELISA method of analysis.

Mouse serum #	Recording 1	Recording 2
Antigen control	Blank	Blank
Antibody control	0.002	0.006
1	0.448	0.477
2	0.459	0.559
3	0.392	0.421
4	0.549	0.694
5	0.519	0.656
6	0.921	1.134
7	1.242	1.189
8	0.921	0.935

Chapter 5.

Discussion

5.1. Choice of a Carrier Molecule for Methoprene

The structure of the methoprene immunogen (45) was designed to produce antibodies specific to the methoprene molecule. The production of specific antibodies against methoprene is dependent on an animal system for which there is only limited control. The immunogen structure, therefore, had to meet a number of requirements to give it the greatest chance of producing antibodies in mice. These requirements were: a) that the methoprene molecule be presented to the animal in its native form; b) that the carrier molecule for methoprene be large enough to be seen as a foreign entity; c) that the carrier molecule have many sites for covalent attachment of methoprene; and d) that the concentration of methoprene on the carrier molecule be high enough to present many molecules of methoprene to the animal's immune system.

Due to its small size, methoprene (molecular weight 310 g/mol) does not cause an immnune response. Generally, molecules of greater than molecular weight 10,000 are used as antigens. Methoprene, therefore, had to be covalently attached to a large carrier molecule in order to meet the above requirements. The carrier molecule used was the protein human serum albumin (molecular weight 69,000, Merck Index, 1976).

Human serum albumin was chosen for a number of reasons. It was used successfully as a carrier molecule for juvenile hormone into rabbits (Lauer et al., 1974a; Baehr et al., 1976; Strambi et al., 1981). It has a maximum of 99 free amine residues available for the covalent attachment of methoprene (Jacobsen et al., 1972; Meloun et al., 1975; Walker, 1976; Dayhoff, 1978). Lysine has been found to be of major importance to the immunoreactivity of human serum albumin (Jacobsen et al., 1972). There is still no consensus on the optimum number of haptens needed to obtain high antibody titer and specificity. Therefore, no effort was made to limit the number of methoprene molecules covalently bound to the protein. Human serum albumin has also been known to remain very soluble in aqeous solution when heavily loaded with haptens.

5.2. The Derivatization of Methoprene

The conjugation of methoprene to human serum albumin required that a reactive functionality on methoprene be formed prior to its reaction with free amine groups on the protein. Methoprene (1) has two places where reactive functionalities may be formed, at either the methoxy end or at the carboxyl end. The place of conjugation to the protein would greatly affect the nature of the antibodies raised against the resulting immunogen.

The methoxy moiety at carbon 11 could have been replaced with a hydroxyl function, followed by further

reaction with, perhaps succinic anhydride, to form a carboxylic acid which could then be coupled to the protein with a coupling reagent. This approach, however, would change the configuration around carbon 11 such that antibodies against the immunogen would no longer recognize 11-methoxy-methoprene, but would recognize 11-hydroxy-methoprene. Such was the case with the juvenile hormone radioimmunoassay for juvenile hormone diol (7) (Strambi et al., 1981). The juvenile hormone epoxide function between carbons 10 and 11 was hydrolyzed to form the diol (7), followed by coupling to the protein via a hemisuccinate derivative (22) (Figure 8). The resulting antibodies only recognized juvenile hormone diols, without distinguishing between the diol homologs (Table 1). This approach was not chosen for methoprene since antibodies against such an immunogen would most likely not recognize methoprene in its native form.

The alternative, then, relied on the derivatization of methoprene at its carboxyl end. The simplest way to form a reactive moiety on methoprene used base hydrolysis of the isopropyl ester at carbon 1 to form the carboxylic acid (35). This acid could have been conjugated to human serum albumin directly by a coupling reagent, or an activated ester of the acid could have been formed with N-hydroxysuccinimide. This approach was taken for juvenile hormone by both Lauer et al. (1974a) and Baehr et al. (1976, 1981). The resulting antibodies recognized juvenile hormone

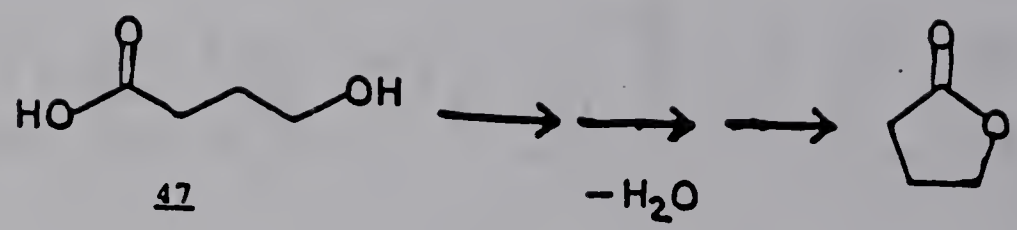
acids and cross reacted significantly with other juvenile hormone homologs (Table 1).

This route was not chosen for methoprene, primarily because it was not desired to have antibodies recognizing methoprene acid (35). The form of methoprene which results in the greatest disruption of the life cycles of insects is the isopropyl ester (1). An immunoassay for methoprene should detect it in its most active form, the ester, to give field applicators accurate information on its presence and ability to continue to control insects in the environment. It was therefore decided that the best way to obtain antibodies against methoprene for subsequent immunoassays was to raise antibodies which would recognize the ester (1). This was accomplished by forming, from methoprene acid (35), a derivative which contained an ester function to mimic native methoprene, and a carboxylic acid function for coupling to the protein carrier. The resulting material, methoprene>spacer (39) was then coupled to human serum albumin.

5.3. The Four Carbon Spacer Group

The synthesis of methoprene>spacer (39) required special consideration. For both an ester function and a terminal carboxylic acid function to be incorporated into 39, the addition of a hydroxy-carboxylic acid was necessary. The material chosen for this addition was 4-hydroxybutanoic acid (47) (Figure 21).

Figure 21. If left unprotected, 4-hydroxybutanoic acid (47) can cyclize with itself to form the 4-butyrolactone (27).


HO
OH
47
$-H_2O$
acid catalyzed
27

The decision to use 4-hydroxybutanoic acid was based on a number of factors. The antibodies raised against an immunogen prepared from juvenile hormone acid had greater affinities for the acid over the native hormone (Lauer et al., 1974a; Baehr et al., 1976). Based on these results, it was hoped that the addition of an ester function to methoprene acid (35), via a spacer group, would enhance the chance of the subsequent immunogen to raise antibodies against native methoprene (1).

5.3.1. Effect of Spacer length

The importance of placing a spacer group between a hapten and its carrier has been shown in studies of immunogenicity of haptens bound to liposomes. Liposomal immunogenicity was dependent on an optimal distance separating the hapten from its hydrophobic anchor within the liposome bilayer (Kinsky et al., 1984). Liposomes which contained no spacer or contained a twelve carbon spacer were non-immunogenic, in contrast to the maximum immune response induced by liposomes containing a six carbon spacer. A similar result was found for hapten bound to avidin, a basic glycoprotein of moledular weight 16,000 (Scott et al., 1984). Hapten-avidin complexes lacking a six carbon spacer group failed to induce an antibody response. Spacer length has also been shown to affect the ability of IgG antibodies to bind to liposome surfaces containing aminoethylthioacetyl (AETA) spacers (spacer length of four atoms) (Hashimoto et

al., 1986). Liposomes containing no AETA spacer did not bind IgG antibodies, in contrast to the significantly higher IgG attachment by liposomes containing one AETA spacer (four atoms) or two AETA spacers (eight atoms). Additionally, these four to eight atom spacer groups, which may contain carbon, nitrogen, or sulfur atoms, do not induce an immune response (Aldwin and Nitecki, 1987; Hashimoto et al., 1986). Such "featureless" structures confer length and flexibility to immunogen structure.

A four carbon spacer group was placed between methoprene and its carrier. It is conclevable that this provided the necessary length to place methoprene above the surface of the protein. It is also concelvable that a spacer length of six or eight carbons may further enhance the immune response against the methoprene immunogen. A study of the effects of spacer length is, however, beyond the scope of this thesis.

5.3.2. Protection of the Four Carbon Spacer Group

The spacer group, 4-hydroxybutanoic acid (47), is a bifunctional material which has a tendency to lose a mole of water under acidic conditions, resulting in formation of the cyclic product, 4-butryolactone (27) (Figure 21). The reaction required to couple 4-hydroxybutanoic acid (47) to methoprene acid (35) used the reagent 1,3-dicyclohexylcarbodiimide (DCC). This reagent acts, in both the coupling reaction and the cyclization process, as a

dehydrating agent and since a 5-membered ring is formed in the latter case, ring formation is likely to be predominant. In fact, preliminary experiments which attempted to couple farnesoic acid (34) with 4-hydroxybutanoic acid, using DCC, yielded the lactone.

The necessity of protecting 4-hydroxybutanoic acid became apparent very quickly. A sequence of protection-deprotection reactions were carried out to prevent the formation of the lactone (27). The preparation of 4-benzyloxybutanoic acid (28) protected the hydroxyl function at 4-C of 4-hydroxybutanoic acid (47) with a benzylether. This allowed for the independent protection of the carboxylic acid function at 1-C.

The choice of a protecting group for the carboxylic acid was of some concern, primarily because the protecting group would have to be removed as the last step to generate methoprene>spacer (39). The reagent used to remove the protecting group would have to be extremely selective in that it could not attack any of the functionalities in methoprene (1), namely the methoxy function at 11-C, the conjugated double bonds between 2-C and 4-C, and the ester function at 1-C.

Because of these restrictions, the protecting group chosen was the (2-trimethylsilyl)ethyl residue. This residue has been used as an N-protecting group which was found to be selectively cleavable with fluoride ion under mildly basic conditions (Carpino and Tsao, 1978). This

residue was also applied as a carboxylic acid protecting group with cleavage under the same mild conditions. 2-(Trimethylsilyl)ethyl esters prepared from 2-(trimethylsilyl)ethanol (29) and the carboxylic acid residues of peptides were stable under workup conditions and during hydrogenation (Sieber et al., 1977). The mild cleavage conditions and the stability of these esters made this protecting group the most attractive. There are many protecting groups available for carboxylic acid protection (Greene, 1981b), but their removal requires hydrogenation, or more acidic or alkaline conditions which may attack the methoprene molecule.

The preparation of the trimethylsilylethyl ester (30) from 4-benzyloxybutanoic acid (28) and 2-(trimethylsilyl)ethanol (29), gave a protected four carbon spacer group which could not yield the lactone (Figure 9b). The next step required the removal of the benzylether protecting the hydroxyl group of 2-(trimethylsilyl)ethyl-4-benzyloxybutanoate (30). This was done with catalytic hydrogenation to give the protected derivative, 2-(trimethylsilyl)ethyl-4-hydroxybutanoate (31) (Figure 9c). This deprotection step generated the free hydroxyl group needed to continue the synthesis of methoprene>spacer (39).

A general carbodiimide coupling reaction was used to prepare protected (trimethylsilyl)ethyl esters of the carboxylic acids of both farnesol (34) and methoprene (35) (Figure 11). Products of this reaction (36 and 37)

contained an ester function on either end of the parent compound, 4-hydroxybutanoic acid (47). The ester function at 1-C was part of the 2-(trimethylsilyl)ethyl carboxylic acid protecting residue. As stated above, this residue is sensitive to attack with fluoride ion. The deprotection reaction to remove the residue was carried out in the presence of tetraethylammonium fluoride as the source of fluoride ion (Figure 12). The carboxylic acids generated contained the parent molecule, 4-hydroxybutanoic acid (47), and a juvnelle hormone analog, either farnesol or methoprene, covalently bound by an ester function.

5.4. The Use of Activated Esters in the Preparation of the Methoprene Immunogen

Two of the most commonly used methods of coupling haptens to proteins are the use of activated ester derivatives of the haptens, and the use of coupling reagents such as carbodiimides. Water soluble carbodiimides such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide methyl p-toluene sulfonate (CMC) or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) have been used to directly couple water soluble carboxylic acids with proteins (Hammock and Mumma, 1980; Strambi et al., 1981). These cabodiimides, however, have the tendancy to cross link proteins, greatly reducing the solubility of the immunogen.

The first attempts at conjugating methoprene>spacer (39) to human serum albumin used EDC as the coupling

reagent. The resulting protein product contained only a trace amount of ^{14}C-labeled methoprene, and was highly insoluble in neutral buffer solutions (unreported results). It was concluded that the lack of solubility of the methoprene derivative (39) under the aqueous, acidic conditions necessary for an EDC coupling resulted in the cross linking of the protein. This method has been used to conjugate a water soluble juvenile hormone diol derivative to human serum albumin (Strambi et al., 1981); however, EDC can not be used when the reactants are not soluble under the appropriate reaction conditions.

Alternatively, activated ester derivatives provide good leaving groups in the presence of nucleophiles. Immunogen prepartion techniques have frequently used activated ester derivatives to couple haptens to nucleophilic determinants on proteins. Carboxylic acid derivatives of N-hydroxysuccinimide have been frequently used as activated esters for protein conjugation. This method of conjugation to proteins has been used for derivatives of pesticides such as allethrin and diflubenzuron (Hammock and Mumma, 1980), and insect hormones such as juvenile hormone (Lauer et al., 1974a; Baehr et al., 1976) and ecdysone (Lauer et al., 1974b).

5.4.1. The N-Hydroxysuccinimide Ester Method

The failure of the direct carbodiimide coupling of methoprene>spacer to human serum albumin prompted the

preparation of the N-hydroxysuccinimide derivative of methoprene>spacer (41) (Figure 13a). The successful use of such derivatives had already been demonstrated for juvenile hormone immunogen preparations (Lauer et al., 1974a; Baehr et al., 1976). However, the pH used for these reactions was very alkaline (pH 9.5). N-hydroxysuccinimide esters have been shown to be stable under acidic conditions, but are sensitive to base hydrolysis; they are also fairly insoluble in aqueous solutions. To be able to solubilize both the ester and the protein, it was necessary to use a mixture of THF:H_2O (1:1) and high pH (9.5). Protein solubility was greatly increased at this alkaline pH in the presence of an organic solvent. These conditions, however, would have caused the hydrolysis of the ester before it could react with amines on the protein, resulting in juvenile hormone immunogens with between 17 and 26 of the available amines reacted (Table 4), as reported by the above mentioned authors.

Prior to repeating this procedure for the preparation of the methoprene immunogen (45), an N-hydroxysuccinimide ester of a related compound, linoleic acid, was prepared. This ester was placed in a number of solutions of THF:H_2O (1:1) with varying pH, as a means of testing the effect of pH on N-hydroxysuccinimide esters. The ester was quickly hydrolysed in pH 9 and above, but that the rate of hydrolysis was far slower if the pH was adjusted to below 9 (personal observations).

To obtain the greatest reaction between the N-hydroxysuccinimide ester of methoprene>spacer (41) and human serum albumin, the reaction conditions were modified so that the pH was lowered to pH 8-8.5, and the concentration of THF:H_2O was slightly greater for THF (1.3:1, v/v) (Figure 16). Over a reaction time of 48 h, a protein conjugate was formed with 53 molecules of hapten per molecule of protein. This immunogen contained more haptens than any previously reported immunogens for either the activated ester methods or the carbodiimide coupling raction (Table 4).

5.4.2. The 1-Hydroxy-2-Nitro-4-Benzenesulfonate Ester Method

The N-hydroxysuccinimide ester coupling method worked quite well for preparation of the methoprene immunogen (45). When it was found, however, that a water soluble activated ester of methoprene could be prepared (Aldwin and Nitecki, 1987), this route was also pursued. The idea of a water soluble activated ester was very appealing since it would allow the reaction between the ester and the protein to occur in an aqueous medium.

The preparation of a water soluble active ester of methoprene first required the synthesis of sodium 1-hydroxy-2-nitro-4-benzenesulfonic acid (43) (HNSA). This was accomplished easily by the nitration of sodium 4-hydroxybenzenesulfonate dihydrate (42) (King, 1921) (Figure 14). The resulting phenol (43) was water soluble. The activated ester of methoprene>spacer (39) was prepared using

a DCC coupling reaction (Figure 13b). This derivative of methoprene (**44**) readily dissolved into aqueous solutions.

Esters prepared from 1-hydroxy-2-nitro-4-benzesulfonate absorb below 300 nm (Aldwin and Nitecki, 1987). Hydrolysis of the activated ester (**44**) by base resulted in the formation of the parent acid, methoprene>spacer (**39**), and the dianion (**46**) (Figures 15). The ability to detect the free yellow dianion (**46**) with spectrophotometry (406 nm) provided a means to calculate the amount of ester formed from the DCC coupling reaction, without further purification of the product. The relation expressed in equation 1 (page 74) was used to calculate the total amount of material present as the ester **44**.

The formation of the methoprene immunogen (**45**) was the result of the reaction between the 1-hydroxy-2-nitro-4-benzenesulfonate ester of methoprene (**44**) and human serum albumin. The generation of the yellow dianion in the presence of nucleophiles (free amine groups on human serum albumin) also allowed for its measurement (Figure 17). The number of amine residues on human serum albumin which had reacted with the activated ester (**44**) was calculated based on the relationship between the amount of free dianion present at the start of the reaction and the amount of free dianion that was liberated at the end of the reaction (Appendix C, page 190). Both methods, direct scintillation counting of the radiolabeled immunogen (**45**) and spectrophotometric measurement of the liberated dianion

(46), compared well with each other (Tables 3 and 5). The rapid progress of the HNSA reaction seemed to allow for 27-34 residues on one molecule of protein to react with the activated ester (Table 5).

The correlation between the two methods for determining the number of haptens covalently bound to the protein provides an alternative to the use of a radiolabled derivative. The spectrophotometric method was useful in that it provided an accurate view of the rate of the reaction over a short period of time. Scintillation counting of the labeled protein product, however, provided a direct means of obtaining the ultimate result.

The ability to synthesize water soluble activated esters of juvenile hormone analogs also has many advantages. For many years research has been hampered by the hydrophobicity of juvenile hormone and many of its analogs, methoprene included (Giese, 1977; Granger, 1982). Now, for the first time, juvenile hormone analog derivatives may be completely solubilized in aqueous solutions. Conjugation of such derivatives to hydrophilic materials such as proteins or enzymes, is greatly facilitated, as demonstrated above. Use of spectrophotometry to monitor reaction rate eliminates the need for labeled tracers. Additionally, any hydrophobic material which can be derivatized to a carboxylic acid could be made water soluble by the formation of the activated esters of 1-hydroxy-2-nitro-4-benzenesulfonate.

5.5. Immunogen Purification and Characterization

Each step in the derivatization process of methoprene was carefully monitored. All intermediates including a number of new compounds were synthesized and were characterized using thin layer chromatography, infrared spectroscopy, nuclear magnetic resonance, and elemental analysis. Appendix 1 contains all spectral data for the intermediates leading to methoprene>spacer (39).

Initially, the juvenile hormone analog, farnesol (31), was also derivatized to form farnesol>spacer (38). Although an immunogen was not prepared from farnesol, reaction conditions leading to farnesol>spacer (38) were successfully applied to the derivatization of methoprene with similar good results. Since the specific reactions employed produced the desired products for two juvenile hormone analogs, it is likely that these same reactions can be applied to other juvenile hormone homologs or analogs if these materials can be derivatized to their carboxylic acid form.

The methoprene immunogen (hapten-carrier protein conjugate) was purified from low molecular weight by-products of the protein coupling reactions with exhaustive dialysis. Dialyzing the protein products against phosphate buffered saline (pH 7.4), followed by dialysis with distilled water, removed all unreacted starting materials and by-products very efficiently. Dialysis with distilled water was continued until no radioactivity was detected in

the dialysis wash. Sephadex (G25-40) column chromatography was also used to purify the protein products from the reaction with the 1-hydroxy-2-nitro-4-benzenesulfonate activated ester (44). The protein product, however,still required dialysis to remove trace amounts of unbound radiolabeled starting material and the yellow dianion by-product.

Characterization of the immunogen after dialysis and lyophilization was done by direct liquid scintillation counting. This quantitative method of measuring the number of moles of methoprene bound per mole of protein carrier provided the most efficient and accurate determination. Other quantitative methods include the monitoring of a change in absorbance of the hapten-protein carrier in a spectral region where the protein itself does not absorb (Hammock and Mumma, 1980), or monitoring of the proteins' reactive groups, such as free amine groups, before and after conjugation (Habeeb, 1966). Neither of these methods was employed due to the possibility that denaturation of the protein may affect the functionalities available for binding. These methods are also less direct, and perhaps less accurate than the use of a radiolabeled tracer.

The methoprene immunogen was subjected to a competitive binding assay to determine if the radiolabeled haptens were covalently or noncovalently bound to the protein carrier (Figure 18). If the label was in the form of noncovalently bound methoprene>spacer, it would have been displaced by

unlabeled methoprene>spacer which was present in an excess of one thousand times. The recovered immunogen would not retain any of its radioactivity after dialysis. This was not the case. The methoprene immunogen lost none of its radioactivity after competition with unlabeled methoprene>spacer, indicating the hapten was covalently bound to the protein carrier, and the protein coupling reactions used to prepare the methoprene immunogen were a success.

5.6. The Choice of Isotope for the Methoprene Immunogen Tracer

Prior to receiving a gift of 5-^{14}C-methoprene, the use of tritium was investigated as the isotope for the methoprene immunogen tracer. Lithium diisopropylamide was used to remove a proton in the `-position (2-C) of the carboxylic acid, methoprene>spacer (39), forming the lithium salt (Lochmann and Lim, 1973). Tritium, in the form of tritiated water, was added to a solution of the lithium salt, thus allowing for the exchange of lithium with tritium in the 2-C position. The tritiated product was stable under workup conditions and column chromatography, and contained 1.3×10^6 counts min^{-1} mg^{-1}. When ^{3}H-methoprene>spacer was used to prepare the methoprene immunogen via a water soluble carbodiimide coupling, the tritium label was lost under the acidic conditions necessary for the reaction. The resulting protein product contained a trace amount of radioactivity,

and at the time, it was unclear as to the cause of the low incorporation of the label. Two explanations were presented: a) the carbodiimide coupling method failed due to a lack of solubility of the reactants; and b) the tritium label exchanged freely with protons, and was removed from the protein product by dialysis. Both explanations were shown to be correct.

The use of ^{14}C-methoprene in the synthesis of methoprene>spacer showed that the carbodiimide coupling reaction was not successful if the reactants were not soluble. Subjecting ^{3}H-methoprene>spacer to both acidic and basic conditions showed that the tritium label readily exchanged with protons under such conditions. This method of labeling a carboxylic acid with tritium did not incorporate the label to prevent it from being easily removed. The most stable form of a radiolabeled material is one that has the isotope directly incorporated into the structure of the compound. Thus, when 5-^{14}C-methoprene became available, it was used in all subsequent methoprene derivatization or protein conjugation reactions.

5.7. Is the Methoprene Immunogen Immunogenic?

The immunization schedule for BALB/c mice injected a minimal amount of immunogen per booster shot per mouse (Figure 19). The production of antibodies in BALB/c mice against the methoprene immunogen was evident by the use of the indirct ELISA method (Figure 20). This preliminary

screening of the serum obtained from tail bleedings of the eight immunized mice showed that the mice had, indeed mounted an immune response against the methoprene immunogen (Table 5). This information was necessary to determine if the mice needed more booster shots of the immunogen, or if they were ready to receive sarcoma cells for the production of ascities fluid. Since the assay showed a strong response to the immunogen from all eight mice (Table 5), it was decided that the mice were ready to receive the cancer cells.

The information provided from this screening showed that the methoprene immunogen could elicit an immune response in mice. The degree of the response is beyond the scope of this study, but will be investigated in the future.

5.8. Immunoassay Development

Once antibodies have been raised in quantity and their specificity for methoprene has been established, a number of immunoassays may be developed. The most widely used immunoassays include radioimmunoassay (RIA), ELISA, or enzyme immunoassay (EIA). The form that the immunoassay for methoprene will take is dependent on a number of factors.

5.8.1. Radioimmunoassay for Methoprene

The radioimmunoassay is one of the most commonly used immunological assays. A radioimmunoassay for methoprene could be developed with a specific antibody against

methoprene and a radiolabeled methoprene derivative with high specific activity. The radiolabeled tracers used in the juvenile hormone radioimmunoassays have used ^{125}I in the form of chloramine T (Baehr et al., 1976, Figure 7), or of an iodinated tyrosine derivative (Strambi et al., 1981, Figure 8).

Recently, methoprene derivatives with very high specific activity have been reported (Prestwich, 1987). ^{3}H-Methoprene was synthesized with a specific activity greater than 70 Ci/mmol. It may be possible to develop a highly sensitive methoprene radioimmunoassay with this derivative. The use of tritium-labeled methoprene would insure that the radioimmunoassay would be detecting only methoprene. Such an immunoassay would be useful in searching for endogenous methoprene, a phenomenon which may exist since high affinity binding sites have been found for methoprene in the nuclei of Manduca sexta epidermal cells (Prestwich, 1987).

5.8.2. The Enzyme-Linked Immunosorbent Assay

The ELISA assay is a widely used, simple, rapid and versatile method of measuring either antibody affinity or antigen concentration. The assay is a discontinuous solid-phase system which usually involves two cycles of three washings and a second antibody step (Steward and Lew, 1985). The antibody or antigen is conjugated to an enzyme capable of converting a chromagenic substrate into a colored

product. The sensitivity of the assay is very high since the enzyme is capable of converting 500,000 substrate molecules to product molecules per minute (DeToma and MacDonald, 1987). A spectrophotometer is used to measure the concentration of enzyme present as a result of a competition reaction. The enzyme label replaces the radiolabel, making the assay more convenient, safer, and less costly for routine use.

An enzyme-linked immunosorbant assay has not been reported for methoprene or any of the juvenile hormones. The indirect enzyme immunoassay method (Figure 20) was used to determine if antibodies had been produced against the methoprene immunogen. This type of immunoassay assay may also be modified to measure antigen concentration.

A more direct method of measuring methoprene concentration would be to conjugate methoprene to an enzyme. This enzyme immunoassay (EIA) first coats the methoprene immunogen-specific antibody to the solid support. A known amount of enzyme-conjugated methoprene is added to the wells along with an unknown amount of solution containing methoprene. The free methoprene would compete with enzyme-conjugated methoprene for binding sites on the absored antibody. Unbound methoprene and enzyme-conjugated methoprene would be washed away, and chromogenic substrate would be added. The absorbance produced is inversely proportional to the amount of free antigen. Recently enzyme immunoassays have been developed for juvenile hormone III

and juvenile hormone III diol (Baehr et al., 1987), and are supposedly equivalent to or better than the radioimmunoassays using iodinated tracers.

Both enzyme-linked immunoassays have advantages and disadvantages. The ELISA method presents the antigen to the antibody on a solid surface. This may be problematic, partcularly for a small molecule like methoprene, which, when coated to a surface, may not present itself to the antibody in a way that the antibody can recognize. The results obtained from the EIA method are dependent on a lack of color. This also becomes a problem if one is assaying a colored sample. The presence of additional color would certainly decrease the sensitivity of this assay.

Despite these problems, enzyme-linked immunoassays have many advantages over RIA. No radiolabeled tracer is required, reducing risk and the use of protective facilities and equipment. The equipment needed for these assays is generally inexpensive and lends itself well to automation. Enzyme-linked immunoassays, with a few modifications can be adapted to kit form, allowing for methoprene assay in the field. Samples may also be analyzed without expensive and time consuming purification procedures.

5.9. Conclusions

The use of immunological assays for the analysis of pesticides has lagged far behind their use in clinical chemistry and endocrinology (Hammock and Mumma, 1980). It

is not likely that immunological assays will replace current analytical methods of pesticide analysis. However, they may aid in the initial detection of pesticides, giving valuable information as to the need to do more testing or to reapply the material for the optimal control.

The immunochemical techniques presented here will be used to develop immunological assays for methoprene. Methoprene was used as a model compound to present the general principles of how a non-immunogenic material can be made immunogenic. The procedures developed have allowed, for the first time, the production of a methoprene immunogen which was found to be immunogenic in BALB/c mice.

The derivatization of methoprene, prior to conjugation to a protein, incorporated a four carbon spacer group via an ester function. The spacer group serves to mimic the ester function of methoprene, and acts as a flexible bridge between methoprene and the protein surface. Although there may be differences between native methoprene and the methoprene derivative conjugated to the protein, it is hoped that the immune system of the immunized animals will be encouraged to raise antibodies which specifically recognize methoprene.

The use of activated esters is a common method of conjugating small molecules to proteins. Two activated esters were used to conjugate methoprene to human serum albumin. The use of the N-hyroxysuccinimide ester of methoprene modified procedures used for the preparation of

immunogens for the juvenile hormones. The modified method produced an immunogen with a high density of methoprene on the protein. This immunogen was subsequently used to immunize mice.

The 1-hydroxy-2-nitro-4-benzenesulfonate ester of methoprene was also synthesized and it enabled the complete solubilization of methoprene in aqueous solutions. This activated ester was coupled to human serum albumin in a fraction of the time needed for the N-hydroxysuccinimide activated ester. Additionally, the reaction was carried out in a neutral buffer solution, as opposed to an aqueous/organic mixture of high pH. The density of methoprene on the resulting immunogen was not as high (31 molecules vs. 53 molecules of methoprene per molecule protein), but was higher than other reported immunogens for juvenile hormone.

The complete synthetic process for the production of the methoprene immunogen is summarized in figure 22. These steps lead to the formation of an immunogen with a four carbon spacer group. The four carbon spacer group was prepared from a lactone, forming a diprotected intermediate which prevented the product from recyclizing to the lactone. The removal of the 2-(trimethlysilyl)ethyl protecting residue used a reagent specific for that residue, insuring that no other functionalities would be attacked. The general method outlined (Figure 22) may be repeated with other carboxylic acids. The discovery that methoprene was

made water soluble when coupled to 1-hydroxy-2-nitro-4-benzenesulfonic acid may be applied to other organic materials to make them water soluble.

The antibodies raised will be used to develop imuunoassays for the detection of methoprene. Such assays, if properly developed, should detect methoprene in the nanogram to picogram range in biological and environmental samples. The assay should also produce results which can be validated with physical-chemical and analytical methods.

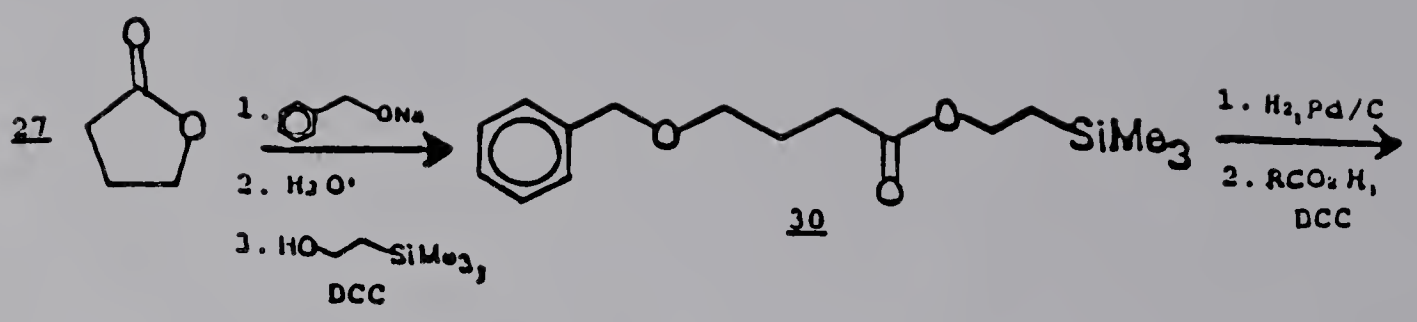
5.10. Summary of Results

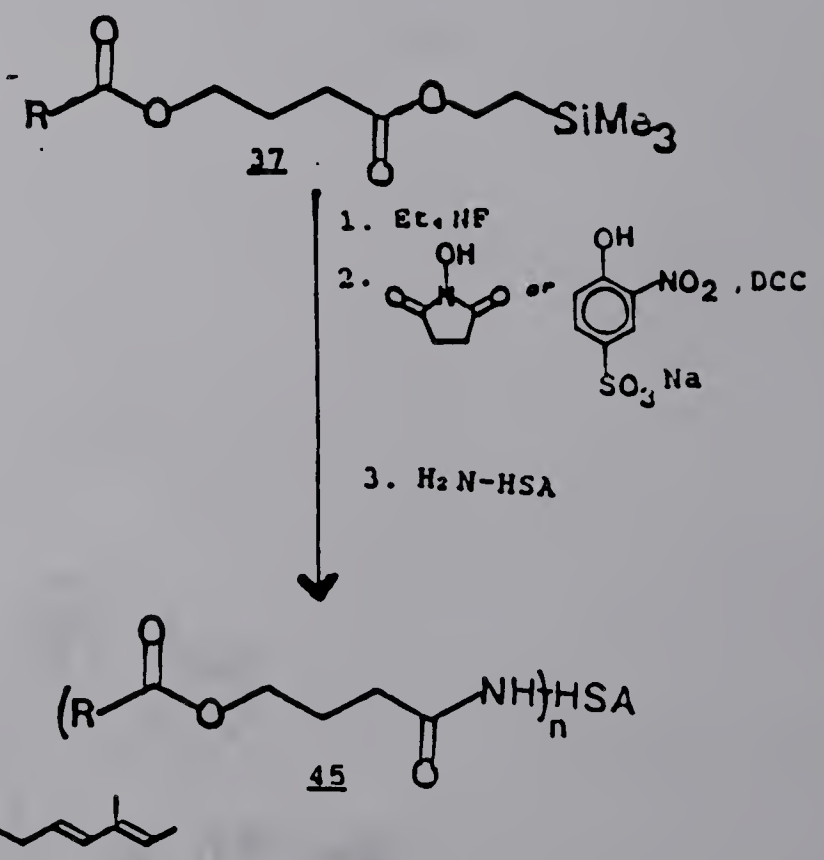
1. Methoprene may be made immunogenic in mice by coupling it to a protein.

2. The incorporation of a four carbon spacer group retained the methoxy and ester functions that are characteristic of the methoprene molecule.

3. Human serum albumin, the protein carrier of choice, allows for the conjugation of at least 53 methoprene molecules per molecule of albumin, and remains soluble at

Figure 22. Summary of synthetic steps required to produce the methoprene immunogen (**45**).


27
1.
2. H3O+
3. HO SiMe3
DCC
30
SiMe3
1. H2, Pd/C
2. RCO2H,
DCC


R
SiMe3
37
1. Et4NF
2.
OH
N
or
OH
NO2
DCC
SO3Na
3. H2N-HSA
(R
NH)nHSA
45
R=

this density of hapten.

4. The two activated ester methods of protein conjugation load many hapten molecules onto the protein under organic/aqueous or aqueous reaction solutions.

5. The protecting groups used to protect the four carbon spacer group were removed with specific reagents under mild conditions.

6. The use of a stable radioisotope such as ^{14}C allows for direct determination of the number of hapten molecules conjugated per molecule protein.

7. The methods developed for the preparation of an immunogen may be applied to other non-immunogenic carboxylic acids.

8. Other water insoluble carboxylic acids may be made water soluble by coupling to 1-hydroxy-2-nitro-4-benzenesulfonic acid.

9. The enyzme-linked immunosorbent assay was successfully used to detect antibodies in mouse serum which were specific for the methoprene immunogen.

Appendices

Appendix A.

Nuclear Magnetic Resonance and Infrared Spectra

Figure A-1. ^{1}H NMR ($CDCl_3$, 200 MHz) of 4-benzyloxybutanoic acid (28).

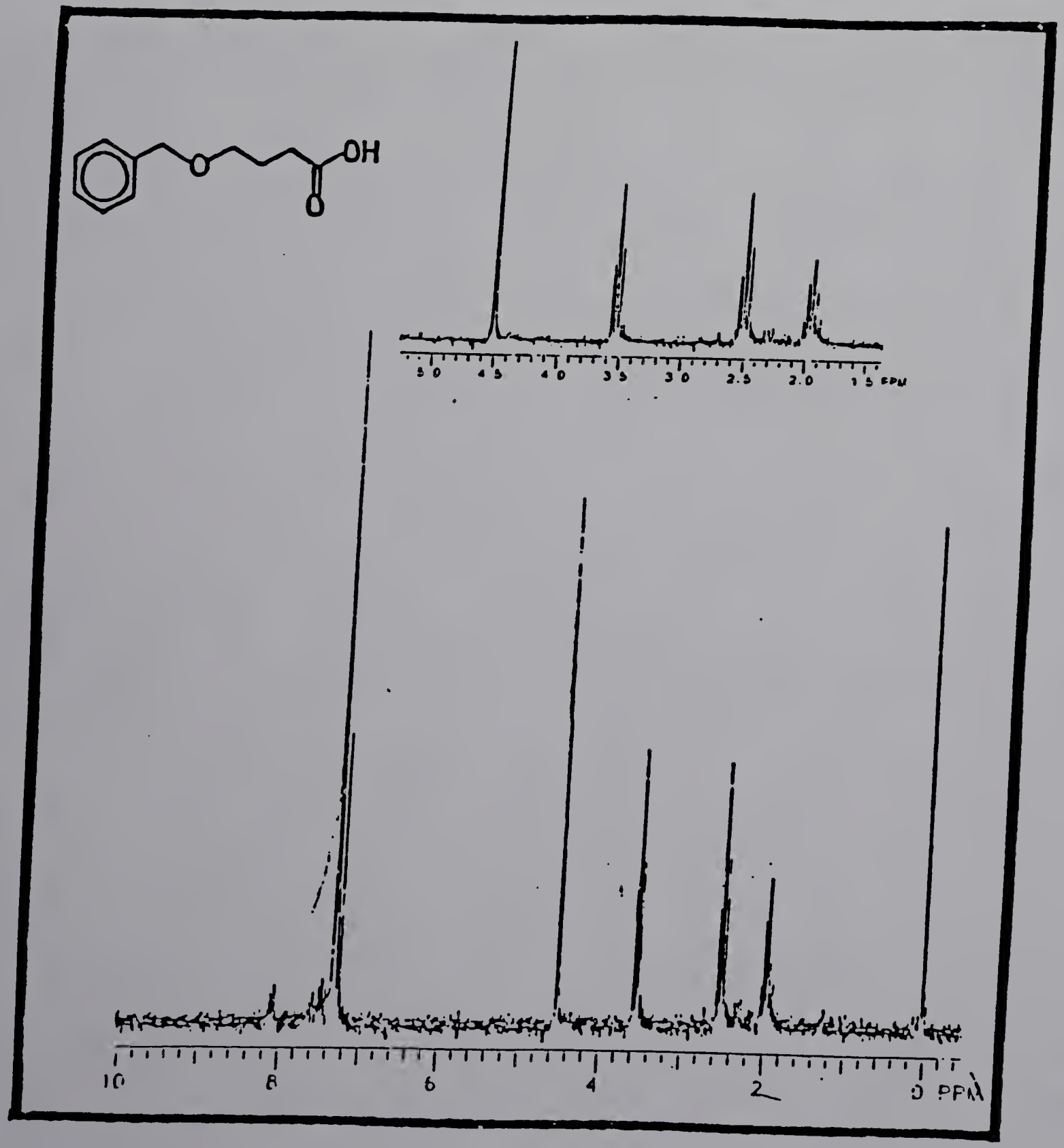

OH
O
O
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5 PPM
10
8
6
4
2
0 PPM

Figure A-2. IR (neat) of 4-benzyloxybutanoic acid (28).

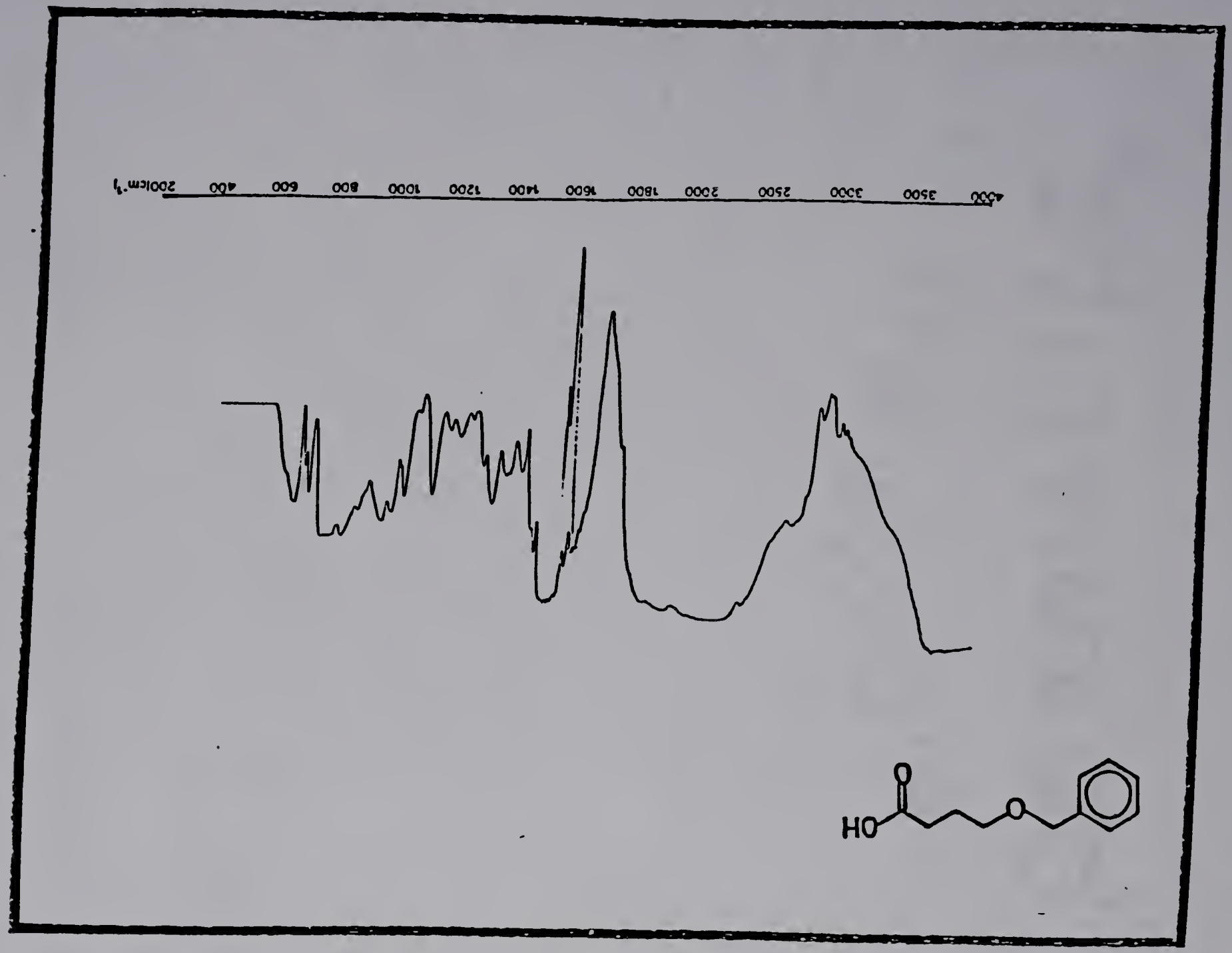

4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200cm⁻¹
HO
O
O

Figure A-3. ^{1}H NMR ($CDCl_3$, 60 MHz) of 2-(trimethylsilyl)ethyl 4-benzyloxybutanoate (30).

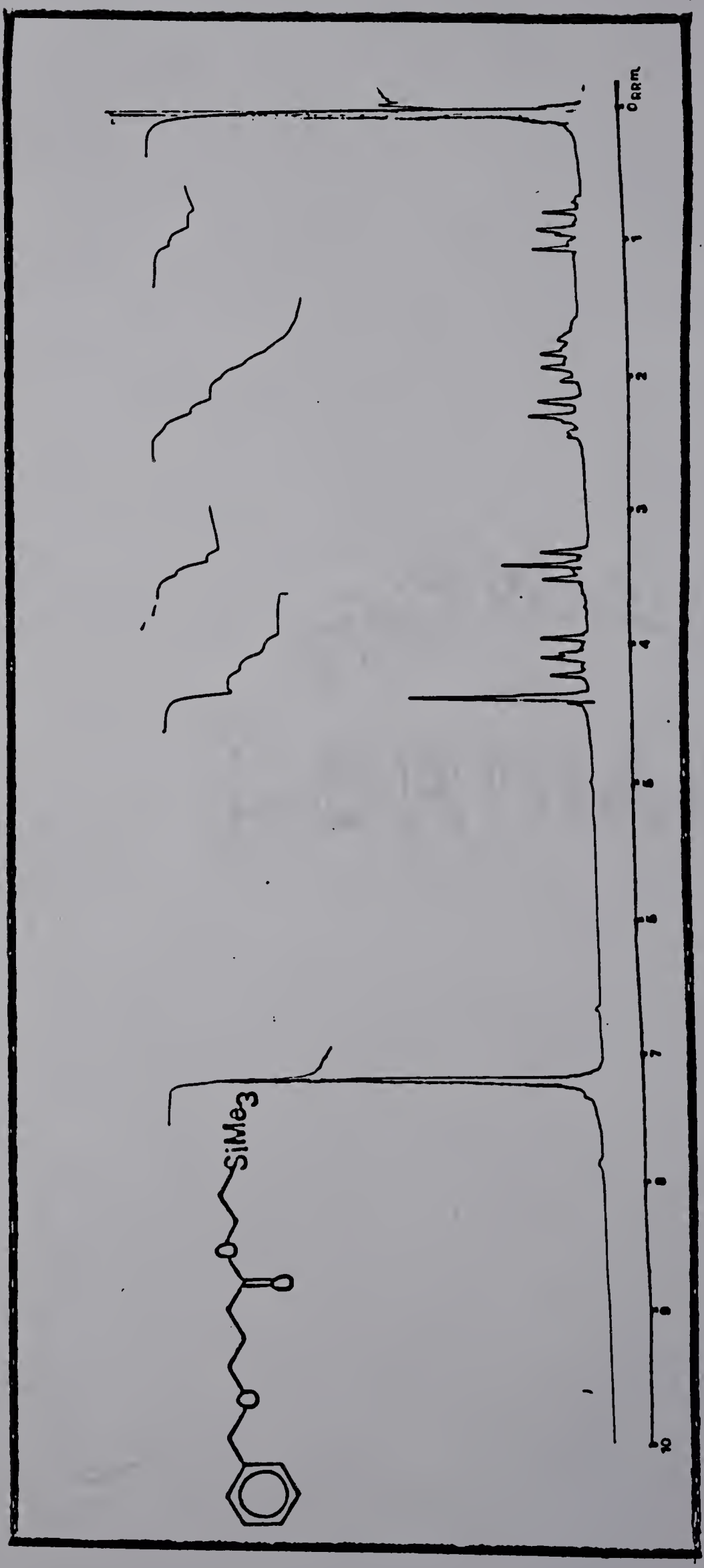

SiMe3
δ ARM
8
7
6
5
4
3
2
1
0

Figure A-4. IR (neat) of 2-(trimethylsilyl)ethyl 4-benzyloxybutanoate (30).

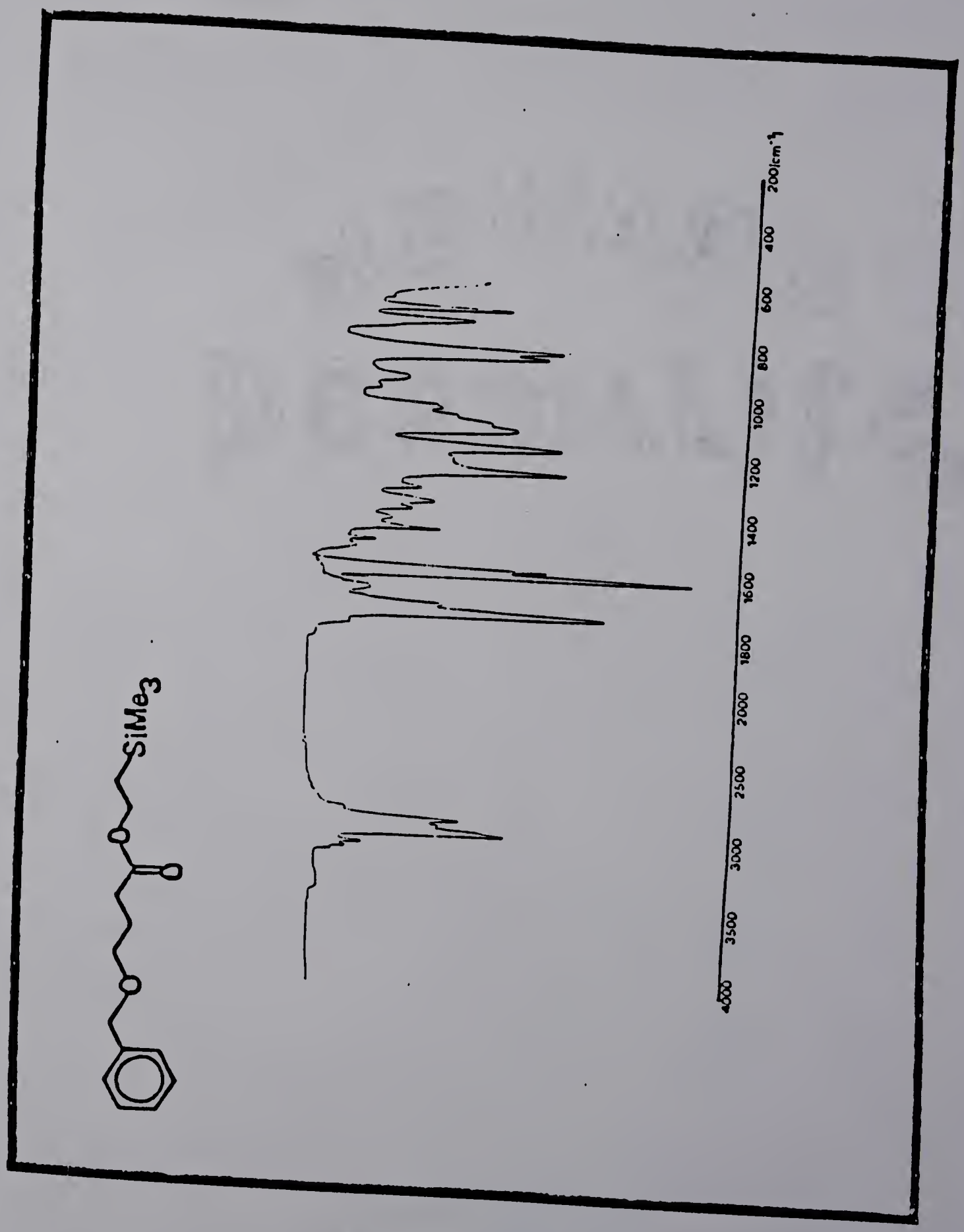

SiMe3
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200 [cm-1]

Figure A-5. ^{1}H NMR ($CDCl_3$, 60 MHz) of 2-(trimethylsilyl)ethyl 4-hydroxybutanoate (31).

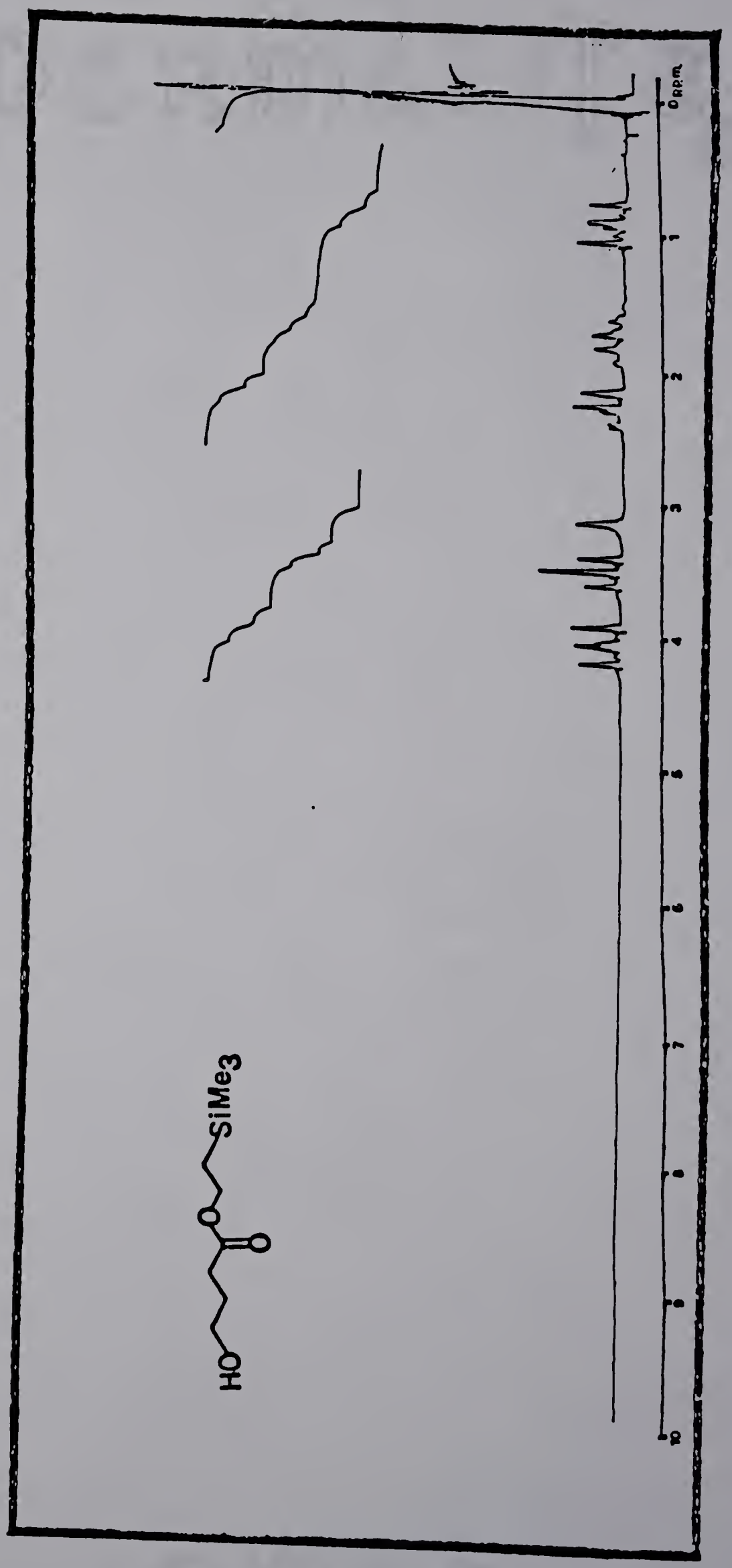

0 PPM
1
2
3
4
5
6
7
8
HO
O
O
SiMe3

Figure A-6. IR (neat) of 2-(trimethylsilyl)ethyl 4-hydroxybutanoate (31).

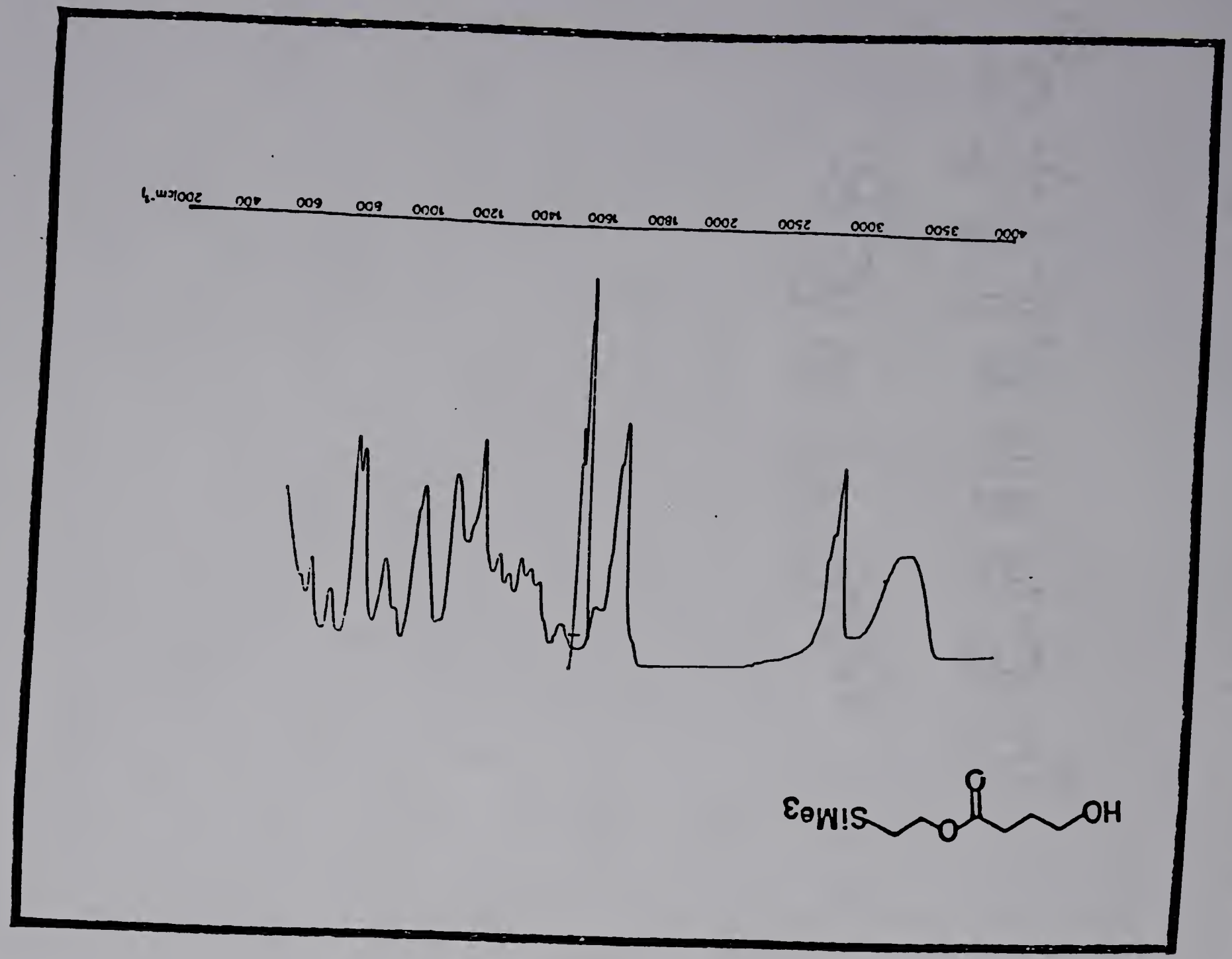

4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 400 200 cm⁻¹
HO
O
O
SiMe3

Figure A-7. ^{1}H NMR ($CDCl_3$, 60 MHz) of 3,7,11-trimethyl-2,6,10-dodecatrienal (33).

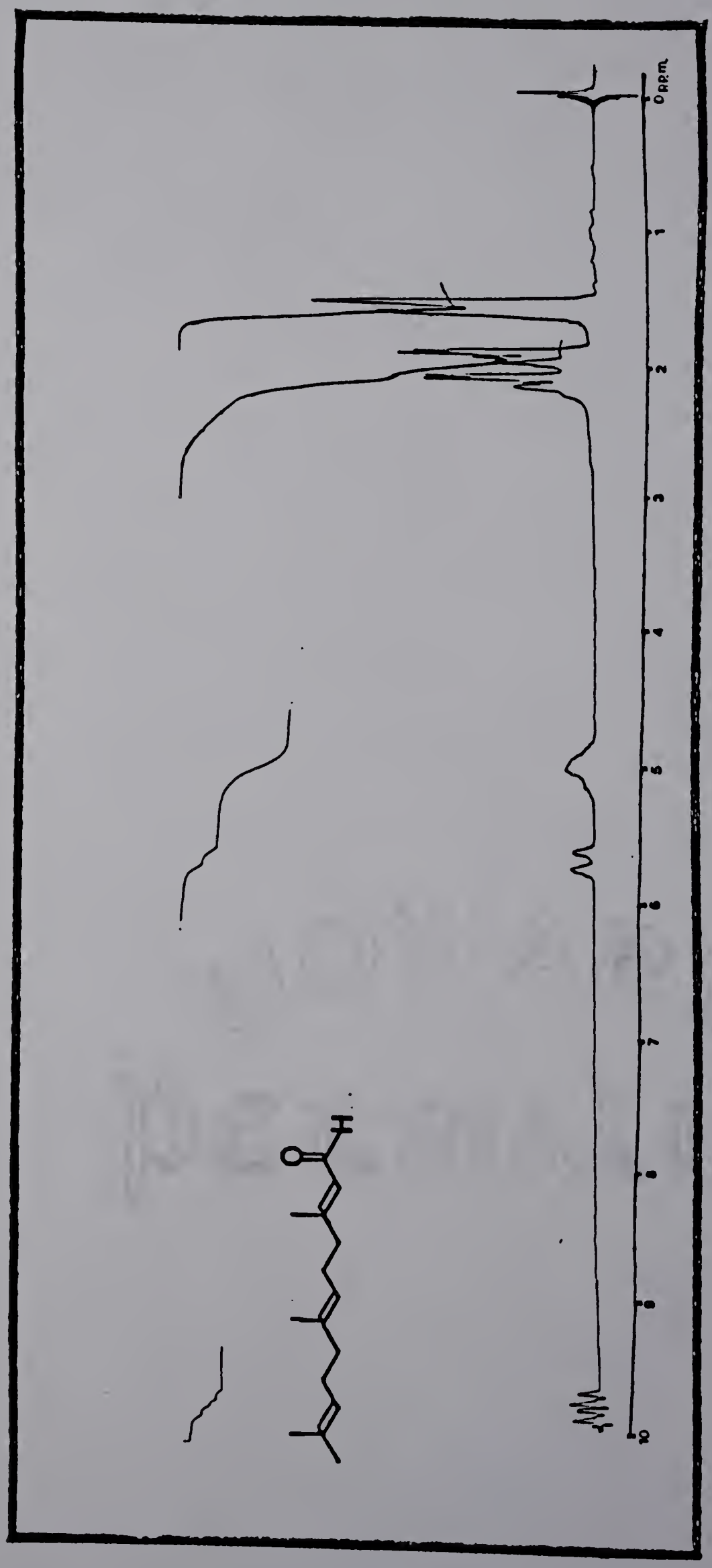

0 p.p.m.
1
2
3
4
5
6
7
8
9
10

Figure A-8. IR (neat) of 3,7,11-trimethyl-2,6,10-dodecatrienal (33).

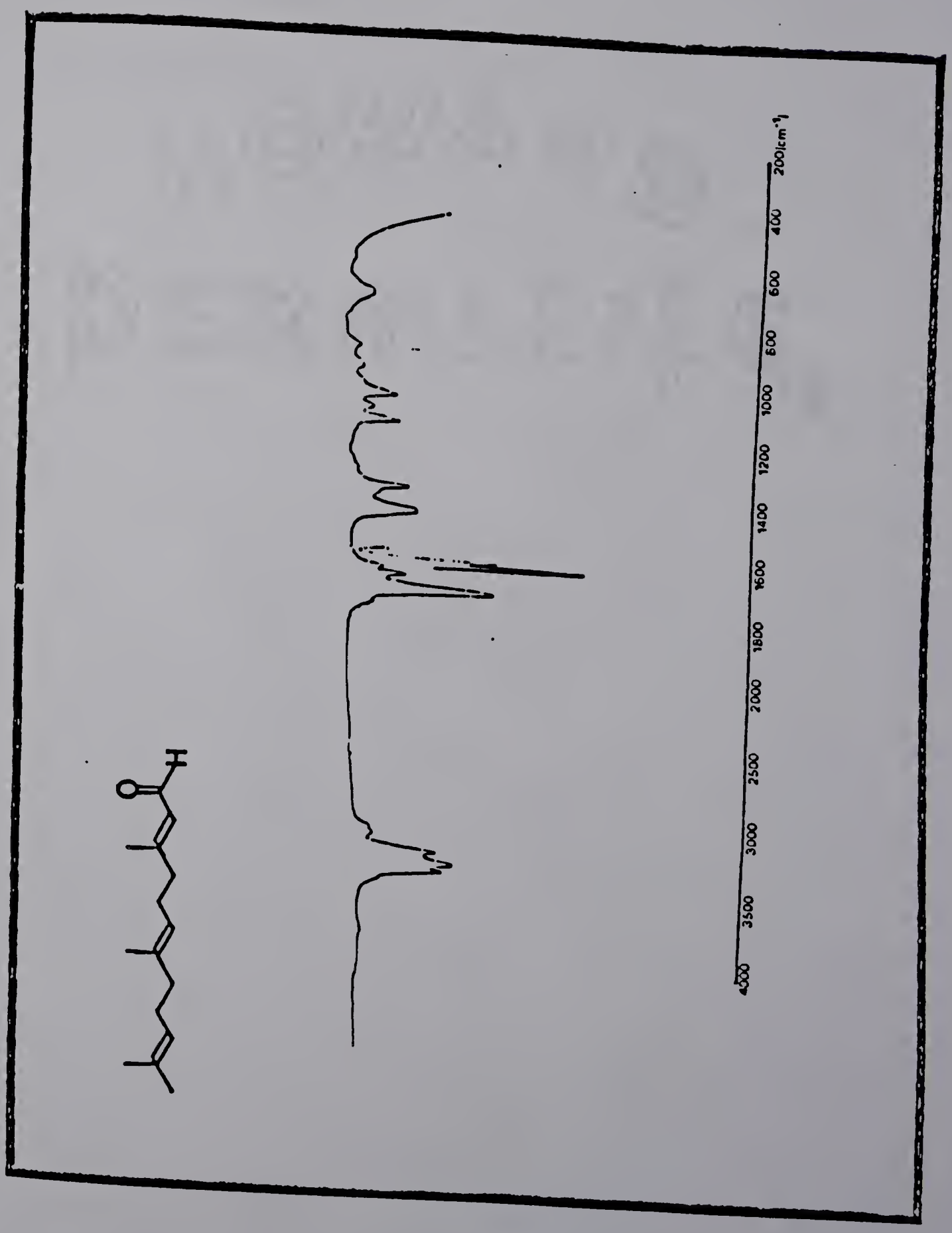

4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)
O
H

Figure A-9. ^{1}H NMR ($CDCl_3$, 60 MHz) 3,7,11-trimethyl-2,6,10-dodecatrienoic acid (34).

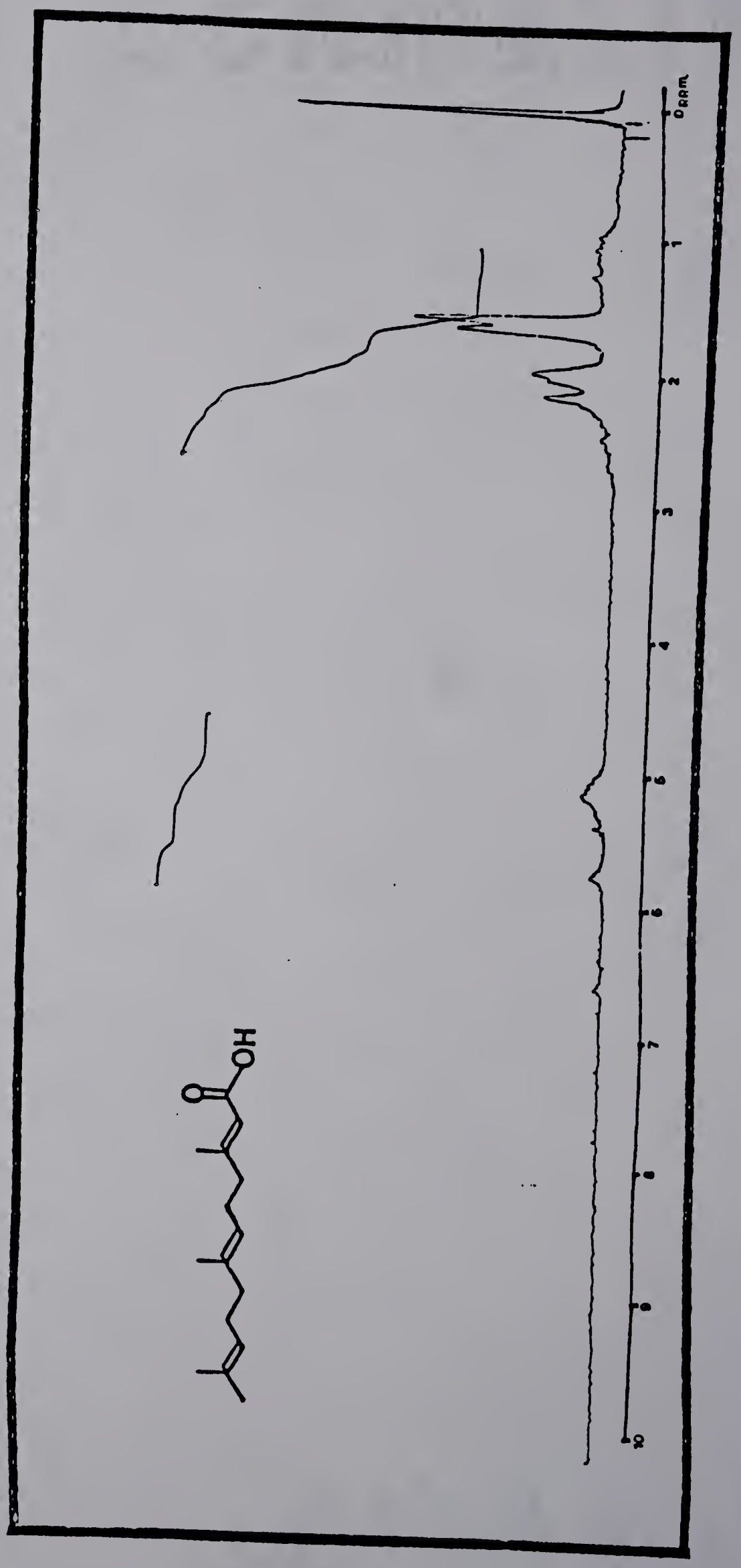

OH
O
0 RAM
1
2
3
4
5
6
7
8

Figure A-10. IR (neat) of 3,7,11-trimethyl-2,6,10-dodecatrienoic acid (34).

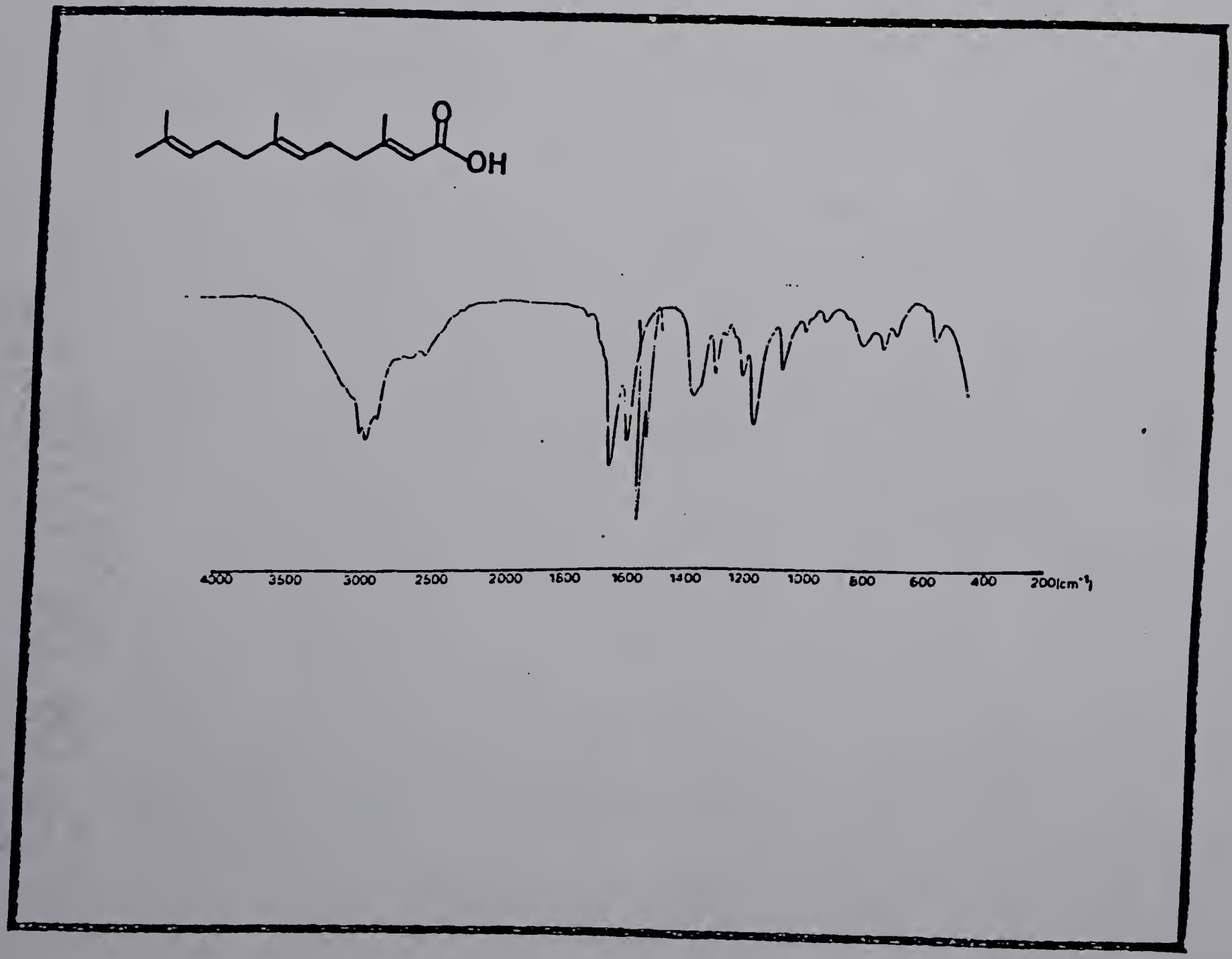

OH
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)

Figure A-11. ^{1}H NMR ($CDCl_3$, 60 MHz) of 2-(trimethylsilyl)ethyl 4-(3,7,11-trimethyl-2,6,10-dodecatrienoyloxy)butanoate (36).

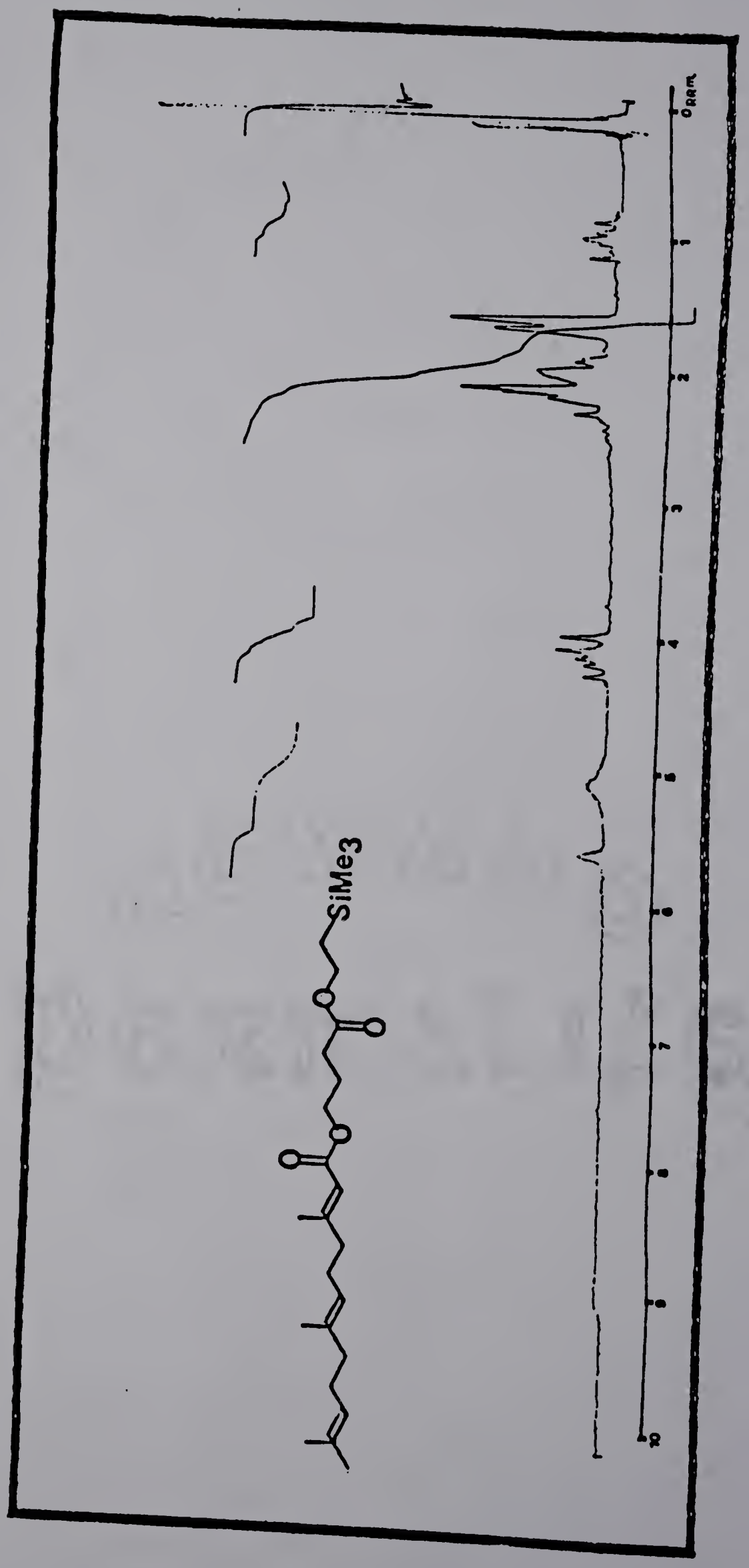

SiMe3
δ PPM

Figure A-12. IR (neat) of 2-(trimethylsilyl)ethyl 4-(3,7,11-trimethyl-2,6,10-dodecatrienoyloxy)butanoate (36).

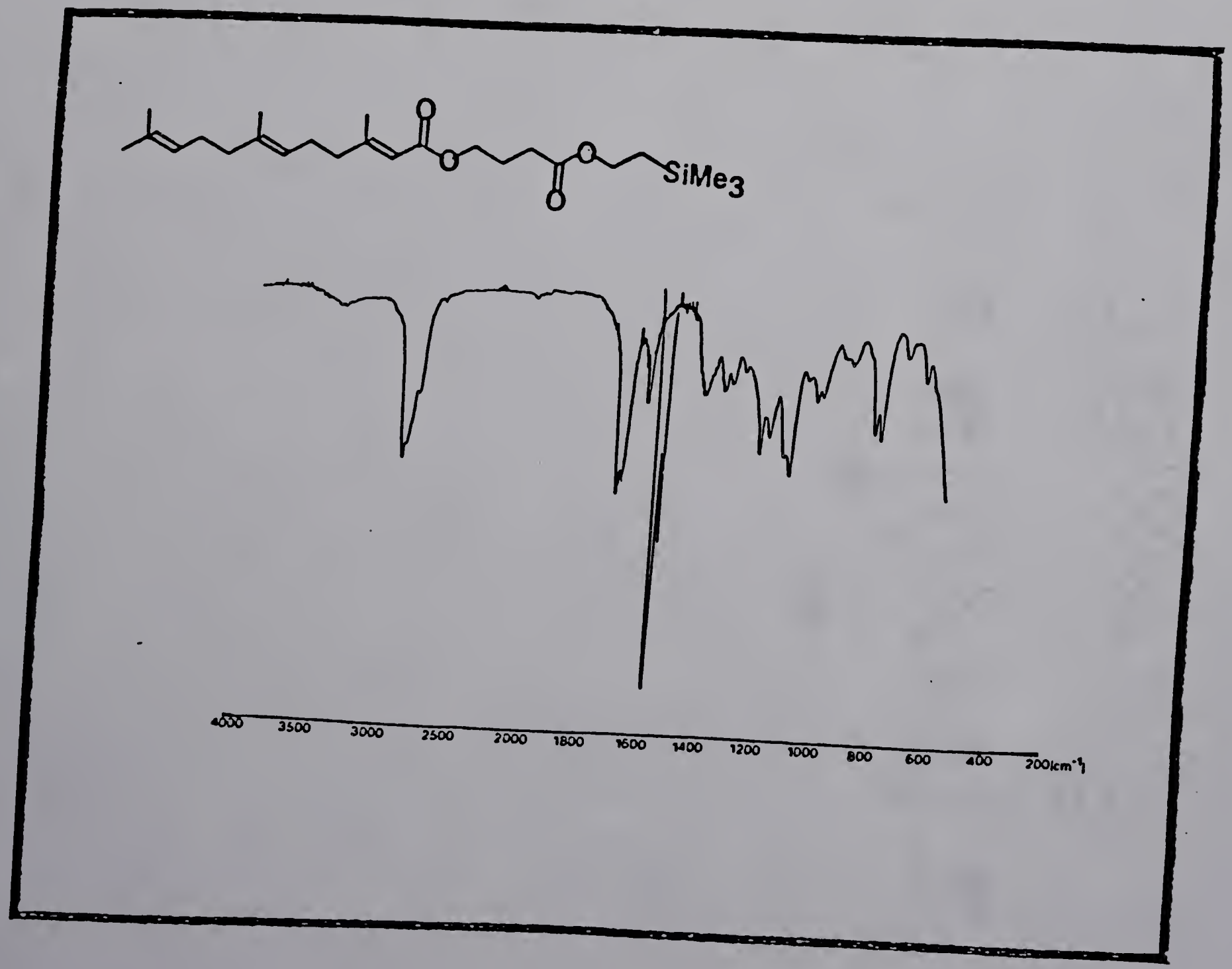
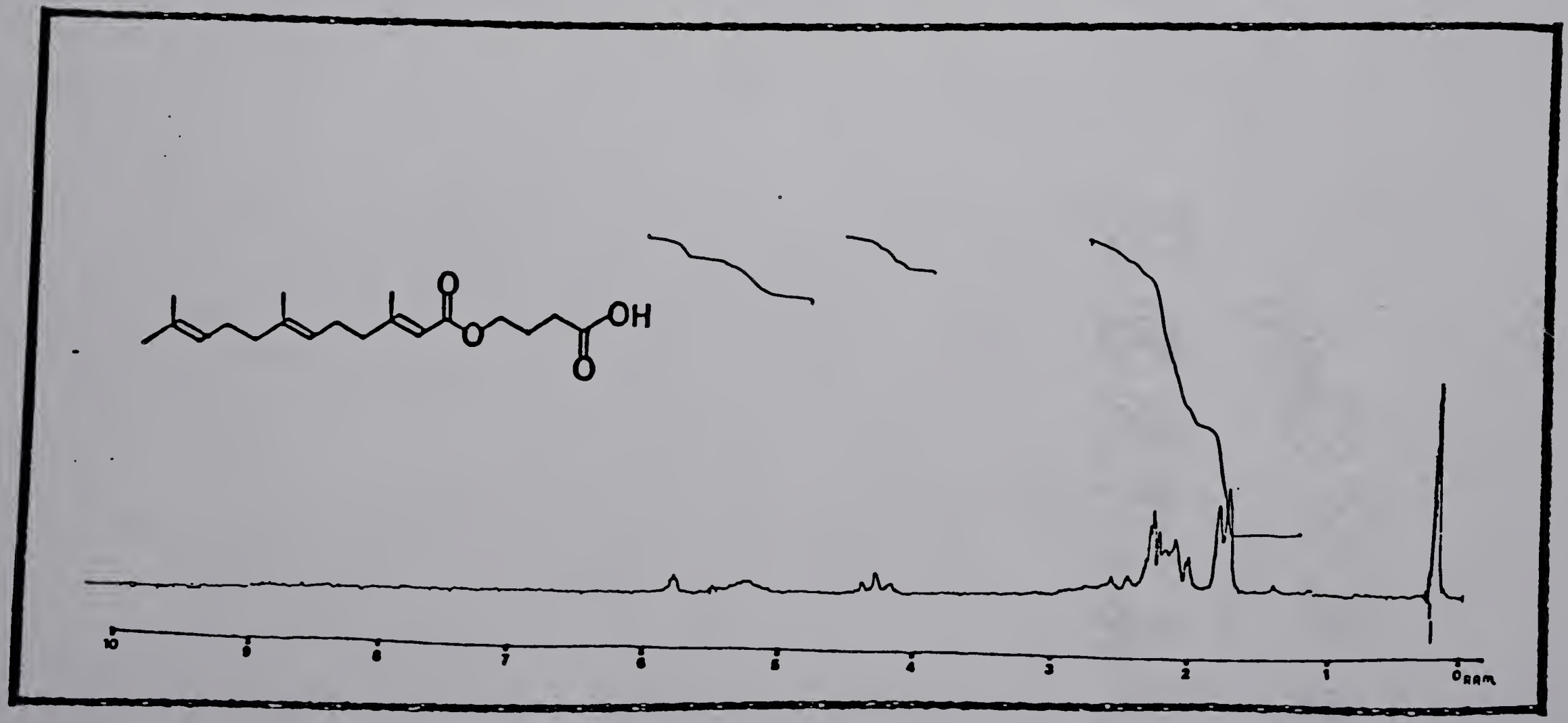

SiMe3
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200cm⁻¹

Figure A-13. ^{1}H NMR ($CDCl_3$, 60 MHz) of 4-(3,7,11-trimethyl-2,6,10-dodecatrienoyloxy)butanoic acid (38).


OH
O
O
O
10
9
8
7
6
5
4
3
2
1
0 PPM

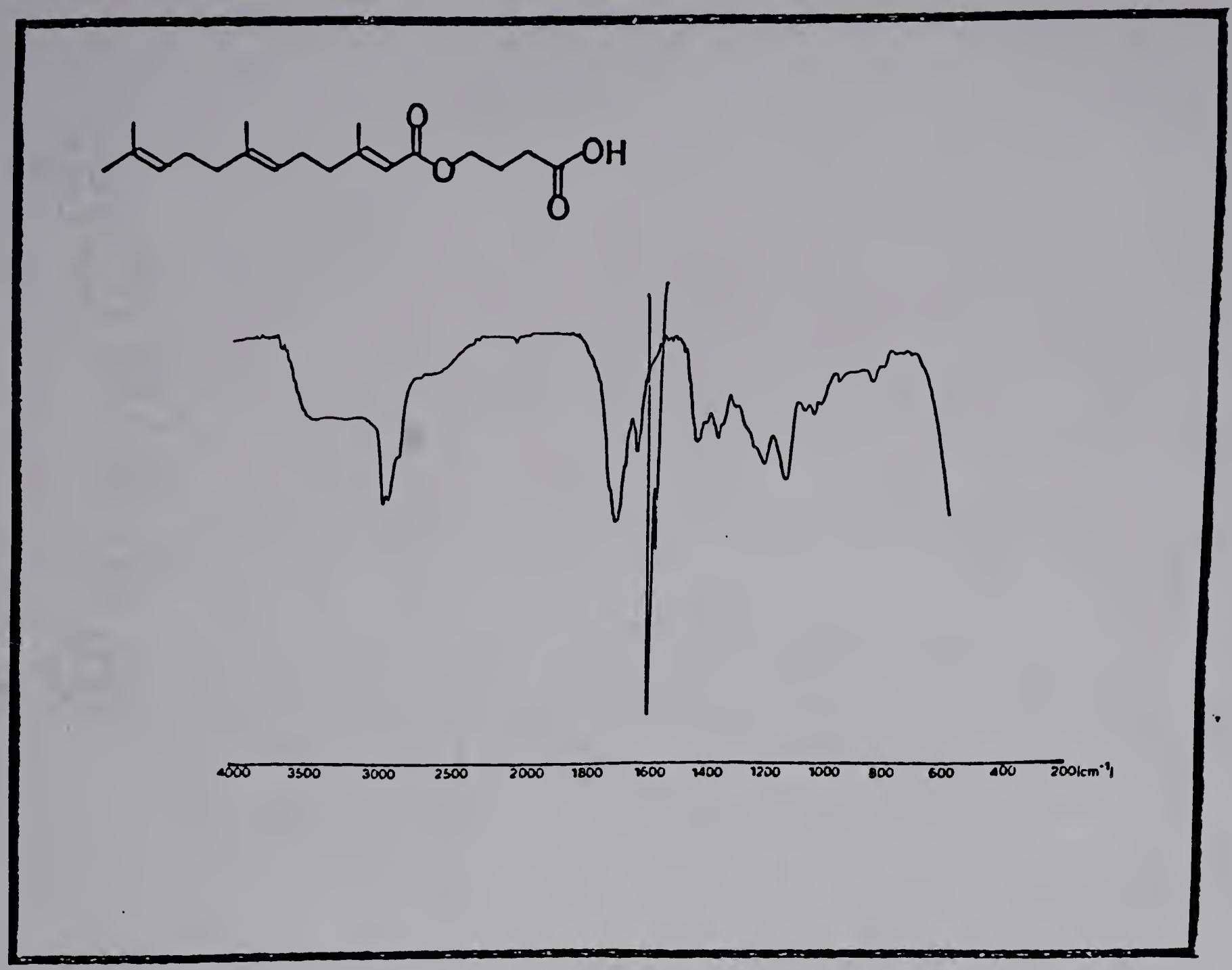

OH
O
O
O
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)

Figure A-14. IR (neat) of 4-(3,7,11-trimethyl-2,6,10-dodecatrienoyloxy)butanoic acid (38).

Figure A-15. ^{1}H NMR ($CDCl_3$, 60 MHz) of (2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid (35).

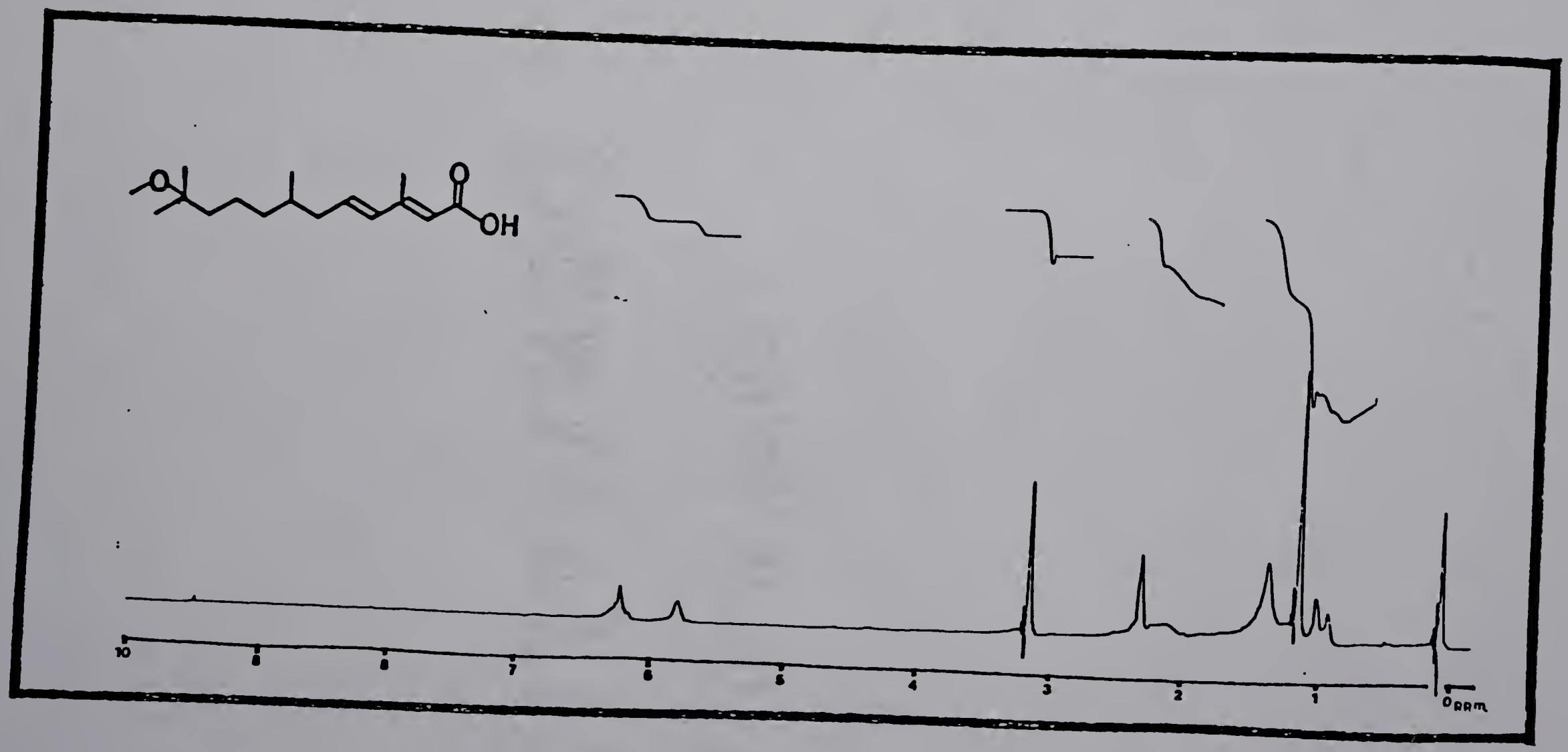

OH
10
9
8
7
6
5
4
3
2
1
0 PPM

Figure A-16. IR (neat) of (2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid (35).

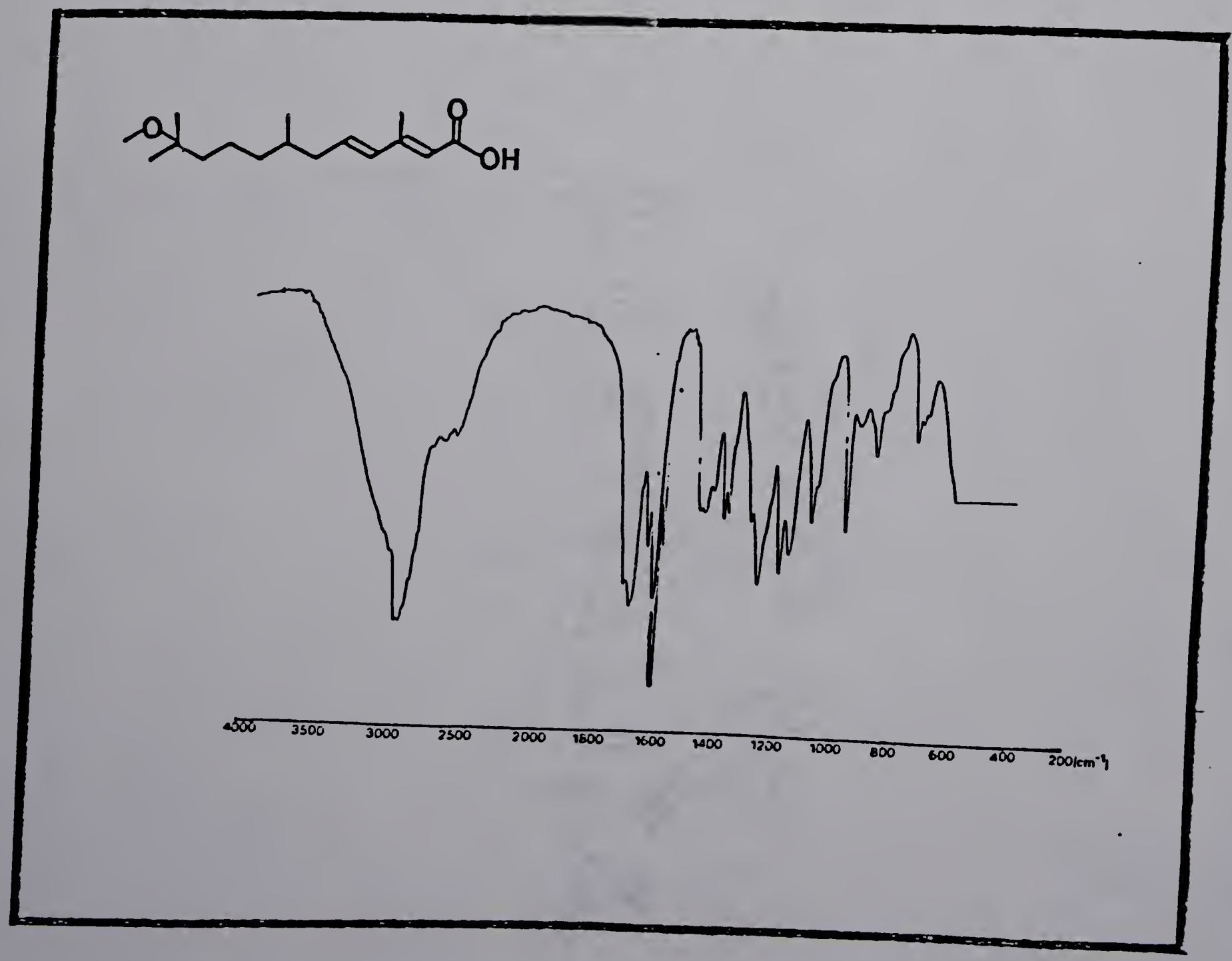

OH
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200/cm⁻¹

Figure A-17. ^{1}H NMR ($CDCl_3$, 60 MHz) of 2-(trimethylsilyl)ethyl 4-(2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy)butanoate (37).

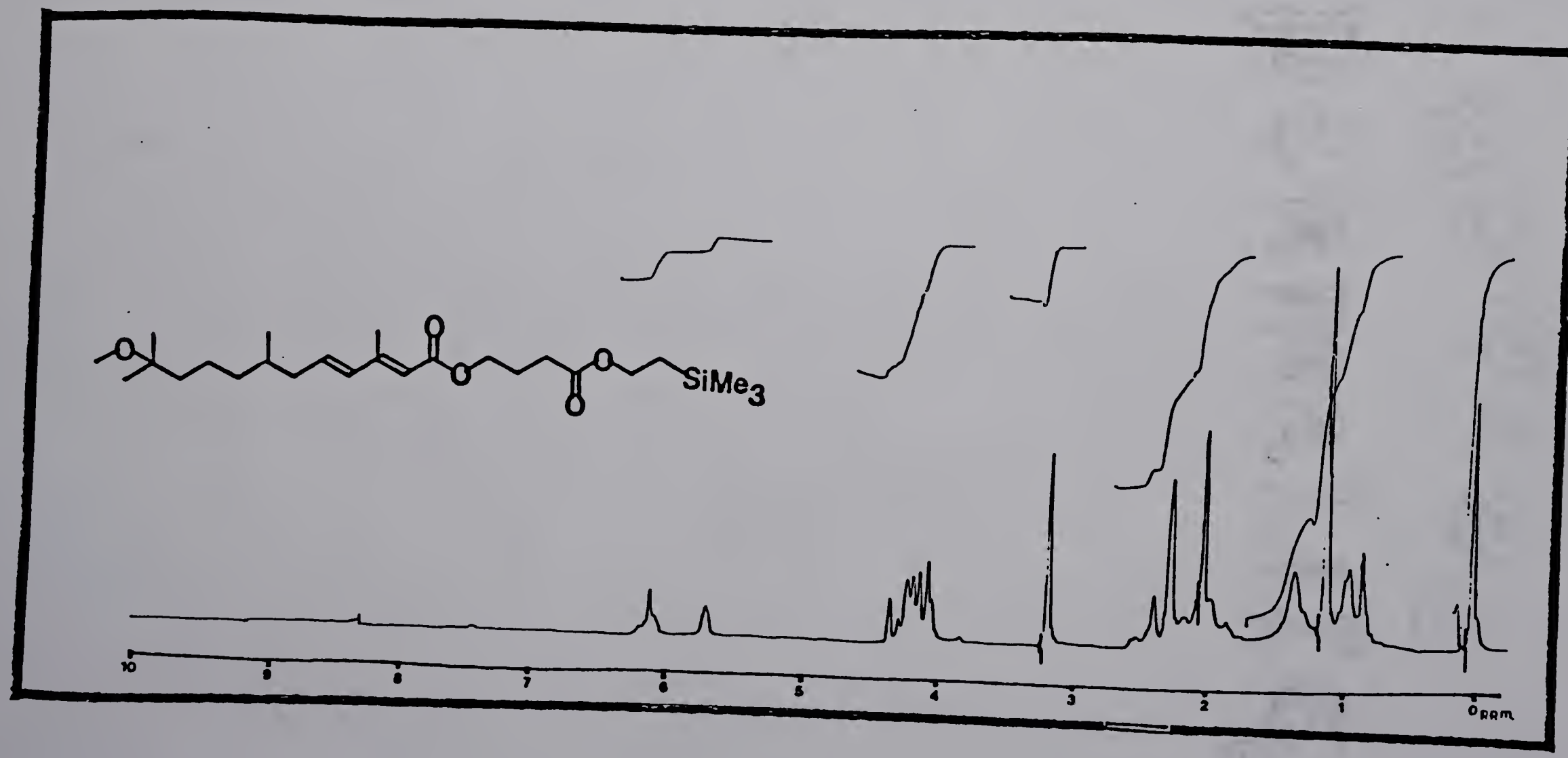

SiMe3
10
9
8
7
6
5
4
3
2
1
0 PPM

Figure A-18. IR (neat) of 2-(trimethylsilyl)ethyl 4-(2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy)butanoate (37).

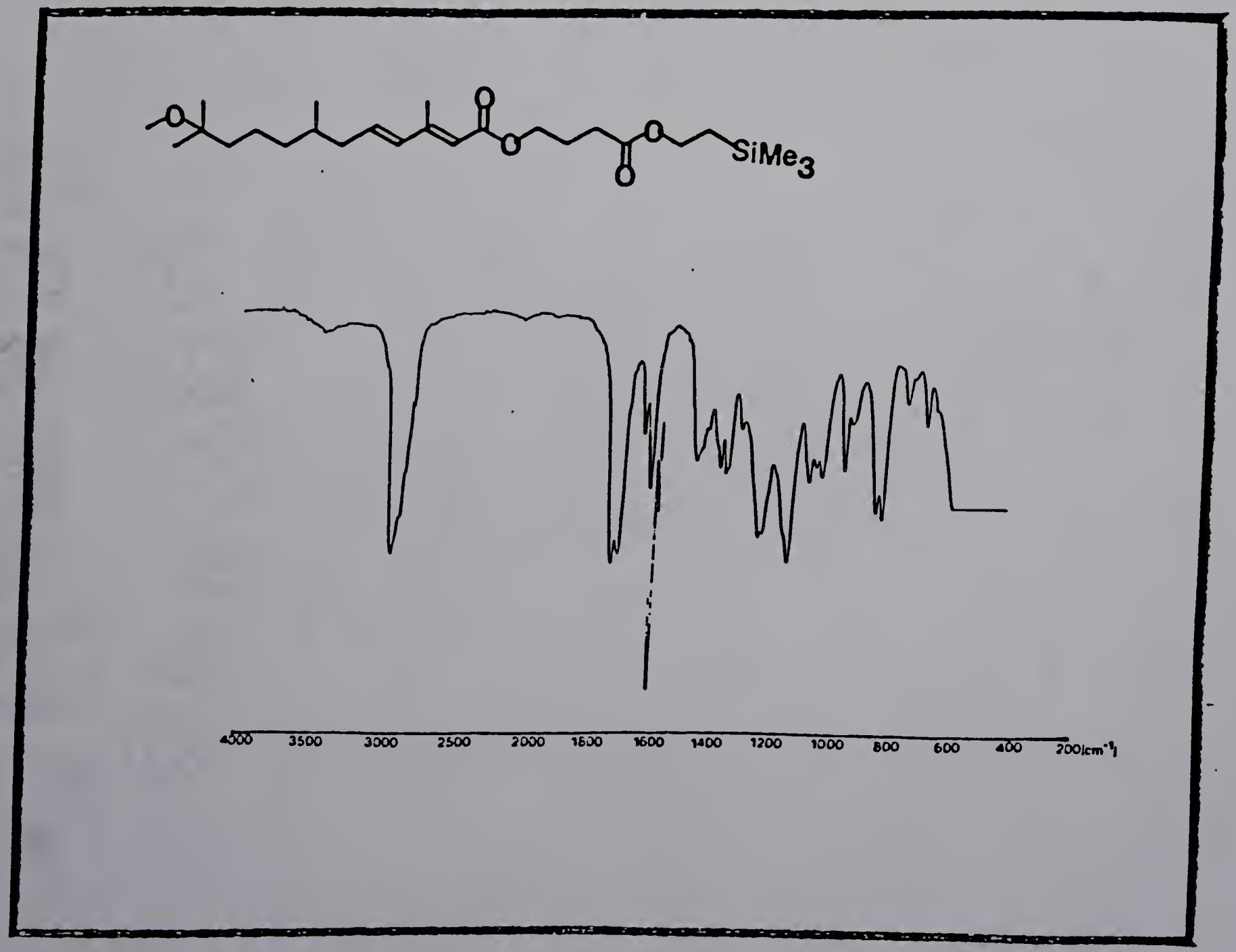

SiMe3
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)

Figure A-19. ^{1}H NMR ($CDCl_3$, 60 MHz) of 4-(2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy) butanoic acid (39).

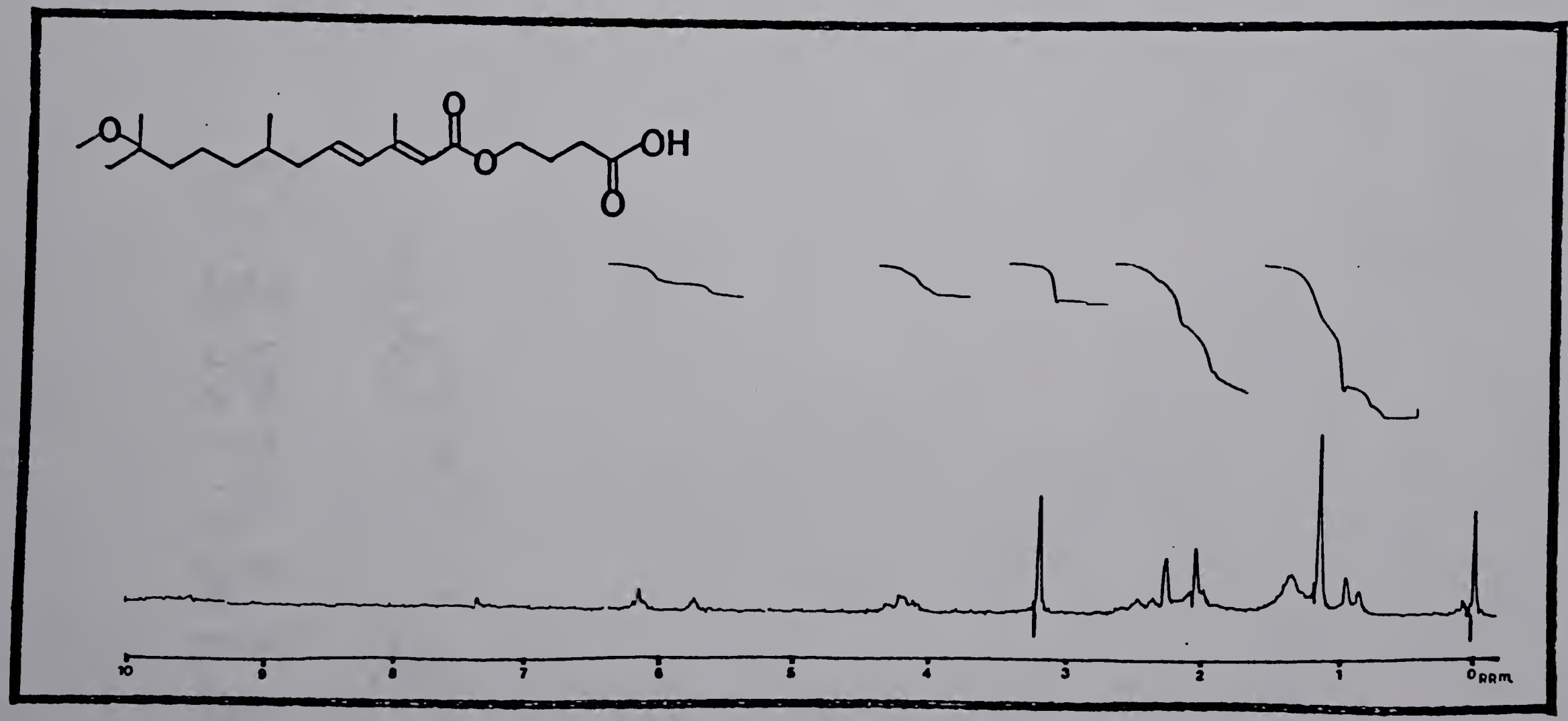

O
O
O
OH
O
10
9
8
7
6
5
4
3
2
1
0 PPM

Figure A-20. IR (neat) of 4-(2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy) butanoic acid (39).

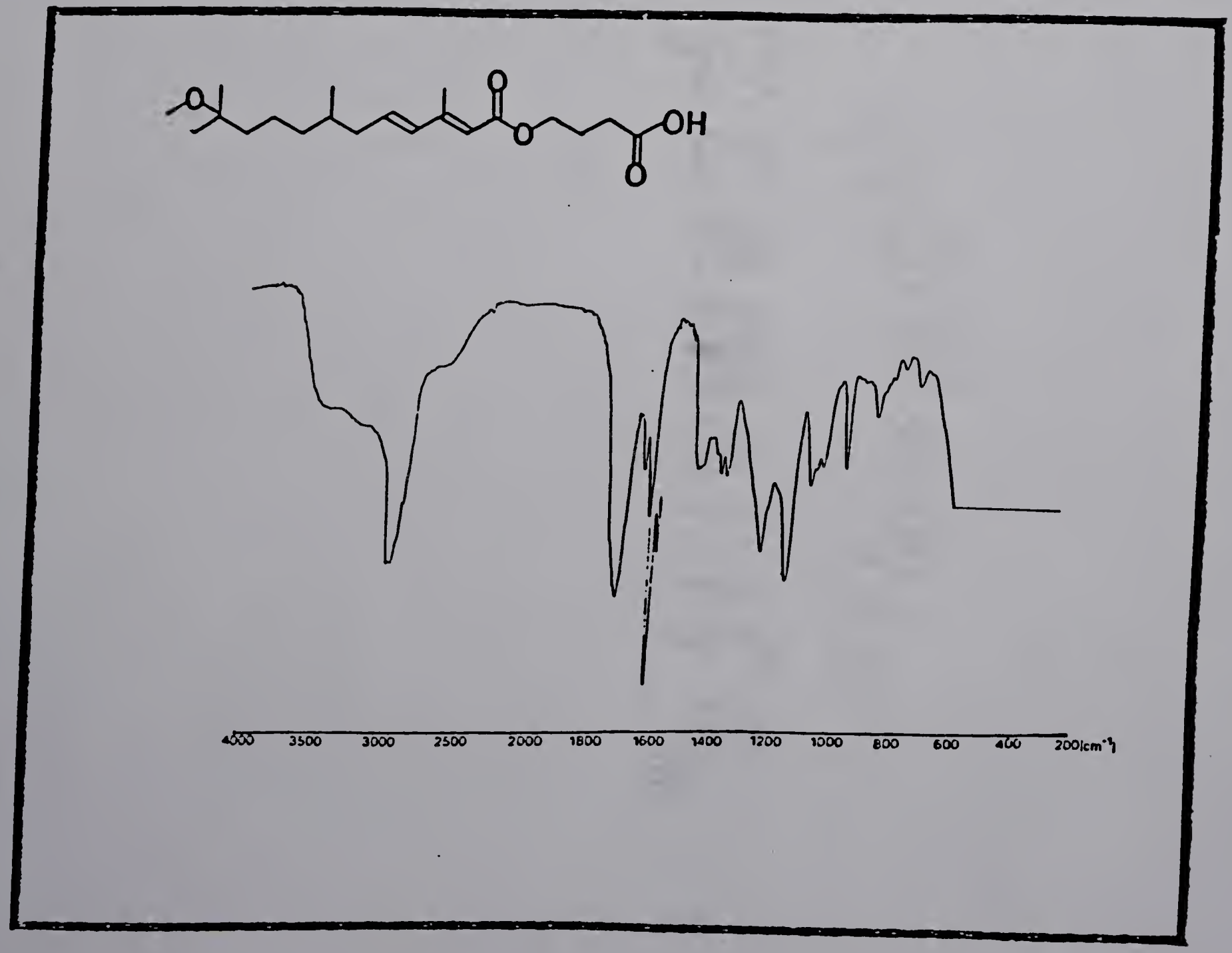

OH
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)

Figure A-21. ^{1}H NMR ($CDCl_3$, 60 MHz) of N-hydroxysuccinimidyl 4-(2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy) butanoate (41).

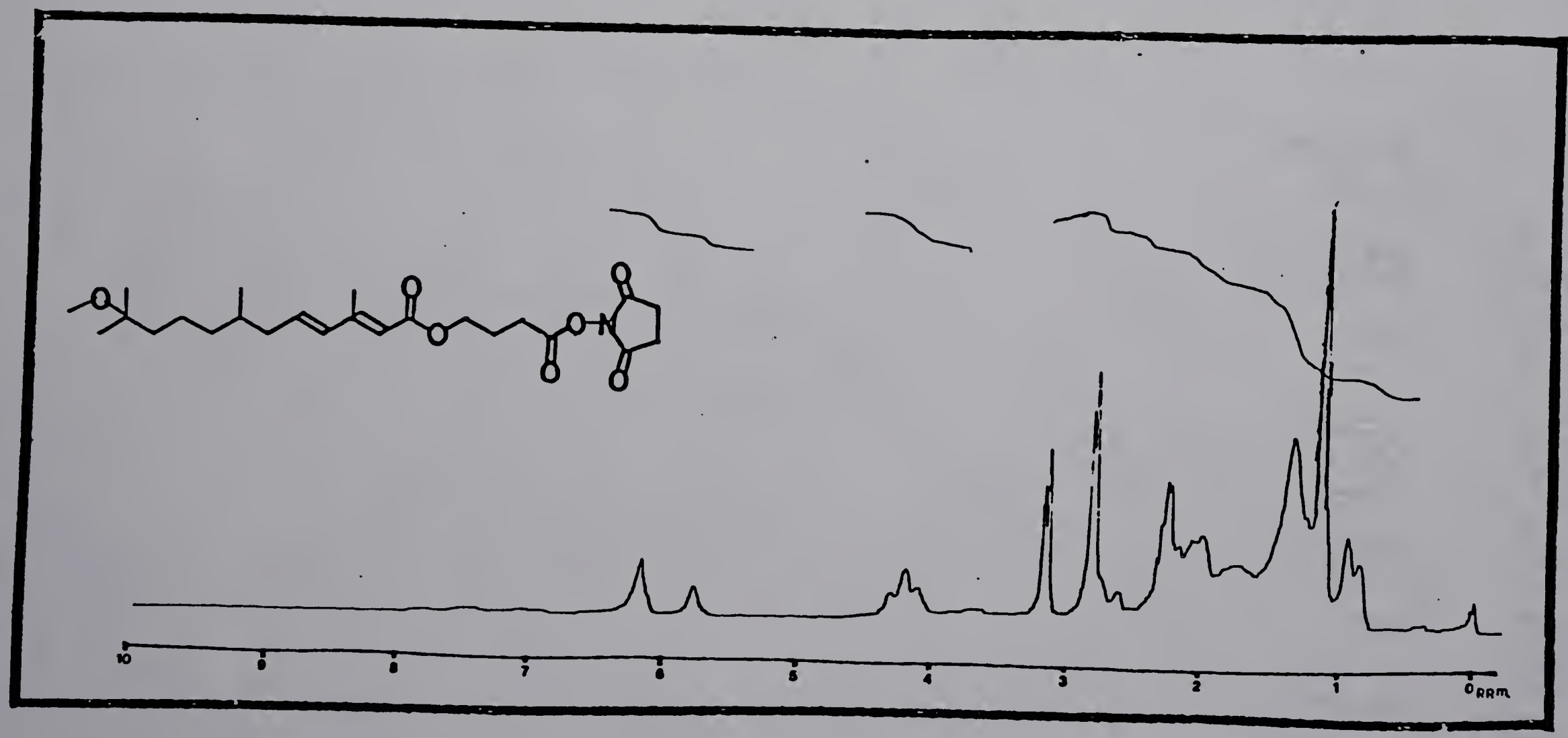

10
9
8
7
6
5
4
3
2
1
0 PPM

Figure A-22. IR (neat) of N-hydroxysuccinimidyl 4-(2E, 4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoyloxy) butanoate (41).

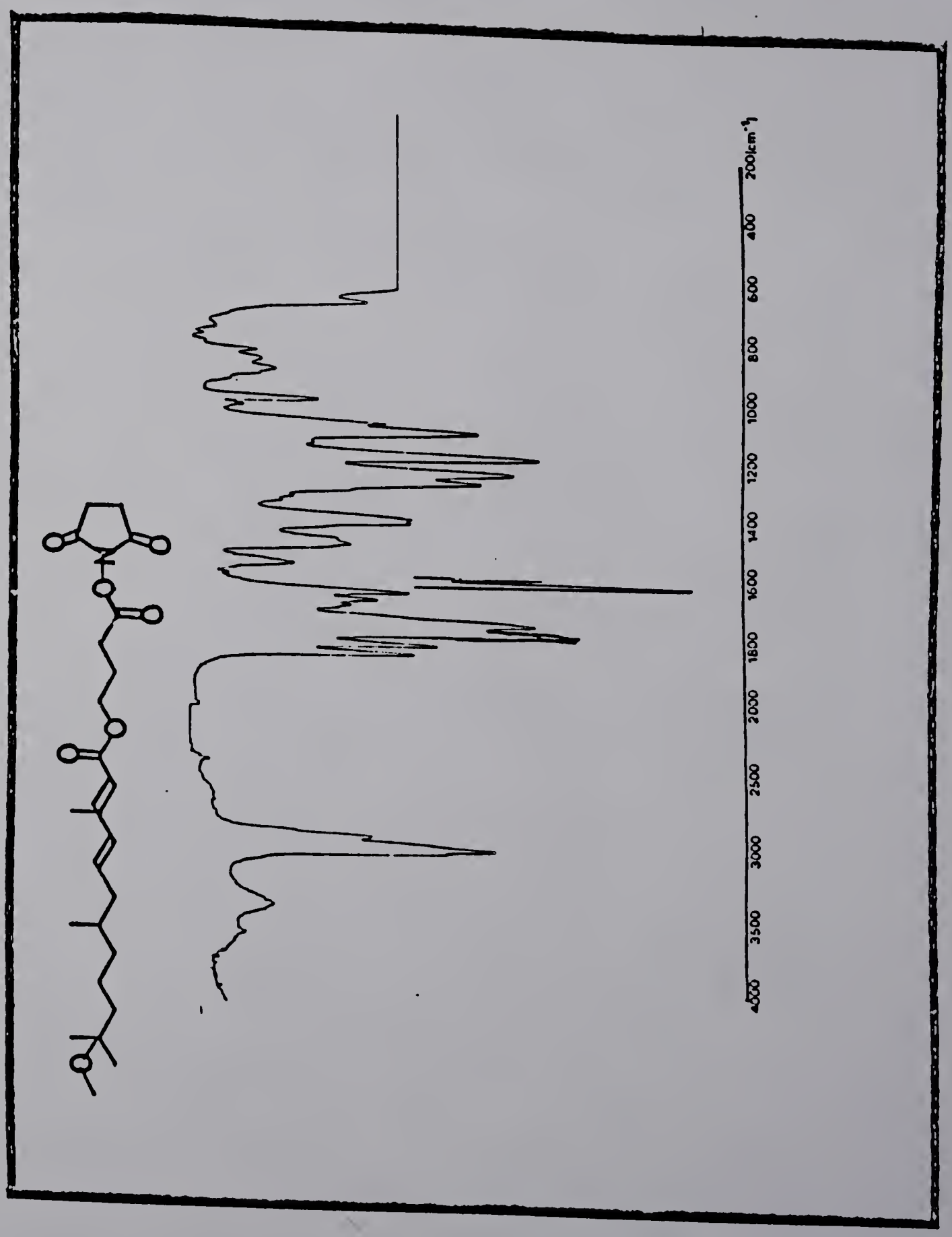

4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)

Figure A-23. ^{1}H NMR (D_2O, 200 MHz) of sodium 1-hydroxy-2-nitro-4-benzenesulfonate (42).

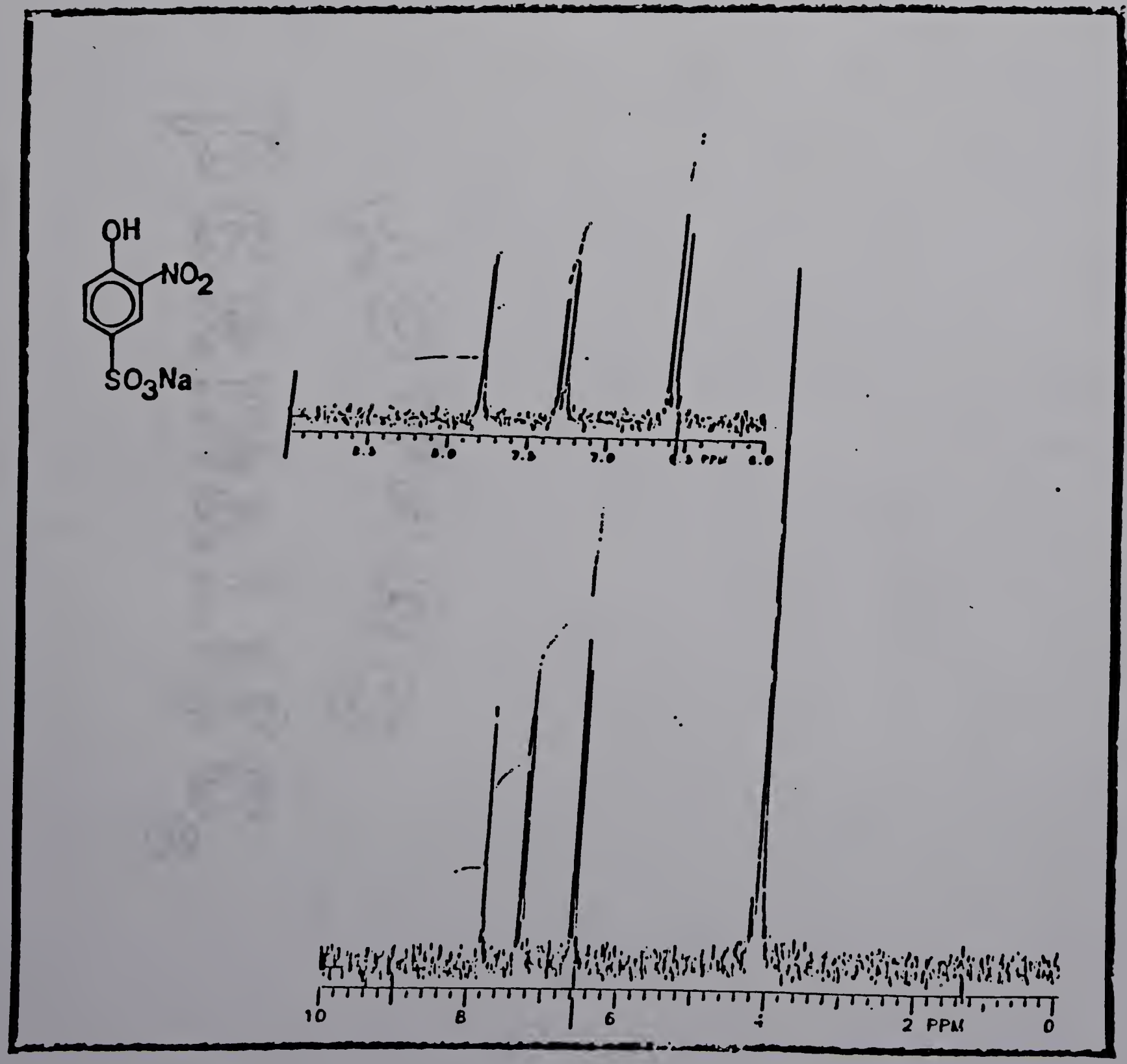
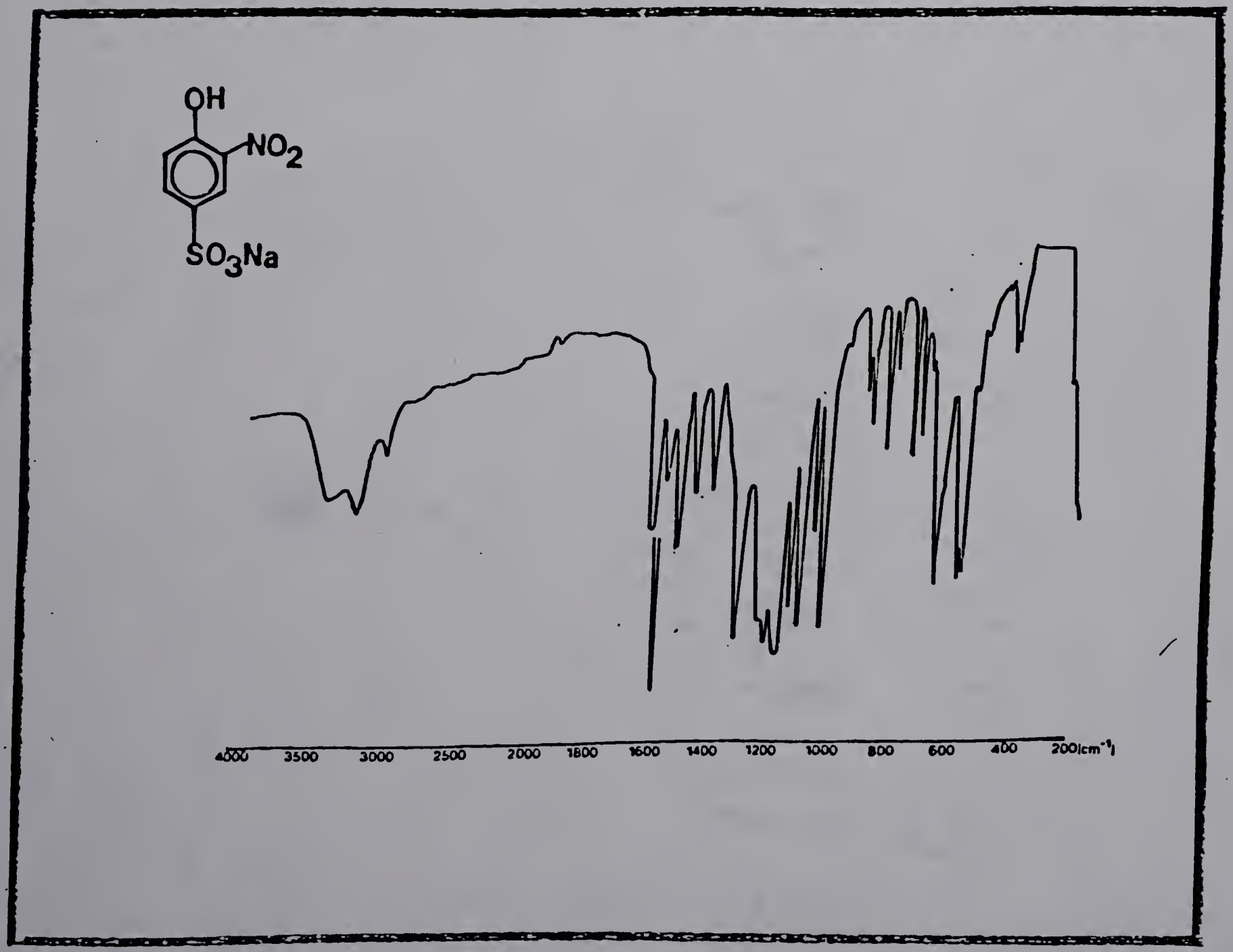

OH
NO2
SO3Na
10
8
6
4
2 PPM
0

Figure A-24. IR (KBr) of sodium 1-hydroxy-2-nitro-4-benzenesulfonate (**42**).


OH
NO2
SO3Na
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
200(cm⁻¹)

Appendix B.

Calculation of the Number of Molecules of Methoprene>Spacer Conjugated per Molecule of Protein Using ^{14}C-Methoprene as a Tracer

354.4 g/mol = Molecular weight methoprene>spacer

69,000 g/mol = Molecular weight human serum albumin

x = counts min^{-1} / mg protein conjugate

y = counts min^{-1} / mg methoprene>spacer

$$\frac{x}{y} \cdot 100 = \%\ \text{methoprene>spacer on 1 mg of protein conjugate}$$

$$\frac{x}{y} = \text{ug methoprene>spacer on 1 mg protein conjugate}$$

$$1 - \frac{x}{y} = \text{ug protein conjugate}$$

Ratio of methoprene>spacer : protein

$$= \frac{\frac{x}{y}}{1 - \frac{x}{y}}$$

$$= \frac{\text{ug methoprene>spacer}}{\text{ug protein conjugate}}$$

$$= \frac{\text{umol methoprene>spacer}}{\text{umol protein conjugate}}$$

Sample Calculation for Trial 1, preparation of the methoprene immunogen via the 1-hydroxy-2-nitro-4-benzenesulfonate (HNSA) activated ester:

x = 2020 counts min^{-1} per mg protein conjugate

y = 14,744 counts min^{-1} per mg methoprene>spacer

$\frac{x}{y} \cdot 100 = 13.7\ \%$, or 137 ug methoprene>spacer on 1 mg protein conjugate

Ratio of methoprene>spacer : protein conjugate

$$= \frac{137\ ug}{1\ mg - 137\ ug}$$

$$= \frac{137\ ug\ methoprene>spacer}{863\ ug\ portein\ conjugate}$$

$$= \frac{0.3864\ umol\ methoprene>spacer}{0.0125\ umol\ protein\ conjugate}$$

$$= \frac{31\ molecules\ methoprene>spacer}{1\ molecule\ protein\ conjugate}$$

Appendix C.

Calculation of the Number of Molecules of Methoprene>Spacer Conjugated per Molecule of Protein Using Spectrophotometry

See Figures 15 and 17, pages 76 and 84 , for chemical equations.

1. Calculation of the percentage HNSA-ester (44).

$A_{406}NaOH$ = Absorbance of the above sample after complete hydolysis with 5N NaOH. This reading records the total amount of free dianion.

$$\% \text{ HNSA-ester} = \frac{A_{406}NaOH - A_{406}}{A_{406}NaOH} \times 100 \qquad \text{Eq. 1}$$

From Table 2, page 77, 140 ug sample of crude HNSA-ester (44).

$$\% \text{ HNSA-ester} = \frac{0.714 - 0.500}{0.714} \times 100$$

$$= 29.97 \%$$

2. Calculation of the number of molecules of methoprene>spacer per molecule of human serum albumin (HSA).

354.4 g/mol = Molecular weight (MW) of methoprene>spacer (39)

577.7 g/mol = Molecular weight of methoprene>spacer-1-hydroxy-2-nitro-4-benzenesulfonate ester (HNSA-ester) (44)

218.2 g/mol = Molecular weight of 1-hydoxy-2-nitro-4-benzenesulfonate (46)

69,000 g/mol = Molecular weight of human serum albumin (HSA)

For 140 ug of crude methoprene>spacer-HNSA (HNSA-ester),

$A_{406} = 0.500$

$A_{406}NaOH = 0.714$

To find the ratio, R, of HNSA-ester to free dianion in 140 ug of crude ester:

Assumptions: 1. Crude methoprene>spacer-HNSA contains m moles dianion + n moles HNSA-ester.

2. A_{406} ' moles free dianion.

3. $A_{406}NaOH$ ' moles total dianion

$$\frac{A_{406}}{A_{406}NaOH} = \frac{m}{n + m} = R \qquad \text{Eq. 2}$$

$$= \frac{0.500}{0.714} = 0.700$$

Solving Eq. 2 for m,

$$m = n \frac{(R)}{1 - R}$$

$$= n \frac{R}{1 - R} = n \frac{0.700}{1 - 0.700} = 2.33\ n$$

Solving Eq. 2 for n,

$$n = m \frac{(1 - R)}{R}$$

$$= m \frac{1 - R}{R} = m \frac{1 - 0.700}{0.700} = 0.429\ m$$

To find n, mol ester in crude HNSA-ester:

$$weight_{crude\ ester} = (MW_{dianion} \cdot m) + (MW_{ester} \cdot n) \qquad \text{Eq.3}$$

Substituting for m and solving for n,

$$n = \frac{weight_{crude\ ester}}{MW_{dianion} \frac{R}{1 - R} + MW_{ester}}$$

$$= \frac{140 \times 10^{-6}\ g}{218.2\ g/mol(2.3) + 577.7\ g/mol}$$

$$= 0.129 \times 10^{-6}\ mol\ HNSA\text{-}ester$$

To find m, mol dianion in crude HNSA-ester:

Substituting for n, and solving for m,

$$m = \frac{weight_{crude\ ester}}{MW_{dianion} + MW_{ester} \frac{1 - R}{R}}$$

$$= \frac{140 \times 10^{-6}\ g}{218.2\ g/mol + 577.7\ g/mol(0.429)}$$

$$= 0.290 \times 10^{-6} \text{ mol dianion}$$

It can further be assumed that from the reaction between the HNSA-ester and human serum albumin (Figure 17),

moles of dianion liberated = moles ester reacted

At $t = 0$, $A_t = A_0$; $\text{mol}_{\text{ester rxd}} = 0$, so $t = t_{\text{rxn end}}$, therefore:

$$A_{end} >> A_0$$

and

$$\text{mol}_{\text{ester rxd}} >> \text{mol}_{\text{dianion start}}$$

$$A_t \text{ ` } \text{mol}_{\text{dianion start}} + \text{mol}_{\text{dianion liberated}}$$

$$\text{ ` } \text{mol}_{\text{dianion start}} + \text{mol}_{\text{ester rxd}}$$

$$A_0 \text{ ` } \text{mol}_{\text{dianion start}}$$

Therefore

$$\frac{A_t}{A_0} = \frac{\text{mol}_{\text{dianion start}} + \text{mol}_{\text{ester rxd}}}{\text{mol}_{\text{dianion start}}}$$

$$= 1 + \frac{\text{mol}_{\text{ester rxd}}}{\text{mol}_{\text{dianion start}}}$$

$$\text{mol}_{\text{ester rxd}} = \text{mol}_{\text{dianion start}}\left(\frac{A_t}{A_0} - 1\right) \qquad \text{Eq.4}$$

To determine the amount of dianion at the start of each reaction,

140×10^{-6} g = 0.290 umol dianion, 65.5×10^{-6} g
and 0.129 umol ester, 74.5×10^{-6} g

For the 15 min reaction, 10.52 mg of the crude ester was used:

$$\frac{10.52 \text{ mg}}{0.140 \text{ mg}}(0.290 \text{ umol}_{\text{dianion start}}) = 21.8 \text{ umol}_{\text{dianion star}}$$

From Table 5,

$$\text{umol}_{\text{ester rxd}} = 21.8 \text{ umol}_{\text{dianion start}}\left(\frac{0.837}{0.750} - 1\right)$$

$$= 2.53 \text{ umol}_{\text{ester rxd}}$$

To determine the number of molecules of methoprene>spacer per molecule protein (from Table 5),

$$= \frac{\text{umol}_{\text{ester rxd}}}{\text{umol protein}}$$

$$= \frac{2.53\ \text{umol}_{\text{ester rxd}}}{0.072\ \text{umol}_{\text{protein}}}$$

$$= \frac{34\ \text{molecules methoprene>spacer}}{1\quad \text{molecule protein}}$$

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