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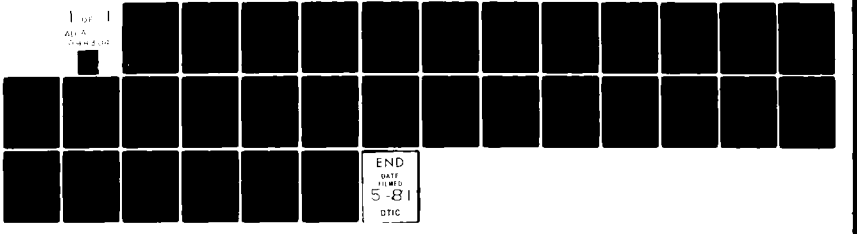

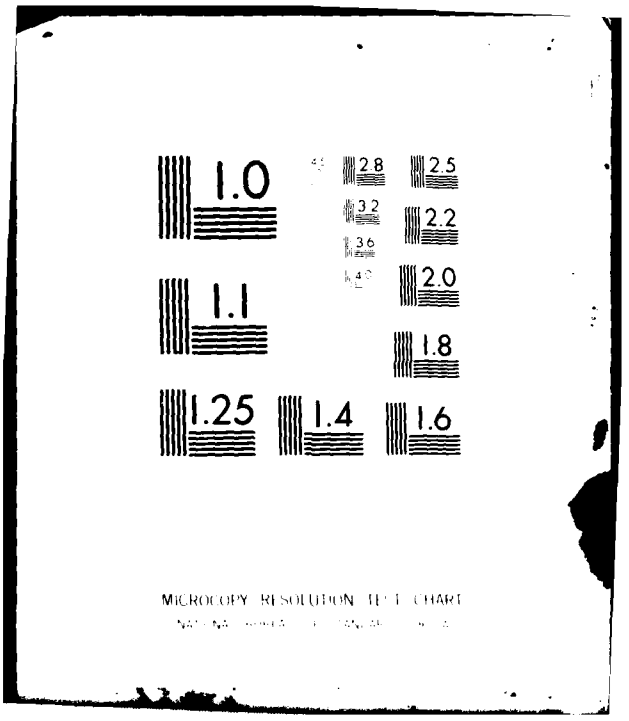
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OFFICE OF NAVAL RESEARCH

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TECHNICAL REPORT NO. 1

# The Synthesis and Evaluation of 15-Keto-$PGI_{B1}$ Analogues.

by

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The ethyl analogue, 3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone, was prepared as a structurally more suitable model for the investigation of the base promoted conversion of 15-keto-PGB1 to an oligomeric mixture termed PGBx. Base treatment of the ethyl analogue results in the conversion to an oligomeric mixture capable of protecting against the loss of oxidative phosphorylation in isolated mitochondria without inhibition at higher concentrations as observed for PGBx. The chemistry of the oligomerization was investigated and has major implications for an understanding of the complexity of the oligomeric mixture PGBx.

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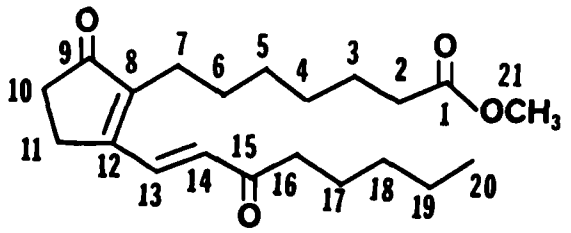
## INTRODUCTION

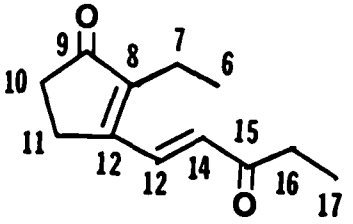
In its current usage, the term $PG_{Bx}$ refers to an oligomeric mixture derived from treatment of 15-dehydro-prostaglandin $B_1$ methyl ester (15-dehydro-$PG_{B1}$, 1) with ethanolic potassium hydroxide.[1] In vitro, $PG_{Bx}$ restores oxidative phosphorylation in degenerated isolated rat liver mitochondria[2] and stimulates the release of $Ca^{+2}$ from sarcoplasmic reticulum and heart mitochondria.[3] In vivo, $PG_{Bx}$ facilitates and significantly increases survival after what otherwise would be lethal episodes of myocardial ischemia in monkeys[4] and restores central nervous system function in dogs after otherwise fatal hypoxia.[5] $PG_{Bx}$ has also been reported to normalize high blood glucose, obesity, and excess appetite of the diabetic mouse.[6,7]

The details of the synthesis and purification of $PG_{Bx}$[8] as well as physical, chemical, and spectral data for $PG_{Bx}$[1] have been reported. $PG_{Bx}$ has been characterized in a general manner as a complex mixture of closely related oligomers formed by an initial reaction at the 13,14-double bond of 15-dehydro-$PG_{B1}$ with retention of the overall prostaglandin skeleton.[1] Due to the inability to resolve the complex mixture into individual components, a more detailed structural assignment was not possible.

Many of the problems associated with the direct structural elucidation of the complex oligomeric mixture termed $PG_{Bx}$ can be attributed to the inherent complexity of oligomers in which the oligomeric unit is a 20 carbon prostaglandin. As an alternative approach, a program was undertaken to define the chemical pathway of oligomerization for structurally simpler analogues of 15-dehydro-$PG_{B1}$ such as 3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone (2) hereafter referred to as the "ethyl analogue." Even though the ethyl analogue contains substantially fewer carbons than 15-dehydro-$PG_{B1}$, the essential conjugated cyclopentenone functionality of 1 is retained in the ethyl analogue leading to the expectation of similar oligomerization pathways.




1 15-Dehydro-$PG_{B1}$ Methyl Ester




2 Ethyl Analogue

In the initial investigation of the base promoted oligomerization of the ethyl analogue, spectroscopic studies indicated that the oligomerization proceeded in a manner similar to that of 15-dehydro-$PG_{B1}$.[9] Somewhat unexpectedly, the bicarbonate soluble fraction of the crude oligomeric mixture was found to exhibit activity in the protection of oxidative phosphorylation.[9] Activity was observed throughout fractions derived from size exclusion chromatography but was concentrated in several of the fractions.[9]

The primary objective of the present program (ONR Contract N00014-80-C-0117) is directed to the investigation of the base promoted oligomerization of the ethyl analogue as a potential new precursor to an oligomeric mixture exhibiting "$PG_{Bx}$" type activity and as a model structurally more suitable than 15-dehydro-$PG_{B1}$ for determination of the chemical pathway involved in oligomerization.

## RESULTS AND DISCUSSION

### I. The Synthesis of 3-(trans-3-Keto-1-Pentenyl)-2-Ethyl-2-Cyclopentenone (2, Ethyl Analogue).

The synthesis of the ethyl analogue was carried out using a route previously developed in our laboratories[9] and is outlined in Figure 1. Intermediates and the final product were purified by preparative liquid chromatography and exhibited satisfactory spectral and analytical data. Like 15-dehydro-$PG_{B1}$, the ethyl analogue degrades slowly upon standing at room temperature but is stable for long periods when stored under nitrogen at -15°C.

### II. The Base Promoted Oligomerization of the Ethyl Analogue.

#### 1. Initial Oligomerization Carried Out Over a Seven Day Period.

The initial oligomerization of the ethyl analogue was carried out over a seven day period to conform to the original experimental conditions under which mitochondrial activity was first observed for the bicarbonate soluble oligomer fraction.[9] It was anticipated that the oligomerization conditions were unnecessarily harsh and would lead to a very complicated oligomeric product and, as such, would represent only a starting point.

The oligomerization was carried out[10] by the addition of 2.5 grams of the ethyl analogue in 50 ml of ethanol to 50 ml of 2 N KOH. The reactants, contained in a lightly stoppered flask, were stirred for 4 hours at 80° and then allowed to stand at room temperature for 7 days. At that time, the reaction mixture was diluted with water, acidified to pH 3 with dilute HCl, and extracted with isobutanol (due to the serious emulsions resulting from the use of isobutanol, ethyl acetate was used for extraction in all subsequent oligomerizations). The combined isobutanol extracts were extracted with 0.1 M $NaHCO_3$ and the combined bicarbonate extracts were acidified to pH 3 and extracted with isobutanol. The combined isobutanol extracts were dried and the solvent was removed under vacuum to give an ca. 65 percent yield of crude bicarbonate soluble oligomer. The original isobutanol extracts were washed with water, dried, and after removal of the isobutanol under vacuum gave ca. 35 percent of a crude bicarbonate insoluble oligomer. Both the crude bicarbonate soluble and insoluble oligomer fractions were further fractionated by size exclusion chromatography over Sephadex LH-20.[10] As also observed for $PG_{Bx}$,[1] distinct separations were not accomplished and fractions were somewhat arbitrarily selected.[10]

Both the bicarbonate soluble and insoluble oligomer fractions from Sephadex LH-20 chromatography were submitted to Dr. Thomas Devlin of Hahnemann Medical

College for evaluation of mitochondrial and ionophoretic activity. The results of the two assays can be summarized as follows:[10,11]

Evaluation of Mitochondrial Activity

1. Activity in protection of oxidative phosphorylation was observed throughout both bicarbonate soluble and insoluble oligomer fractions but was generally higher for bicarbonate soluble fractions.

2. Activities for ethyl analogue oligomer fractions were generally lower than for "standard $PG_{Bx}$" (e.g. highest activity for two of the bicarbonate soluble fractions was ca. 50 percent that of "standard $PG_{Bx}$" when expressed as weight of sample required for 50 percent protection.

3. No inhibitory activity was observed for the bicarbonate soluble ethyl analogue oligomer fractions even at concentrations much higher than those resulting in almost total inhibition by "standard $PG_{Bx}$."

4. Because of the lack of inhibition, an even greater percent protection than that afforded by "standard $PG_{Bx}$" was observed for several of the bicarbonate soluble oligomer fractions at higher concentrations.

5. The activity observed for the bicarbonate insoluble fractions appears to represent the first observation of protection of oxidative phosphorylation by a neutral oligomeric material.

Evaluation of Ionophoretic Activity

1. Varying degrees of ionophoretic activity were observed throughout the bicarbonate soluble and insoluble oligomer fractions. Although the activities were generally lower than that of "standard $PG_{Bx}$," several bicarbonate soluble fractions exhibited activities higher than "standard $PG_{Bx}$."

2. The ionophoretic activity appeared to generally correlate with oligomer size but a direct relationship between ionophoretic activity and mitochondrial activity was not evident from the data.

Activities for the protection of oxidative phosphorylation of the bicarbonate soluble fractions were also determined by Dr. H. Shmukler of the NADC Laboratories. At oligomer concentrations for which "standard $PG_{Bx}$" exhibits maximum protection, the bicarbonate soluble oligomer fractions derived from the ethyl analogue exhibited activities that followed the same pattern as those determined by Dr. Devlin but were generally lower [10,12]

Although both bicarbonate soluble and insoluble oligomer fractions derived from the ethyl analogue exhibited "$PG_{Bx}$" like activities in the *in vitro* oxidative phosphorylation and ionophoretic assays, the oligomeric fractions were clearly very complicated

mixtures. This was not unexpected because of the 7 day oligomerization period employed. Rather than continue the investigation of such complicated mixtures, attention was focused on the chemistry involved in the formation of oligomers from the ethyl analogue in an effort to develop more favorable oligomerization conditions.

2. Chemistry of the Ethyl Analogue Oligomerization.

With indications in the initial oligomerization of potential for the ethyl analogue as a precursor to an oligomeric mixture with "$PG_{Bx}$" like *in vitro* activity, a more detailed investigation of the oligomerization chemistry was undertaken in an effort to maximize *in vitro* activity while reducing the severity of oligomerization conditions. Since higher activities had been observed for the bicarbonate soluble oligomer fraction, special attention was directed to maximizing the conversion to a bicarbonate soluble oligomer. More specifically, the second phase of our investigation of the oligomerization of the ethyl analogue was directed to:

1. Optimizing the conversion to bicarbonate soluble oligomer while reducing the severity of the oligomerization conditions.

2. Defining the particular functionality responsible for inducing bicarbonate solubility.

3. Determining the chemical pathway of oligomer formation.

A. Optimization of the Bicarbonate Soluble Fraction.

An extended series of reaction conditions were explored[10] in an effort to maximize the conversion to a bicarbonate soluble oligomer while reducing the severity of the oligomerization conditions. The critical observation derived from this series of reactions was the previously unsuspected role of oxygen in the conversion to a bicarbonate soluble oligomer. With an understanding of the oxygen requirement, the simple expedient of carrying out the oligomerization open to the atmosphere with efficient stirring resulted in the conversion to oligomeric mixtures which were greater than 80 percent bicarbonate soluble in periods as short as 3 - 5 hours at 50°. The results for the *in vitro* evaluation of protection of oxidative phosphorylation for a typical oligomerization carried out at 50° for 5 hours are given in Table 1. The activities (as a percent of "standard $PG_{Bx}$" activity) are uniformly higher than those of the 7 day oligomerization while the percent conversion to a bicarbonate soluble oligomer has been significantly improved. No inhibition was observed even at 120 ug, a concentration in which almost total inhibition is observed for "standard $PG_{Bx}$."

With rigorous exclusion of oxygen during the oligomerization, the oligomer obtained was typically less than 5 percent bicarbonate soluble reinforcing the essential role of oxygen in formation of bicarbonate soluble oligomer. For such bicarbonate insoluble oligomers, some *in vitro* protection of oxidative phosphorylation was observed throughout all fractions derived from size exclusion chromatography (Table 2) but at a substantially lower level than observed for bicarbonate soluble oligomer fractions (Table 1). As before, no inhibition was observed at oligomer concentrations for which virtually complete inhibition was observed for "standard $PG_{Bx}$."

In summary, the ethyl analogue can be converted to an oligomeric mixture that is greater than 80 percent bicarbonate soluble by treatment with 1 N KOH at 50° at sharply reduced reaction times (e.g. 3 - 5 hours) if carried out open to the atmosphere with efficient stirring. Although lower than $PG_{Bx}$, in vitro activity in protection of oxidative phosphorylation is present in all fractions but is substantially higher in several. Because of the absence of inhibition, an even greater protection of oxidative phosphorylation than obtainable for "standard $PG_{Bx}$" can be obtained at higher oligomer concentrations.

B. Determination of Functionality Responsible for Imparting Bicarbonate Solubility.

Oligomerization of 15-dehydro-$PG_{B1}$ methyl ester by treatment with 1 N KOH would also result in the hydrolysis of the ester functionality resulting in bicarbonate solubility. However, the oligomerization of the ethyl analogue to a bicarbonate soluble oligomer was unexpected since the ethyl analogue contains no functionality that would impart bicarbonate solubility upon simple oligomerization through the 13,14-double bond. In an effort to further define the chemical changes involved in the formation of a bicarbonate soluble oligomer from the ethyl analogue, the functionality responsible for bicarbonate solubility, i.e. acid functionality, was characterized by a combined chemical and spectroscopic approach.

Solubility in dilute bicarbonate solution indicates a functionality somewhat more acidic than carbonic acid (pK = 6.37). The pK values for the various bicarbonate soluble fractions from the 7 day oligomerization are in the same range as $PG_{B1}$[10] but 2-alkyl-1,3-cyclopentanediones such as 2-ethyl-1,3-cyclopentanedione also fall in this pK range.[10]

The presence of a carboxyl ($-CO_2H$) functionality is most clearly supported by an examination of the infrared (IR) spectra of both bicarbonate soluble and insoluble oligomer fractions obtained from oligomerizations carried out in the presence and absence of oxygen respectively. In the bicarbonate soluble oligomer, the presence of both carboxyl ($-CO_2H$) and hydroxyl (-OH) functionalities is evident from examination of the 3300 $cm^{-1}$ (O-H) region of the IR spectrum (Figure 2). By contrast, the bicarbonate insoluble oligomer obtained under oxygen-free conditions exhibits only the hydroxyl O-H absorption as indicated in the 3300 $cm^{-1}$ region. This interpretation can be most clearly demonstrated by a comparison of the IR spectra of 13,14-dehydro-$PG_{B1}$, which contains the $-CO_2H$ and -OH functionalities, and 13,14-dehydro-$PG_{B1}$ methyl ester in which only the -OH functionality is present in the 3300 $cm^{-1}$ region (Figure 3).

This interpretation is further substantiated by treatment of the bicarbonate soluble oligomer with diazomethane in an effort to convert the $-CO_2H$ functionality to the corresponding methyl ester. After treatment, the resultant product is bicarbonate insoluble indicating reaction at the functionality responsible for bicarbonate solubility. That the reacting functionality was the $-CO_2H$ is supported by the absence of the $-CO_2H$ absorption in the IR of the methylated product. Further support is evident from the $^1H$ NMR spectrum of the methylated product which exhibits an $CO_2\underline{CH_3}$ methyl ester absorption at 3.70 $\delta$ which compares favorably with a value of 3.67 $\delta$ for the ester

methyl of 15-dehydro-$PG_{B1}$. The $^{13}C$ NMR spectrum of the methylated product exhibits a cluster of absorptions centered at 51.8 ppm which compares favorably with the ester methyl carbon in 15-dehydro-$PG_{B1}$ methyl ester which appears at 51.4 ppm.

Taken together, the reaction with diazomethane and the IR and NMR spectral data provide virtually conclusive evidence for the $-CO_2H$ group as the functionality responsible for imparting bicarbonate solubility.

C. A Potential Oligomerization Pathway for the Ethyl Analogue.

When the progress of the oligomerization of the ethyl analogue, either with the exposure to or exclusion of oxygen, is monitored by UV spectroscopy, an immediate loss of the 296 nm absorption characteristic of the fully conjugated enone system is observed along with the simultaneous appearance of a new absorption at 238 nm. The 238 nm absorption is characteristic of the fully substituted cyclopentenone chromophore which would result from the loss of the 13,14-unsaturation. After this point, a striking difference becomes evident in the course of the oxygen-exposed and oxygen-free oligomerizations. In the oxygen-free oligomerization, the 238 nm absorption remains quite sharp and decreases only slightly in intensity during the course of a typical 5 hour oligomerization. By contrast, in oligomerizations carried out open to the atmosphere, the initially formed 238 nm absorption continues to decrease over the course of the 5 hour oligomerization to give a broad tailing absorption with $\lambda$ max still at 238 nm which is characteristic of the bicarbonate soluble oligomeric product (Figure 4).

As noted in the previous discussion, the bicarbonate soluble oligomer exhibits both -OH and $-CO_2H$ absorptions in the IR spectrum while the bicarbonate insoluble oligomer obtained under oxygen-free conditions exhibits only the -OH absorption in the IR. If the oligomerization reaction is quenched immediately, the bicarbonate insoluble oligomer obtained does not exhibit an -OH absorption in the IR. If the bicarbonate insoluble oligomer obtained under oxygen-free conditions is resubjected to the original oligomerization conditions but with exposure to the atmosphere (oxygen), it is converted in good yield into a bicarbonate soluble oligomer exhibiting both the -OH and $-CO_2H$ functionalities in the IR.

The oligomer mixture obtained from immediately quenching the oligomerization reaction can be resolved into distinct peaks representing dimers, trimers, etc., by size exclusion chromatography on Sephadex LH-20. Both the bicarbonate soluble and bicarbonate insoluble oligomer products obtained from 5 hour oligomerizations in the presence and absence of oxygen respectively, give the broad unresolved band characteristic of $PG_{Bx}$ when subjected to size exclusion chromatography on Sephadex LH-20.

Several aspects of the chemistry involved in the oligomerization of the ethyl analog suggest an oligomerization pathway involving several distinct stages. As indicated in the UV studies, the initial chain forming reaction, involving reaction at the 13,14-double bond, takes place very rapidly. The 238 nm absorption which is formed is characteristic of a fully substituted cyclopentenone chromophore which would be formed upon an addition reaction at the 13,14-double bond. A possible second step occurring over a longer period of time following the initial reaction at the 13,14-double bond could involve aldol condensations leading to more complicated mixtures as evident

in size exclusion chromatography and which would exhibit an -OH absorption in the IR. The bicarbonate insoluble mixture can be converted into a bicarbonate soluble oligomer by resubjection to reaction conditions in the presence of oxygen providing some additional support for the suggested second step. The third reaction which results in the formation of a $-CO_2H$ functionality leading to bicarbonate solubility takes place over a longer period of time as evident from the gradual decrease of the 238 nm absorption to give the broad trailing absorption characteristic of the bicarbonate soluble oligomer. The IR spectrum of this material which indicates the presence of the $-CO_2H$ and -OH functionalities reflects the major structural change occurring in this stage of the oligomerization.

Of the three steps suggested above for the oligomerization of the ethyl analogue to a bicarbonate soluble oligomer, the first and third steps have stronger experimental support while the second step involving a possible aldol condensation is based on somewhat more tenuous evidence. Having defined the oligomerization as taking place in several distinct stages, the investigation was then directed to a more complete understanding of each stage.

i. Step 1 - The Initial Chain Forming Oligomerization Step.

Under typical $PG_{Bx}$ oligomerization conditions involving treatment of the ethyl analogue with 1 N KOH, the 296 nm UV absorption characteristic of the fully conjugated enone system immediately disappears and is replaced by a new absorption at 238 nm. The 238 nm absorption is characteristic of the fully substituted cyclopentenone system which would be formed by the loss of the 13,14-double bond of the ethyl analogue. In earlier work, it had been established that the "13,14-"unsaturation of the $PG_B$ series and related analogues was susceptible to nucleophilic addition of the Michael type.[13-16] In a Michael addition, the function of the base (in the present case, KOH) is catalytic in nature so that the rate of the reaction can be decreased by the simple expedient of decreasing the concentration of ethanolic KOH. In practice, similar oligomeric mixtures containing some unreacted ethyl analogue and substantial amounts of a dimeric component could be obtained either by reaction with 1 N KOH followed by an immediate acid quench or by reaction with 0.05 N KOH with reaction times of 1 - 2 minutes before termination of the oligomerization with an acid quench.

a. Lower Molecular Weight Oligomeric Mixtures

The lower molecular weight oligomeric mixtures obtained in this manner are bicarbonate insoluble and exhibit the general spectral features associated with $PG_{Bx}$. It was determined by field desorption mass spectrometry that dimer through octomer oligomers were present in the mixture.[17] The lower molecular weight mixture could be resolved into discrete fractions representing dimers, trimers, etc., by size exclusion chromatography on Sephadex LH-20 as illustrated in Figure 5. After

separation, each peak was independently analyzed by field desorption mass spectrometry[17] for determination of oligomer weight, i.e. dimer, trimer, tetramer... content, as indicated in Figure 5. Preparative separations of the individual oligomer fractions were accomplished using two 2.5 x 90 cm Sephadex LH-20 columns in series.

b. Structural Elucidation of the Dimeric Compounds.

As indicated above, separation of the dimer fraction from the lower molecular weight oligomeric mixture could be accomplished by size exclusion chromatography. Analysis of the dimer fraction using a DuPont Zorbax Sil 4.66 mm i.d. x 25 cm column indicated 7 major dimeric components referred to in the following discussion as Dimers 1 - 7 (Figure 6). The isolation of the 7 individual dimeric components was accomplished by chromatography using a 10 mm i.d. x 25 cm Altex LiChrosorb Si 60 column. Confirmation that each of the isolated components was truly a dimer was provided by high resolution mass spectrometric measurement[18] of the molecular ion indicating a molecular formula of $C_{24}H_{32}O_4$ ($C_{12}H_{16}O_2$ x 2) for each component. Structural elucidation of the individual components was accomplished by a combination of UV, IR, MS, and $^{13}C$ and 360 MHz $^{1}H$ NMR analysis. The NMR assignments were facilitated by the previous establishment of unequivocal $^{13}C$ and $^{1}H$ assignments for the ethyl analogue and the 13,14-saturated derivative, which serve as model components of the dimers, by sequential analysis of a series of increasingly complex model compounds.[9,19] Also the deuterium exchange experiments carried out in the course of the NMR study served to define the nucleophilic site on the cyclopentenone ring.

Analysis of the UV data indicated two distinct type of dimers. Dimers 1 - 4 (Figure 7) exhibited UV maxima at 296 and 238 nm, characteristic of the fully conjugated and 13,14-saturated systems, while Dimers 5 - 7 exhibited only a 238 nm absorption characteristic of the fully substituted cyclopentenone system. This interpretation was further supported from analysis of the IR spectral data. Dimers 1 - 4 have conjugated C=C absorptions at 1585 and 1640 $cm^{-1}$ which are characteristic of the fully conjugated and 13,14-saturated ethyl analogue systems respectively. Dimers 5 - 7 exhibit only the conjugated C=C absorption at 1640 $cm^{-1}$, characteristic of the 13,14-saturated ethyl analog, and a new carbonyl absorption at 1750 $cm^{-1}$ which is characteristic of the fully saturated cyclopentanone system. This information, taken together with a detailed analysis of the 360 $^{1}H$ and $^{13}C$ NMR spectral data, provide the basis for the

structural assignments for Dimers 1 - 7 given in Figure 7. Dimers 1 - 4 as well as Dimers 5 - 7 result from a Michael addition of the first ethyl analogue unit to the 13,14-unsaturation of the second analogue unit.

c. Chemical Pathway for Dimer Formation.

The key to an understanding of the chemistry of dimer formation is the recognition that in the Michael addition reaction the ethyl analogue possesses two active nucleophilic sites (C-10 and C-16) as well as two active nucleophilic acceptor sites (C-13 and C-14). Since each addition results in the formation of a C—C bond with the creation of two new chiral centers, the formation of closely related stereoisomers becomes a major factor. Dimers 1 and 2, a diastereomeric pair, result from the addition of the C-10 nucleophile of one ethyl analogue unit to C-14 of the 13,14-double bond of a second ethyl analogue unit (Figure 8). Dimers 3 and 4, a second diastereomeric pair, are derived from the addition of the C-10 nucleophile of the first analogue unit to C-13 of the 13,14-double bond of the second analogue unit. Dimers 1 - 4 are observed in ca. equal amounts and each retains a 13,14-unsaturation that is capable of acting as the receptor in the nucleophilic addition of a 3rd analogue unit by Michael addition.

Dimers 5 - 7 are formed by a double Michael addition across the 13,14-unsaturation that is initiated by the addition of the C-16 nucleophilic center (Figure 9). Initial addition of the C-16 nucleophile of the first analogue unit to C-13 of the 13,14-double bond of the second unit leads to a double addition and the formation of cyclopentanones of the type labeled C. The initial addition of the C-16 nucleophile to C-14 of the 13,14-unsaturation of the second analogue unit results in the formation of cyclopentanones of the type D. In both adducts C and D, 4 chiral centers have been created with the resultant possibility of 8 diastereomers each for adducts C and D. However, since all 4 chiral centers in both cyclopentanones C and D are capable of epimerization under the reaction conditions, it is likely that only the most stable diastereomers of the C and D type would predominate. It is clear that Dimers 5 - 7 are adducts of the C or D type but a more complete assignment of stereochemistry is in progress. In the case of Dimer 7, a crystal structure determination is being made. Dimers 5 - 7 no longer retain a 13,14-unsaturation because of formation by a double Michael addition and would be unable to form trimers by acting as the receptor for the nucleophilic Michael addition of the 3rd analogue unit as is the case of Dimers 1 - 4. However, Dimers 5 - 7 could produce trimers by serving as the nucleophile in a Michael addition to a 3rd analogue unit. Trimers

formed in this manner would be distinguishable by lack of residual 13,14-unsaturation. Since Dimers 5 - 7 each possess three potential nucleophilic sites, trimer formation by this route could rapidly lead to very complex mixtures of closely related structural isomers and stereoisomers.

D. Implications of the Ethyl Analogue Oligomerization Studies for the $PG_{Bx}$ Structural Problem.

At present the most definitive characterization of $PG_{Bx}$ is a complex mixture of closely related oligomers resulting from initial reaction at the 13,14-double bond.[1] Isolation of individual components of the $PG_{Bx}$ mixture has resisted the combined research efforts of several research groups for a number of years without a clear chemical explanation for the separation difficulties encountered in this seemingly intransigent oligomeric mixture. An extension of the chemistry observed in the formation of low molecular weight oligomeric mixtures containing isolable dimers from the ethyl analogue provides a firm chemically based explanation, as well as expectation, of the difficulties inherent in separation of the $PG_{Bx}$ oligomeric mixture. The chemistry observed for the ethyl analogue, because of its close structural relationship to 15-dehydro-$PG_{B1}$, should provide an excellent model for the chemistry involved in $PG_{Bx}$ oligomerization. The key element in such an extension is the recognition of Michael addition as the primary chemical pathway for oligomer formation and that two nucleophilic and two acceptor sites are operational.

The complexity of an oligomeric mixture derived from the ethyl analogue by Michael addition can be most readily illustrated if several simplifying assumptions are made:

1. The oligomer chain grows by a sequential Michael addition of ethyl analogue (EA) units as nucleophiles to the residual 13,14-unsaturation of the oligomer chain, i.e. EA + EA → Dimer + EA → Trimer + EA → Tetramer + EA → etc.

2. Only the C-10 nucleophilic site is active in the chain forming process leading to an oligomer which always retains a residual 13,14-unsaturation for continued growth by nucleophilic addition of subsequent analogue units. Oligomer formation resulting from nucleophilic addition of C-16 is not considered since it is assumed, as in dimer formation, that "double addition" would result leaving no residual 13,14-unsaturation for subsequent nucleophilic addition of ethyl analogue units in the manner described in assumption 1.

3. In the addition of subsequent ethyl analogue units, both C-13 and C-14 of the 13,14-unsaturation would act as active receptor sites with equal facility for addition of the C-10 analogue nucleophile as observed in dimer formation.

With such assumptions, which are simplifying assumptions, the number of closely related structural isomers and stereoisomers would increase very rapidly with each

subsequent analogue unit added to the growing chain. From the ethyl analogue monomer 4 "active" dimers are formed (Dimers 5 - 7 are regarded as "inactive" for chain growth, cf. assumption 2 above) of the types 1 - 4 (Figure 8). Each of the 4 dimers would then add an additional analogue unit through the C-10 enolate (cf. to assumption 2 above) by addition at both the C-13 and C-14 carbons of the 13,14-double bond (cf. 3 above) resulting in the formation of 4 trimers (2 pairs of diastereomers) for a total of 16 predicted trimers (8 diastereomeric pairs). Each of the 16 trimers would then add an additional analogue unit in the manner just described to give 4 tetramers or a total of 64 tetramers from the 16 trimers. In summary, 4 dimers → 16 trimers → 64 tetramers → 256 Pentamers → 1024 hexamers → etc. Even at the tetramer stage, "a complex mixture of closely related oligomers," i.e. 32 pairs of diastereomers, that would be virtually inseparable because of the similarity in physical properties, would be predicted. Even though it is unlikely that all the oligomeric components would be formed in equal amounts, the difficulties of completely separating an oligomeric mixture of even one half or 32 of the predicted tetramers having such similar properties would be virtually insurmountable.

It should also be re-emphasized that the oligomerization sequence outlined above represents, in a sense, the "best possible case." This follows since Dimers 5 - 7 were not considered as precursors active in continued chain growth even though each of the dimers possesses three different nucleophilic sites which are potentially capable of further nucleophilic addition in Michael fashion to the 13,14-unsaturation of a third ethyl analogue unit to form trimers. The total exclusion of the C-16 nucleophilic attack from all phases of oligomer chain formation also represents a major and an experimentally unsubstantiated assumption. An oligomeric product derived from 15-dehydro-$PG_{B1}$ methyl ester affords an additional magnitude of complication since the slightly incomplete hydrolysis of the ester functionality, each of which is different in a given oligomer, would give rise to a myriad of new and slightly different compounds that would be virtually inseparable. The worst possible complication in the oligomerization of 15-dehydro-$PG_{B1}$ would result if the already complicated oligomerization pathway described above proceeding through Michael type addition actually represents only the first chemical stage in the formation of an "active oligomer" as appears to be the case for the ethyl analogue.

The oligomerization pathway described above and the implications for the oligomerization of 15-dehydro-$PG_{B1}$ to $PG_{Bx}$ are based primarily on a study of the oligomerization of the ethyl analogue to low molecular weight oligomeric mixtures for which the dimeric components were isolated and characterized. However, the preliminary investigation of the oligomerization of 15-dehydro-$PG_{B1}$ to low molecular weight oligomeric mixtures suggests a very similar pathway to that followed by the ethyl analogue. Our present investigation of the trimeric and tetrameric components derived from oligomerization of both the ethyl analogue and 15-dehydro-$PG_{B1}$ to low molecular weight oligomeric mixtures should provide further experimental verification of the oligomerization pathway outlined above.

If the implications discussed above regarding the oligomerization of 15-dehydro-$PG_{B1}$ to $PG_{Bx}$ are correct, it is unlikely that attempts to characterize $PG_{Bx}$ by isolation and structural elucidation of individual components will be successful. This contention is supported by the previous unsuccessful efforts of several research groups using this approach over a number of years. In addition, even if one or two components of the

$PG_{Bx}$ mixture could be isolated and characterized, the relationship of the individual components to the complex oligomeric mixture would still remain uncertain.

As an alternative approach, the determination of the chemistry of oligomer formation based on structurally simpler models such as the ethyl analogue should serve to define the general structural characteristics of such complex mixtures of closely related oligomers. Moreover, a recognition of the various routes that are operable in the oligomerization of 15-dehydro-$PG_{B1}$ should allow for the incorporation of structural modifications in the precursor that would eliminate non-productive oligomerization pathways. Such modifications would result in an overall simplification in the structure of the resultant oligomeric mixture along with an enhancement of the biological activity.

Table 1. Protection of Oxidative Phosphorylation for Oligomer Fractions from Sephadex LH-20 Chromatography of Bicarbonate Soluble Oligomeric Mixtures Derived by Treatment of the Ethyl Analogue with 1 N KOH for Five Hours at 50° with Exposure to Oxygen.*

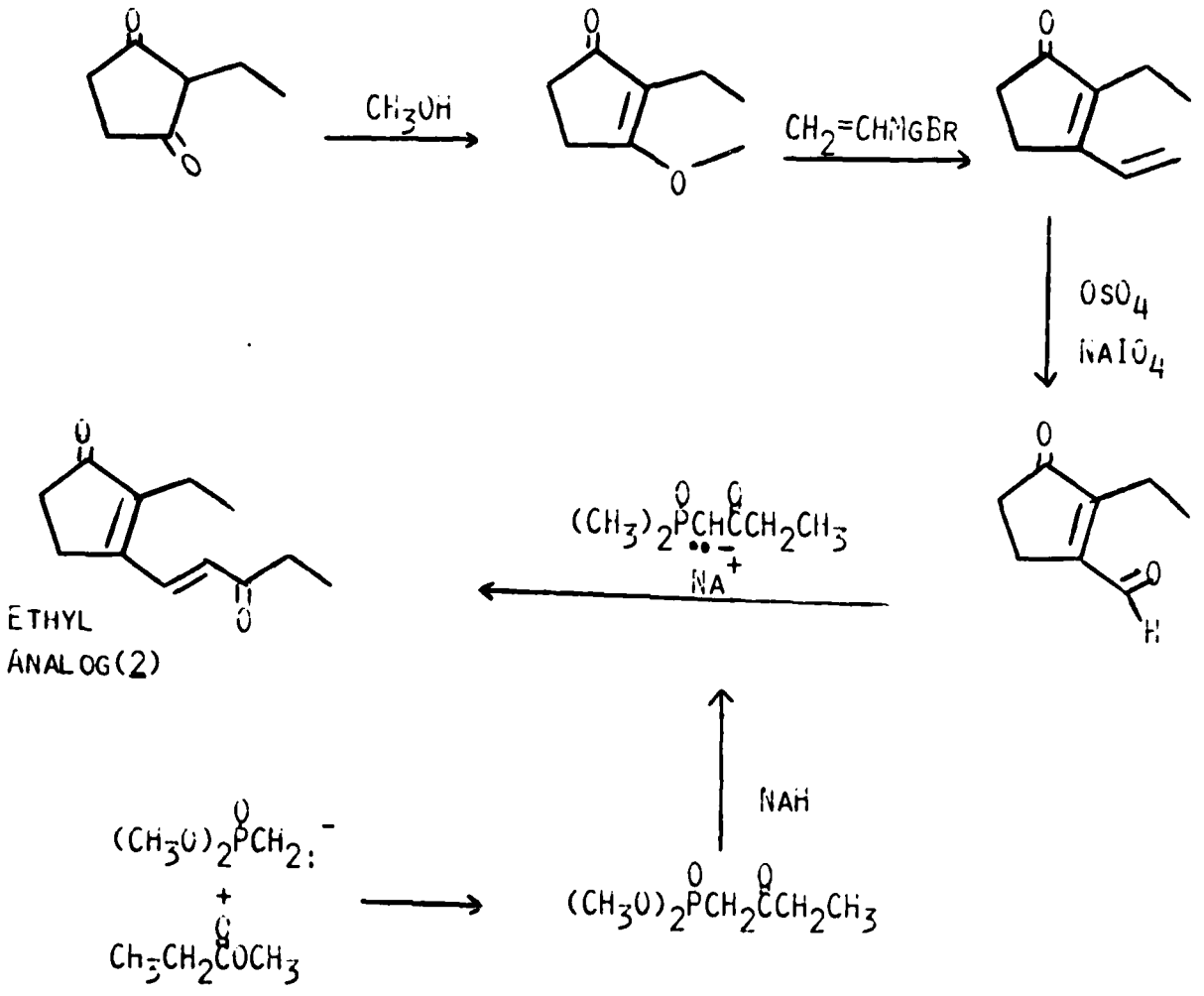
| Sample | % Activity in Comparison to $PG_{Bx}$ | Inhibition |
|---|---|---|
| EtA-BI-12 | 40% | No inhibition at 120 ug |
| EtA-BII-12 | 50% | No inhibition at 120 ug |
| EtA-BIII-12 | 55% | No inhibition at 120 ug |
| EtA-BIV-12 | 45% | No inhibition at 120 ug |
| EtA-BV-12 | 35% | No inhibition at 120 ug |

*Data determined by Dr. T. Devlin, Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, PA

Table 2. Protection of Oxidative Phosphorylation for Oligomer Fractions from Sephadex LH-20 Chromatography of Bicarbonate Insoluble Oligomeric Mixtures Derived by Treatment of the Ethyl Analogue with 1 N KOH for Five Hours at 50° under Oxygen-Free Conditions.*

| Sample | % Activity in Comparison to $PG_{Bx}$ | Inhibition |
|---|---|---|
| EtA-NI-12 | 25% | No inhibition at 120 ug |
| EtA-NII-12 | 25% | No inhibition at 120 ug |
| EtA-NIII-12 | 20% | No inhibition at 120 ug |
| EtA-NIV-12 | 20% | No inhibition at 120 ug |

FIGURE 1. SYNTHESIS OF 3-TRANS-(3-KETO-1-PENTENYL)-2-ETHYL-2-CYCLOPENTENONE (ETHYL ANALOG)



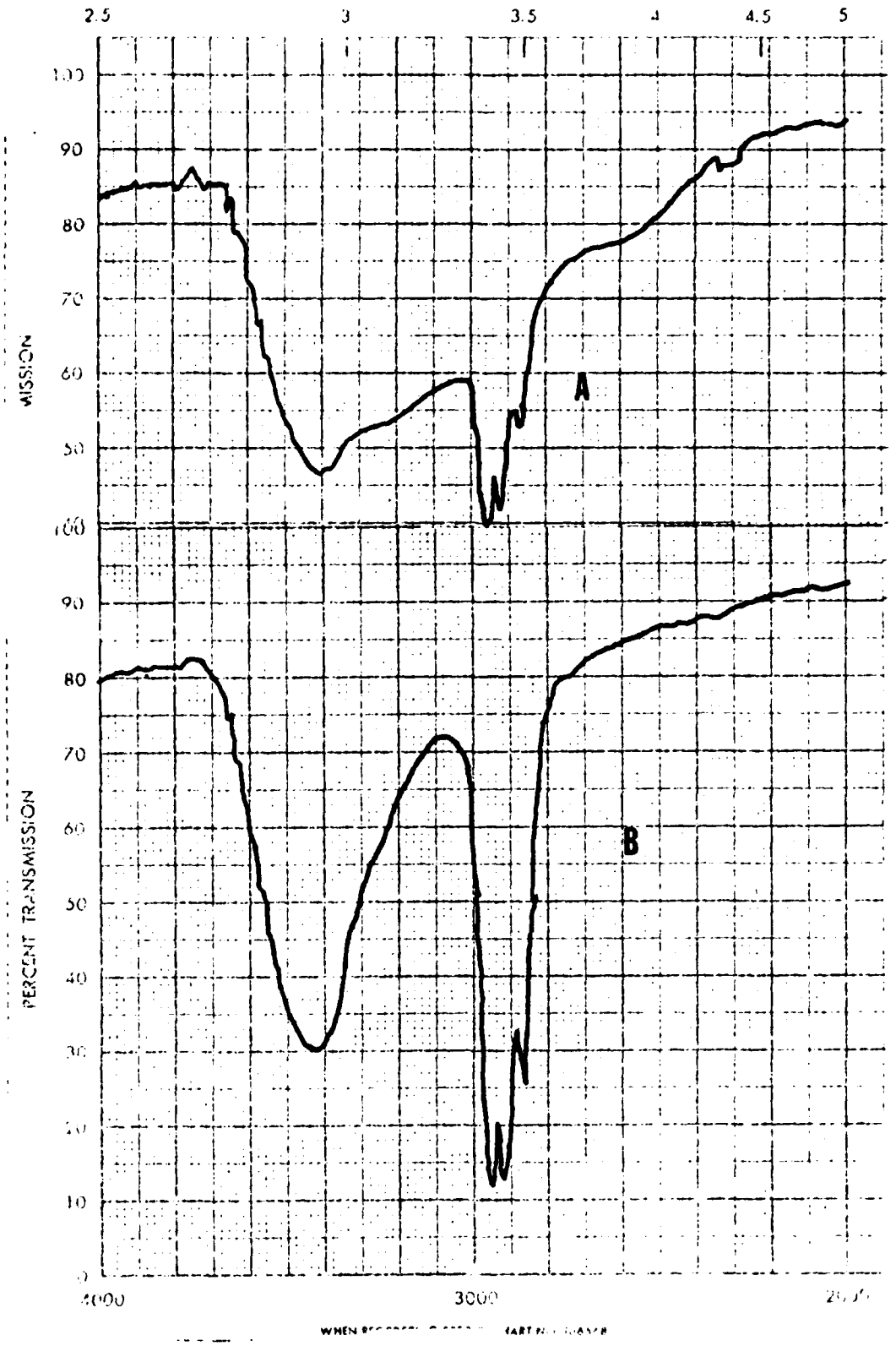



Figure 2. Comparison of the 4000 - 2000 $cm^{-1}$ Infrared Spectral Region for Bicarbonate Soluble (A) and Bicarbonate Insoluble (B) Oligomeric Mixtures Derived by Treatment of the Ethyl Analogue with 1 N KOH for 5 Hours at 50° in the Presence and Absence of Oxygen Respectively.

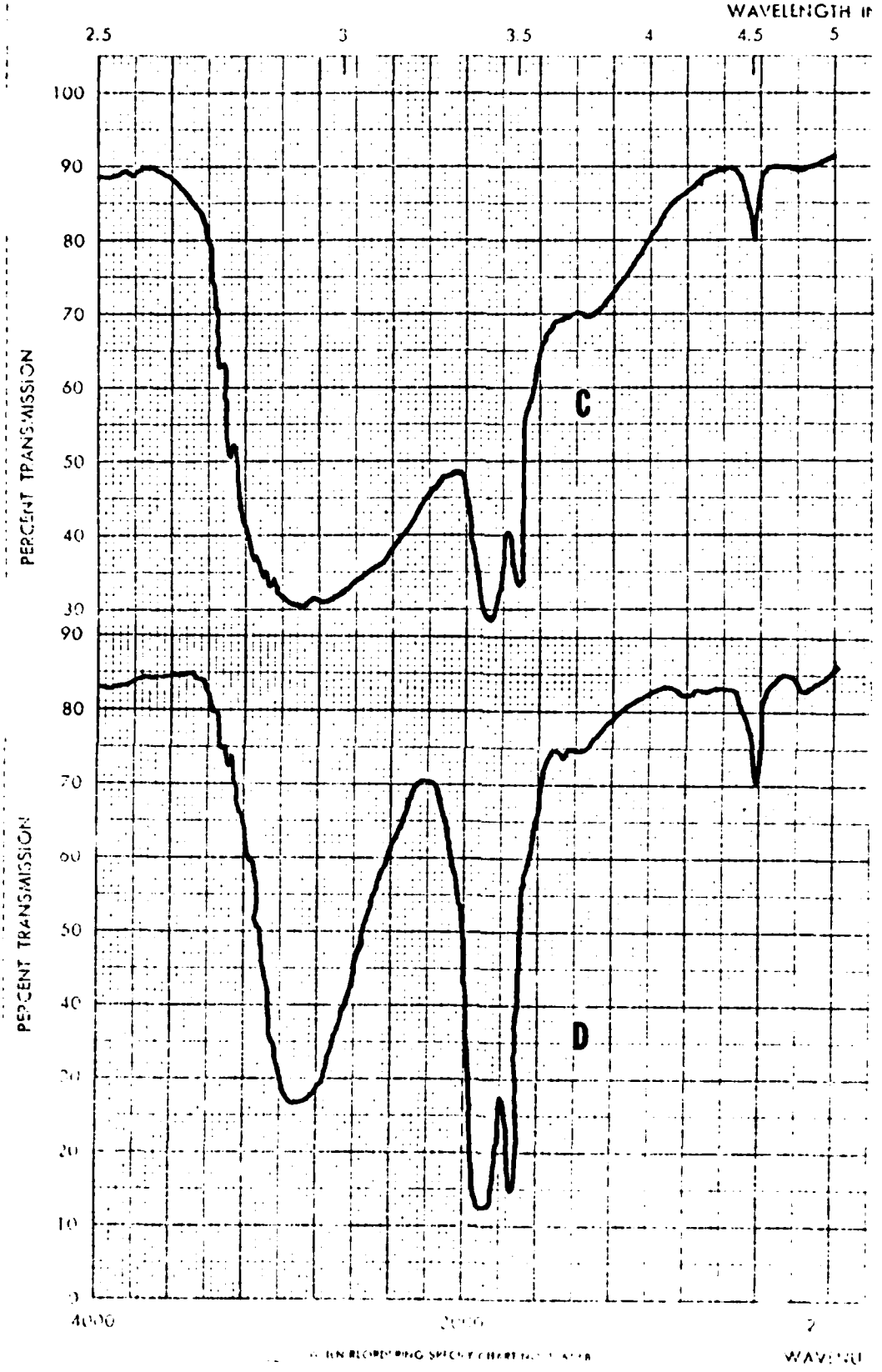



**Figure 3.** Comparison of the 4000 - 2000 $cm^{-1}$ Infrared Spectral Region of 13,14-Dehydro-$PG_{B1}$ Methyl Ester (D).

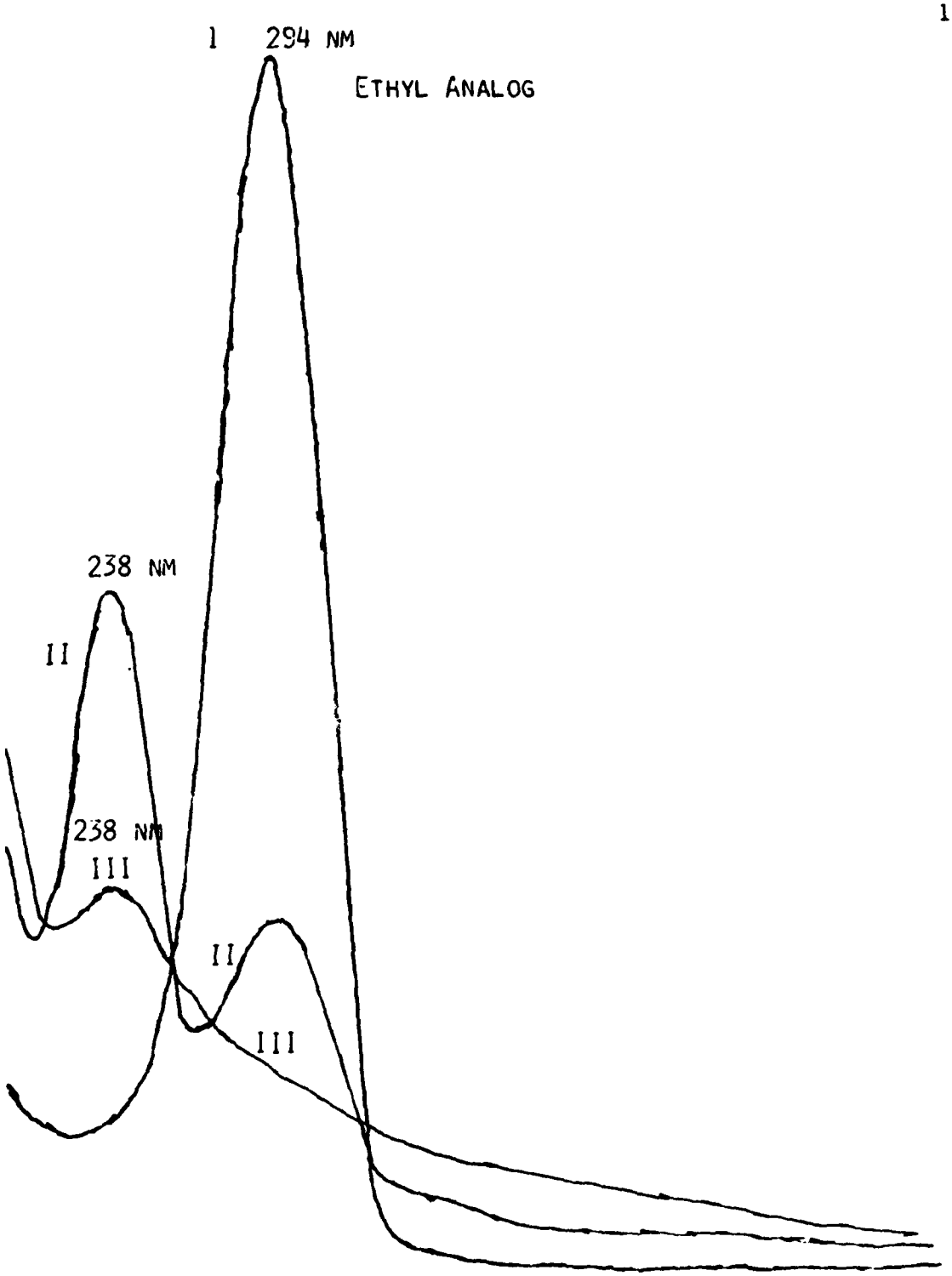



FIGURE 4. CHANGE IN ULTRAVIOLET SPECTRUM WITH TIME FOR OLIGOMERIZATION OF ETHYL ANALOG WITH KOH. I- ETHYL ANALOG II- SPECTRUM IMMEDIATELY AFTER ADDITION OF KOH. III- CHANGE IN SPECTRUM WITH CONTINUED STIRRING AT $50^{0}$.

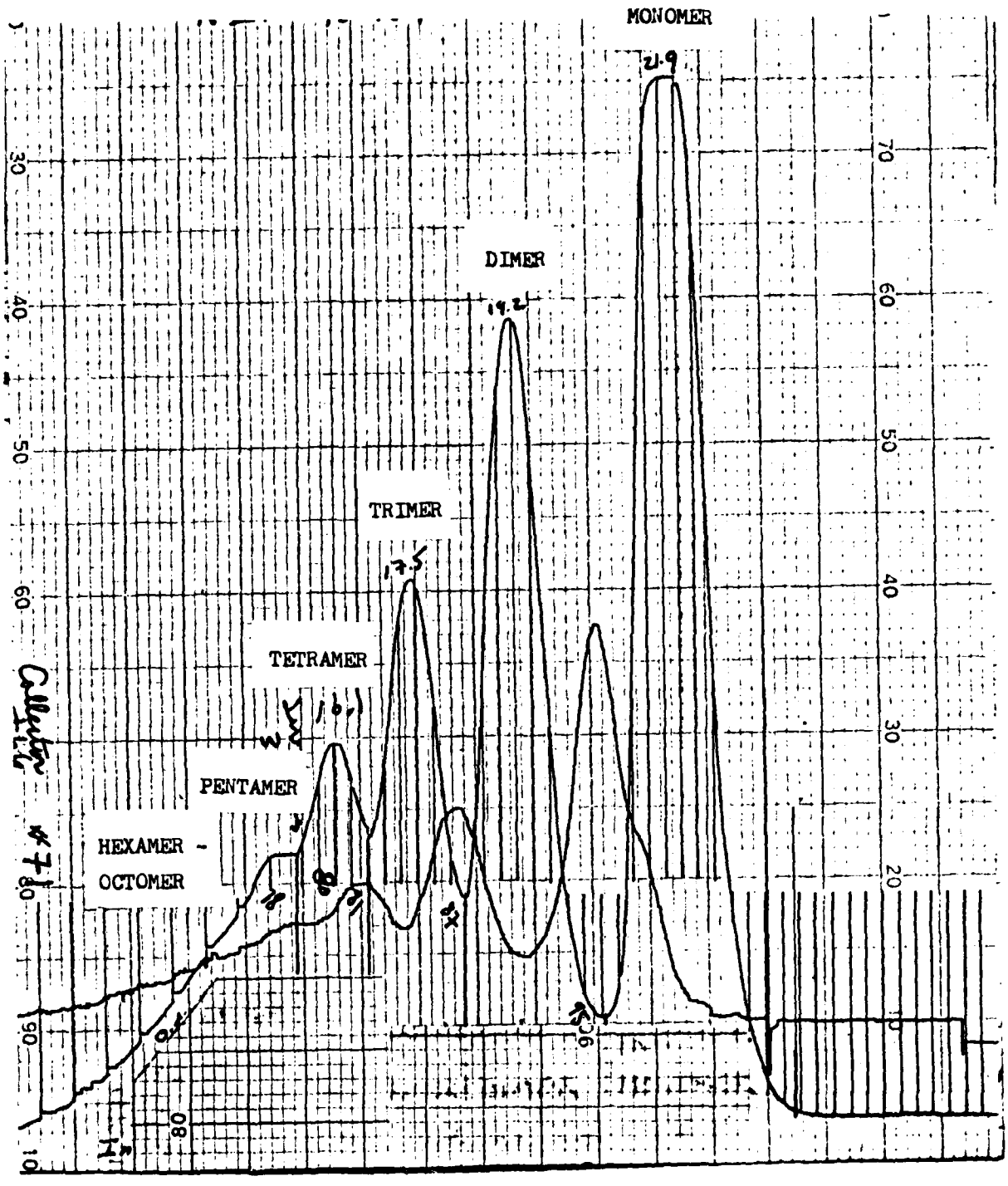



Figure 5. Size Exclusion Chromatography of Lower Weight Distribution Oligomeric Mixtures Derived from the Ethyl Analogue by Brief Exposure to Ethanolic KOH.

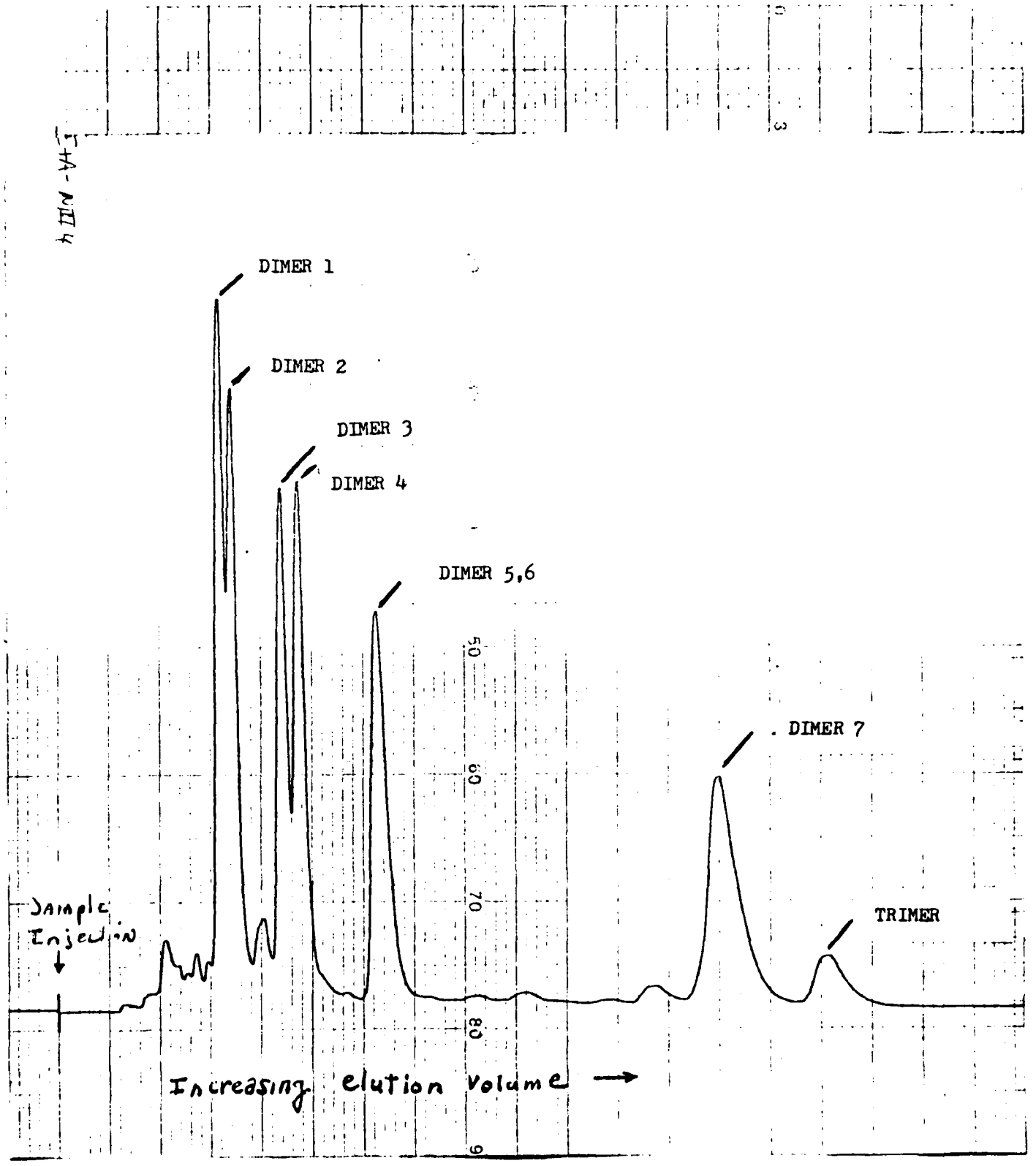
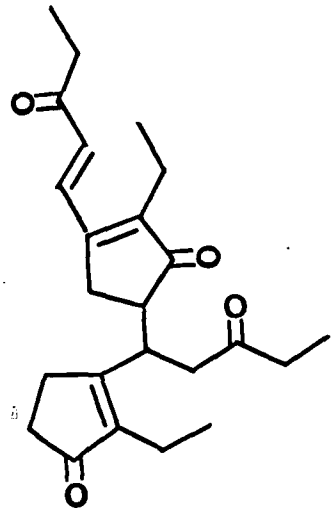
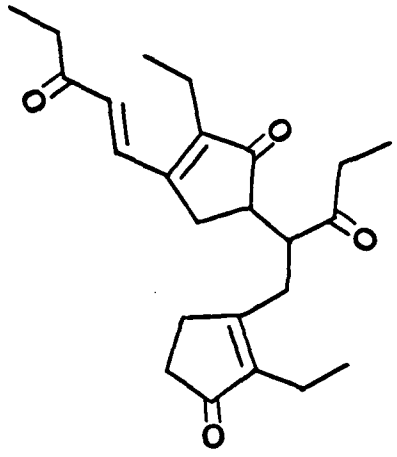


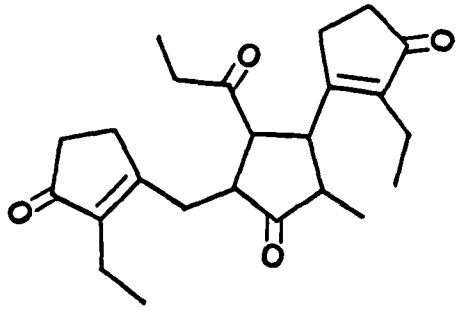

**Figure 6.** Chromatographic Separation of the Dimeric Component of Lower Weight Distribution Oligomeric Mixtures Derived from the Ethyl Analogue by Brief Treatment with Ethanolic KOH.



DIMERS 3,4



DIMERS 1, 2



DIMERS 5-7

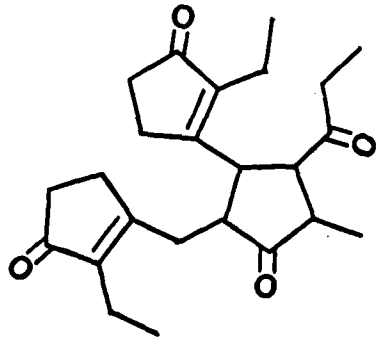


Figure 7. Structural Assignments for Dimeric Components of Lower Weight Distribution Oligomeric Mixtures Derived from the Ethyl Analogue by Brief Treatment and Ethanolic KOH.

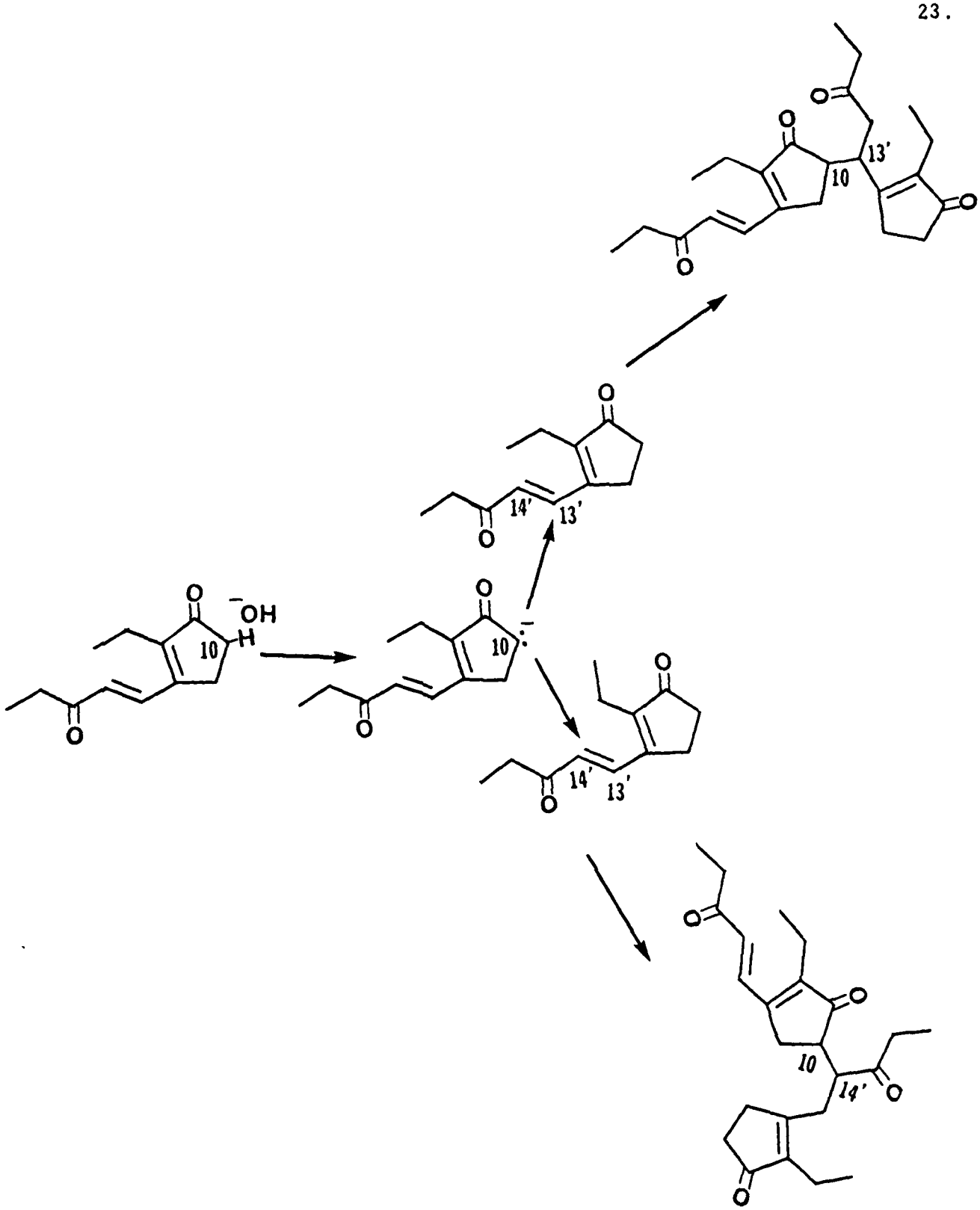



Figure 8. Proposed Mechanistic Pathway for Formation of Dimers 1 - 4 by a Single Michael Addition Initiated by C-10 Nucleophilic Attack.

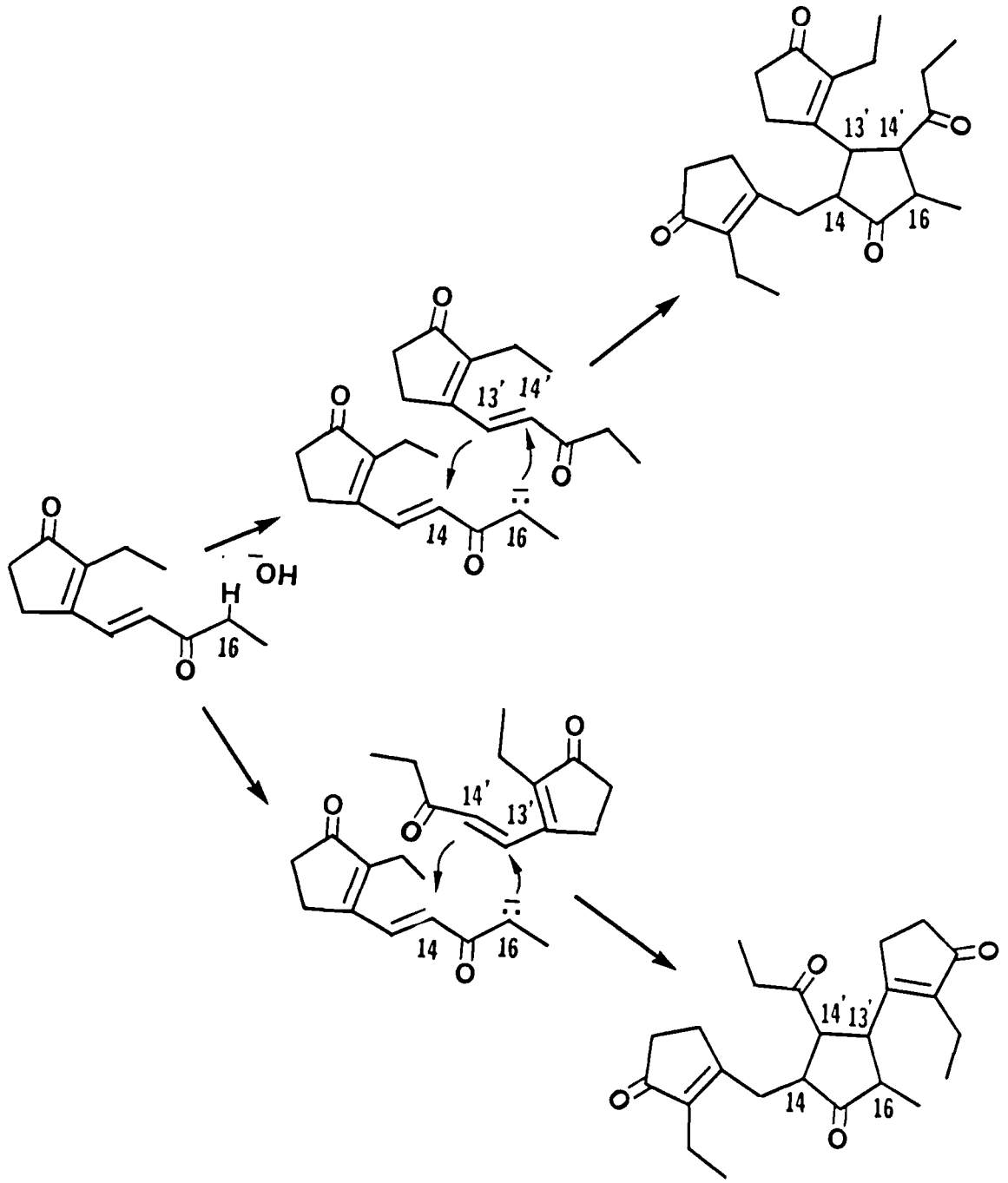



Figure 9. Proposed Mechanistic Pathway for Formation of Dimers 5 - 7 by a Double Michael Addition Initiated by C-16 Nucleophilic Attack.

## EXPERIMENTAL

### 1. Conversion of 3-(trans-3-Keto-2-Pentenyl)-2-Ethyl-2-Cyclopentenone (Ethyl Analogue) into a Higher Molecular Weight Distribution Bicarbonate Soluble Oligomeric Mixture by Treatment with Ethanolic Potassium Hydroxide with Exposure to Atmospheric Oxygen.

General

The following two examples should be regarded as being carried out under a representative set of conditions rather than an exclusive set of conditions that will result in the conversion of the ethyl analogue into the described oligomeric mixture. This is anticipated since changes in the concentrations of either the ethyl analogue or potassium hydroxide solution as well as the reaction temperature and time are interdependent so that a change in one of the above variables can be compensated by a corresponding change in the other variables. However, exposure to sufficient atmospheric oxygen is required for maximum conversion to a bicarbonate soluble oligomeric mixture under the reaction conditions described below. This can be readily accomplished if the reaction is carried out open to the atmosphere accompanied by vigorous stirring of the reaction mixture.

Example 1.

To 500 mg of 3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone (ethyl analogue) in 10 ml of absolute ethanol contained in an unstoppered round bottom flask maintained at 50°C in a constant temperature bath is added 10 ml of a 2 M potassium hydroxide solution to give a final concentration of 1 M ethanolic potassium hydroxide. Reaction is evident immediately and the progress of the conversion to a bicarbonate soluble oligomeric mixture is monitored by ultraviolet spectroscopy. When the conversion is judged sufficiently complete (ca. 3 - 6 hours), the reaction is quenched by the addition of dilute hydrochloric acid and the pH is adjusted to 3. The quenched reaction mixture is diluted with water and extracted several times with ethyl acetate. The combined ethyl acetate extracts are extracted with 0.1 M $NaHCO_3$. The combined bicarbonate extracts are acidified with dilute hydrochloric acid to pH 3 and extracted with ethyl acetate. The combined ethyl acetate extracts are washed with water, dryed, and the ethyl acetate is removed under vacuum to yield ca. 80 - 85 percent crude bicarbonate soluble oligomer.

The crude oligomeric mixture is chromatographed on Sephadex LH-20 using methanol as the carrier solvent. Resolution of the oligomeric mixture is not observed so that five somewhat arbitrarily selected fractions are collected. Activity in the protection of oxidative phosphorylation in isolated mitochondria is observed throughout all five fractions but is highest in fractions 2 and 3. No inhibition is observed at higher concentrations of the oligomer (Table 1).

Example 2.

Alternatively, an equivalent weight of a higher molecular weight bicarbonate insoluble oligomeric mixture, prepared under oxygen-free conditions, can be subjected to the same experimental conditions as described above to give a greater than 70 percent conversion to a bicarbonate soluble oligomeric mixture.

2. Conversion of 3-(trans-3-Keto-1-Pentenyl)-2-Ethyl-2-Cyclopentenone (Ethyl Analogue) into a Higher Molecular Weight Distribution Oligomeric Mixture that is Bicarbonate Insoluble by Treatment with Ethanolic Potassium Hydroxide with Exclusion of Oxygen.

General

The following experimental procedure should not be regarded as a unique set but as a representative set of conditions for conversion of the ethyl analogue into a bicarbonate insoluble oligomeric mixture. This is anticipated since changes in the concentrations of either the ethyl analogue or potassium hydroxide solution as well as the reaction temperature and time are interdependent so that a change in one of the above variables can be compensated by a corresponding change in the other variables. However, oxygen free reaction conditions are required for maximum conversion to a bicarbonate insoluble oligomeric mixture under the reaction conditions described below. This is accomplished by carrying out the conversion under nitrogen after effectively removing residual oxygen by the freeze-thaw technique.

Example 1.

Solutions 500 mg of the ethyl analogue in 10 ml of absolute ethanol and 10 ml of 2 M potassium hydroxide which are contained in separate reaction flasks are rigorously de-oxygenated using the freeze-thaw technique under nitrogen. After warming to room temperature, the 2 M potassium hydroxide solution is transferred under positive nitrogen pressure to the ethyl analogue solution which is maintained at 50° C in a constant temperature bath. Reaction is evident immediately and the progress of the reaction is monitored by ultraviolet spectroscopy. When the reaction is judged complete (ca. 3 - 6 hours), the reaction is quenched by the addition of dilute hydrochloric acid and is adjusted to pH 3. The quenched reaction mixture is diluted with water and extracted several times with ethyl acetate. The combined ethyl acetate extracts are extracted several times with 0.1 M $NaHCO_3$ and then washed with water until neutral. After drying, the ethyl acetate is removed under reduced pressure to yield ca. 90 - 95 percent of a crude bicarbonate insoluble mixture.

The crude oligomeric mixture is chromatographed on Sephadex LH-20 using methanol as the carrier solvent. Clean separation of the oligomeric mixture into distinct peaks is not observed so that four somewhat arbitrarily selected fractions are collected. Activity in the protection of oxidative phosphorylation in isolated mitochondria is observed throughout all the fractions. No inhibition at higher concentrations is observed (Table 2).

3. Conversion of 3-(trans-3-Keto-1-Pentenyl)-2-Ethyl-2-Cyclopentenone (Ethyl Analogue) into a Low Molecular Weight Distribution Oligomeric Mixture Containing a Substantial Dimeric Component by Treatment with Ethanolic Potassium Hydroxide

General

The following two examples are representative of experimental conditions suitable for conversion of the ethyl analogue into a low molecular weight distribution oligomeric mixture containing a substantial dimeric component. Since this oligomerization

takes place by a Michael Reaction, the hydroxide functions as a catalyst. To obtain a low molecular weight distribution oligomeric mixture, very short reaction times are required at higher hydroxide concentrations whereas somewhat longer reaction times are necessary at lower hydroxide concentrations to obtain the desired conversion. The use of reaction temperatures lower than room temperature requires a correspondingly longer reaction time. In general, the oligomerization is carried out until 20-30 percent of unreacted starting material remains and then the reaction is quenched by the addition of dilute acid. The progress of the reaction is monitored with time by ultraviolet spectrometry.

Example 1.

To 1 gram of 3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone in 20 ml of absolute ethanol is rapidly added 20 ml of a 2 N potassium hydroxide solution to give a final concentration of 1 N KOH. Reaction is evident immediately and sufficient dilute hydrochloric acid is rapidly added to bring the pH to 3. The quenched reaction mixture is diluted with water and extracted several times with ethyl acetate. The combined ethyl acetate extracts are first extracted with 0.1 N $NaHCO_3$ and then washed with water until neutral. After drying, the ethyl acetate is removed in vacuo to yield an essentially quantitative recovery of crude oligomeric mixture.

The crude oligomeric mixture is chromatographed on Sephadex LH-20 using methanol as the carrier solvent. Distinct separation of the oligomeric mixture into monomer, dimers, trimers, tetramers, and higher oligomers. Isolation of individual components, e.g. individual dimers, requires further chromatography on both normal and reverse phase columns (Figures 5 and 6).

Example 2.

To 1 gram of 3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone in 20 ml of absolute ethanol is rapidly added 20 ml of 0.4 N KOH to give a final concentration of 0.2 N KOH. After mixing, the reaction mixture is stirred for one minute at room temperature[1] and then the reaction is quenched by the addition of sufficient dilute hydrochloric acid to bring the pH to 3. The quenched reaction mixture is diluted with water and extracted several times with ethyl acetate. The combined ethyl acetate extracts are first extracted with 0.1 N $NaHCO_3$ and then washed with water until neutral. After drying, the ethyl acetate is removed in vacuo to yield an essentially quantitative recovery of crude oligomeric mixture.

The crude oligomeric mixture is separated according to the procedure outlined in Example 1.

1. The reaction time is initially determined by monitoring the progress of the oligomerization by ultraviolet spectrometry. When 20-30 percent of the starting material remains, the reaction is quenched by the addition of dilute hydrochloric acid.

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