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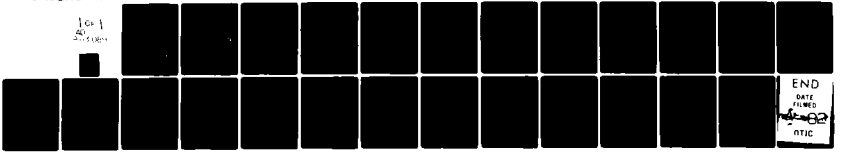

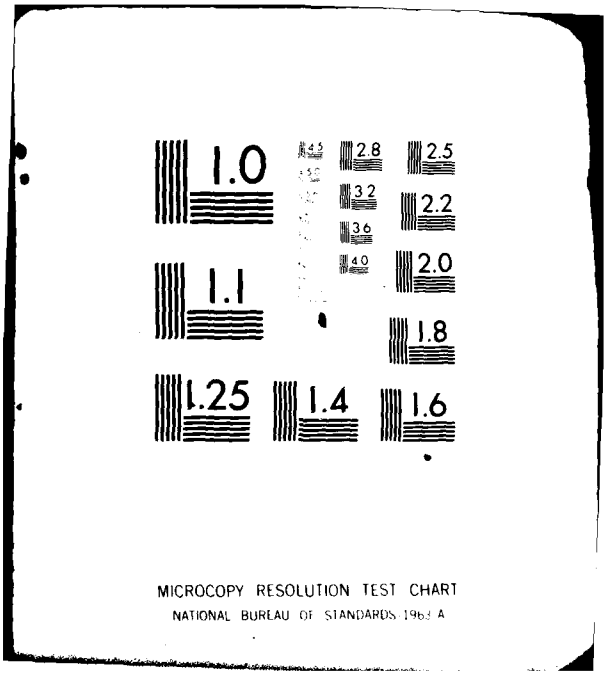
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TECHNICAL REPORT NO. 2

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

by

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Philadelphia, PA 19131

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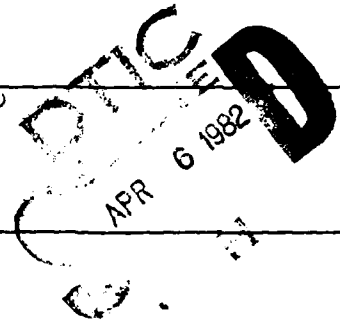


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3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone, ethyl analog; 15-dehydro-PGB$_1$, 15-dehydro-PGB$_1$ analogs; oligomerization of; structural elucidation of; oligomerization pathway of; protection of oxidative phosphorylation; PGBx structural insights

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The initial reaction pathway for the oligomerization of 15-dehydro-PGB$_1$ was determined by the investigation of structurally simpler analogues. This information was used to i) provide structural insights into the complex oligomeric mixture termed PGBx, ii) develop mild reaction conditions for the conversion of 15-dehydro-PGB1 into less complicated oligomeric mixtures still retaining mitochondrial activity, and iii) design structural modifications that would result in the formation of less complicated oligomeric mixtures that still retain the unique mitochondrial protective effect.

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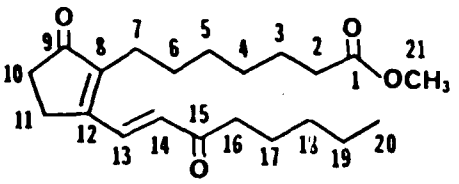
## I. OBJECTIVES

The primary objective of our research program during the period covered by Technical Report No. 2 was the elucidation of the oligomerization pathway of 15-dehydro-PGB$_1$ (1) through the investigation of structurally simpler analogs such as 2. The information derived from this investigation was used to: i) provide structural insights into the complex oligomeric mixture termed PGBx, ii) develop mild reaction conditions for the conversion of 15-dehydro-PGE$_1$ into less complicated oligomeric mixtures still retaining the unique mitochondrial protective effect characteristic of PGBx, and iii) design structural modifications for the prostaglandin precursor that would result in the formation of less complicated oligomeric mixtures which would still retain the activities unique to PGBx.

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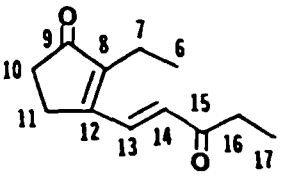


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## II. SUMMARY

The structurally simpler analog 2 was converted with high efficiency into oligomeric mixtures under conditions similar to those used for conversion of 15-dehydro-PGB$_1$ methyl ester into the complex oligomeric mixture termed PGBx. Of particular note, the bicarbonate soluble oligomeric mixture derived from 2 possessed a significant protective effect in the mitochondrial assay without the accompanying concentration dependent inhibition observed for standard PGBx. The functionality responsible for the unanticipated bicarbonate solubility was characterized as the -$CO_2H$ group, the formation of which was dependent on the presence of oxygen in the reaction system. An evaluation of the spectral data provided evidence that the oligomerization pathway involved several distinct steps.

The initial oligomerization step of 2 was investigated and found to result in the formation of a lower weight oligomeric mixture containing dimer through octamer components. The six individual dimer components were isolated and fully characterized. From this data it was evident that the initial oligomer forming reaction was taking place by a Michael addition pathway involving multiple nucleophilic and acceptor sites. As a consequence of the multiple pathways, the oligomeric mixtures that are formed rapidly become very complex. We have now confirmed that 15-dehydro-PGE1 undergoes oligomerization in the same manner as analog 2 providing additional support for the validity of analog 2 as an appropriate model. Our work with the analog 2 suggests that under the very vigorous reaction conditions used in the formation of PGBx a strong possibility exists that the complex mixture of oligomers originally formed may be further complicated by subsequent reactions. A consideration of this structural complexity strongly suggests that the direct isolation and structural elucidation of individual active components of the PGBx mixture would not be a feasible approach and that a complete characterization in the classical sense will not be possible.

As a result of the analog studies, a procedure was developed to permit the direct formation of lower molecular weight oligomeric mixtures of the free acid from 15-dehydro-PGB1 under very mild conditions. Unlike PGBx, these mixtures were readily separable into oligomeric components such as dimers, trimers, etc. by Sephadex LH-20 chromatography. Although the dimer component was inactive, the trimer and higher components exhibited activities on the same order or higher than standard PGBx in the mitochondrial assay. The isolation and characterization of the trimer components will provide for the first time a series of pure oligomer components of known structure which exhibit activity in the protection of oxidative phosphorylation.

With a knowledge of the multiple reaction sites involved in oligomer formation, it has been possible to design structural modifications for the precursor that reduce the number of available reaction sites. Such a reduction would lead to the formation of considerably less complicated oligomeric mixtures. Various analogs of 1 have been prepared that: i) eliminate the double addition products resulting from C-16 enolate addition, ii) eliminate or substantially favor addition to only one of the two acceptor sites, and iii) sharply reduce the number of closely related stereoisomers in the oligomeric mixture.

## III. CONCLUSIONS

We remain convinced that the most appropriate approach to the structural elucidation of the very complex oligomeric mixtures such as PGBx will be found in the determination of the general reaction pathway through the study of strucurally simpler analogs. Our identification of the reaction pathway with the multiple reaction sites involved in the oligomer forming step provides the basis for an understanding of the complex nature of PGBx, the direct structural elucidation of which has resisted the efforts of a number of research groups. We have been able to apply the understanding of the reaction pathway to develop a method for the preparation of low molecular weight oligomeric mixtures from 15-dehydro-PGB1 under very mild conditions. In these mixtures oligomers as small in size as trimers exhibit activities on the same order or higher than PGBx in the mitochondrial assay. The isolation and characterization of the individual trimers will provide the first opportunity to establish structure-activity relationships as a probe of the biological mechanism responsible for this unique mitochondrial activity. Having an understanding of the multiple reaction centers has also enabled us to design structural modifications that reduce the number of reaction sites with a consequent reduction in the complexity of the oligomeric mixtures obtained. In summary, the understanding which we have gained concerning the details of the oligomerization process has provided us with the means necessary to develop modified prostaglandins as precursors to less complicated oligomeric mixtures of structurally defined low molecular weight oligomeric components retaining the unique mitochondrial activity of PGBx.

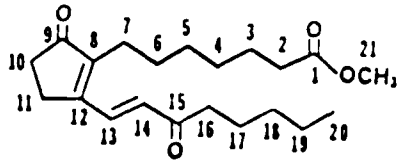
## IV. INTRODUCTION AND BACKGROUND

Currently, the term PGBx refers to a complex mixture derived from 15-dehydro-prostaglandin B1 (15-dehydro-PGB1) methyl ester, 1, upon treatment with 1 M ethanolic potassium hydroxide.[1] A number of unique *in vitro* and *in vivo* activities

have been demonstrated for certain fractions (standard PGBx) of this complex mixture. Standard PGBx protects against the loss of phosphorylating activity during aging in vitro of rat liver mitochondria[2] and functions as a potent "water soluble" ionophore[3] which stimulates the release of $Ca^{+2}$ from sarcoplasmic reticulum and heart mitochondria[3a]. In vivo, PGBx 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]. PGBx also provides a significant measure of protection against the severest forms of cardiac ischemia in various isolated segments of canine heart[6] and protects isolated anoxic rat heart[7]. Such demonstrated unique properties of PGBx strongly suggest potential future application in treatment of incidents of cerebral and myocardial ischemia and as a therapeutic agent for hemorrhagic traumatized combat casualties.

Although substantial progress has been made in defining many remarkable biological properties of PGBx, relatively little has been conclusively established concerning the structural details of PGBx or the chemistry involved in the formation of the active site(s). The lack of any definitive structural detail has hindered the development of a more detailed understanding of these unique biological properties. PGBx has been generally described as a complex mixture of closely related oligomers formed by an initial reaction at the 13,14-unsaturation of 1 with the retention of the overall prostaglandin skeleton.[8] Recent attempts by a number of research groups to resolve this complex oligomeric mixture into individual components retaining activity have proven unsuccessful precluding a more definitive structural assignment.

As an alternative approach to the direct structural elucidation of PGBx, our present program (N00014-80-C-0117) was undertaken in an effort to define the chemical pathway by which oligomerization takes place through the investigation of structurally simpler analogues of 15-dehydro-$PGB_1$ such as the analog 2. Many of the problems associated with the direct structural elucidation of the complex mixture termed PGBx are associated with the inherent complexity of oligomeric mixtures in which the oligomeric unit is a 20 carbon prostaglandin. Although the analog 2 contains substantially fewer carbons than 15-dehydro-$PGB_1$, the essential conjugated cyclopentenone functionality of 1 is retained in 2 leading to the expectation of similar oligomerization pathways.



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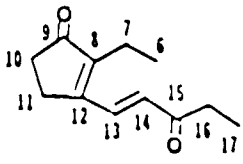

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## V. RESULTS AND DISCUSSION

### A. Oligomerization of the Analog 2

#### 1. Oligomerization of Analog 2 with 1 M KOH

As previously detailed,[9] the analog 2 was readily converted into oligomeric mixtures upon treatment with 1 M KOH under conditions similar to those used in the conversion of 15-dehydro-$PGB_1$ methyl ester (1) to the complex oligomeric mixture termed PGBx. If oxygen was rigorously excluded from the reaction system, the expected bicarbonate insoluble oligomer was obtained. In the presence of oxygen, a high conversion to a bicarbonate soluble oligomeric mixture could be effected. Individual fractions of the bicarbonate soluble oligomeric mixture exhibited ca. 50 percent of the activity of "standard PGBx" at concentrations where maximum protection of oxidative phosphorylation by PGBx was observed. However, an even greater percent protection of oxidative phosphorylation than that afforded by "standard PGBx" was observed at higher concentrations of the analog oligomer since a concentration dependent inhibition was not present.

The conversion of the analog 2 into a bicarbonate soluble oligomeric mixture was postulated to proceed in several distinct stages.[9] The very rapid initial step leading to chain formation appeared to involve reaction at the 13,14-unsaturation. The oxygen mediated reaction resulting in the formation of the $-CO_2H$ functionality responsible for imparting bicarbonate solubility to the oligomeric mixture appeared to be taking place in a subsequent reaction.

The structural similarity of 15-dehydro-$PGB_1$ to the analog 2 supported the operation of similar reaction pathways in the initial chain forming step. Likewise it seemed reasonable that the oxygen mediated reaction resulting in the formation of the $-CO_2H$ functionality in oligomeric mixtures derived from the analog 2 would also occur under the conditions used for the conversion of 15-dehydro-$PGB_1$ methyl ester into PGBx. The presence of the oxygen mediated reaction reaction is easily recognized in the conversion of the analog 2 to bicarbonate soluble oligomeric mixtures since the appearance of the $-CO_2H$ functionality represents a major structural transformation other than the formation of oligomer chains by carbon-carbon bond formation at the 13,14-unsaturation of analog 2. A similar oxygen mediated transformation would not be nearly so evident in PGBx formation since a $-CO_2H$ functionality formed in this manner would be masked by the presence of the $-CO_2H$ groups formed as the result of the simple hydrolysis of the $CO_2CH_3$ ester functionality present in the precursor 15-dehydro-$PGB_1$ methyl ester. The direct comparison of the oligomerization reactions of 1 and 2 carried out in the presence of oxygen strongly suggested that a similar oxygen mediated reaction was taking place in both cases. A more detailed consideration of a possible oxygen mediated reaction taking place in the conversion of the prostaglandin 1 to PGBx is discussed in Section B.

2. Oligomerization of Analog 2 with 0.005 M KOH

Since the investigation of the oligomerization of analog 2 under conditions similar to those used in the conversion of 1 into PGBx (1 M KOH) indicated the operation of several distinct reactions, an attempt was made to isolate the initial oligomerization reaction. Our earlier spectroscopic studies suggested that the initial carbon-carbon bond forming reaction leading to chain formation proceeded through addition to the 13,14-unsaturation via a Michael reaction. Since the function of the base (i.e. KOH) in the Michael reaction is catalytic, the rate of this reaction can be reduced by simply lowering the base concentration.

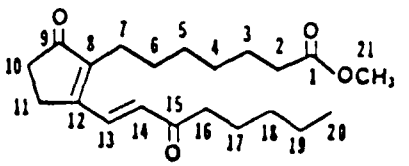
By a reduction in the KOH concentration to 0.005 M, this initial chain forming step could be isolated for detailed investigation.

Treatment of analog 2 with 0.005 M ethanolic KOH at room temperature resulted in an immediate reaction with a new 238 nm UVmax replacing the 296 nm absorption of 2. If the reaction mixture was quenched after 1-2 minutes, the crude product obtained after extraction with ethyl acetate contained 20-25 percent of unreacted 2. Analysis of the crude product by field desorption mass spectrometry (FDMS) indicated an oligomeric mixture of the formula $(C_{12}H_{16}O_2)_n$ with n = 1-8. Separation of the oligomeric mixture into dimer, trimer, tetramer, pentamer, and hexamer-octamer fractions, as confirmed by FDMS analysis of each fraction, was accomplished by size exclusion chromatography on Sephadex LH-20 using $CH_3OH$ as the eluting solvent.

Dimers

HPLC analysis of the dimer fraction (Zorbax Sil, 40% EtOAc/$C_6H_{12}$) revealed six major components which are referred to in the following discussion, in order of increasing retention times, as Dimers 1-6. Separation of the six dimers was effected using a 10 mm x 25 cm LiChrosorb column with 30% EtOAc/$C_6H_{12}$. A molecular formula of $C_{24}H_{32}O_4$, i.e. $(C_{12}H_{16}O_2)_2$, was established for each of the six dimers by HRMS measurement of the molecular ion.

Two distinctly different dimer types were indicated by the spectral data. Dimers 1-4 exhibited UVmax at 296 and 238 nm and conjugated C=C IR absorptions at 1585 and 1640 $cm^{-1}$. Dimers 5 and 6 had a single UVmax at 238 nm, a conjugated C=C IR absorption at 1640 $cm^{-1}$, and a new absorption at 1750 $cm^{-1}$ which is characteristic of a cyclopentanone C=O. Whereas Dimers 1-4 exhibited an intense fragment ion at m/e 192, corresponding to $C_{12}H_{16}O_2$, only a weak m/e 192 ion was present in Dimers 5 and 6. In the 360 MHz $^1H$ NMR spectra of Dimers 1-4, absorptions corresponding to a 13,14-unsaturation and four distinct methyl triplets were present. In contrast, Dimers 5 and 6 exhibited no olefinic protons, three methyl triplets, and a methyl doublet. In the $^{13}C$ NMR Spectra of Dimers 1-4, two methine carbons were evident indicating the formation of a single C-C bond. The presence of four methine carbons in Dimers 5 and 6 along with a carbonyl carbon at 214 ppm indicated the formation of two C-C bonds leading to a cyclopentanone ring. This data taken with a detailed consideration of $^1H$ and $^{13}C$ NMR data led to the structural assignment of Dimers 1-6 found in Figure 1.

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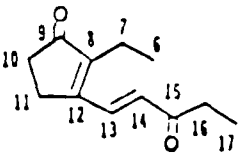


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Dimers 1-6 are formed by base catalyzed Michael addition in which two nucleophilic (C-10 and C-16) and two acceptor (C-13 and C-14) sites of the analog 2 are active (Figure 1). The presence of multiple reaction sites coupled with the formation of two new chiral centers for each new bond formed

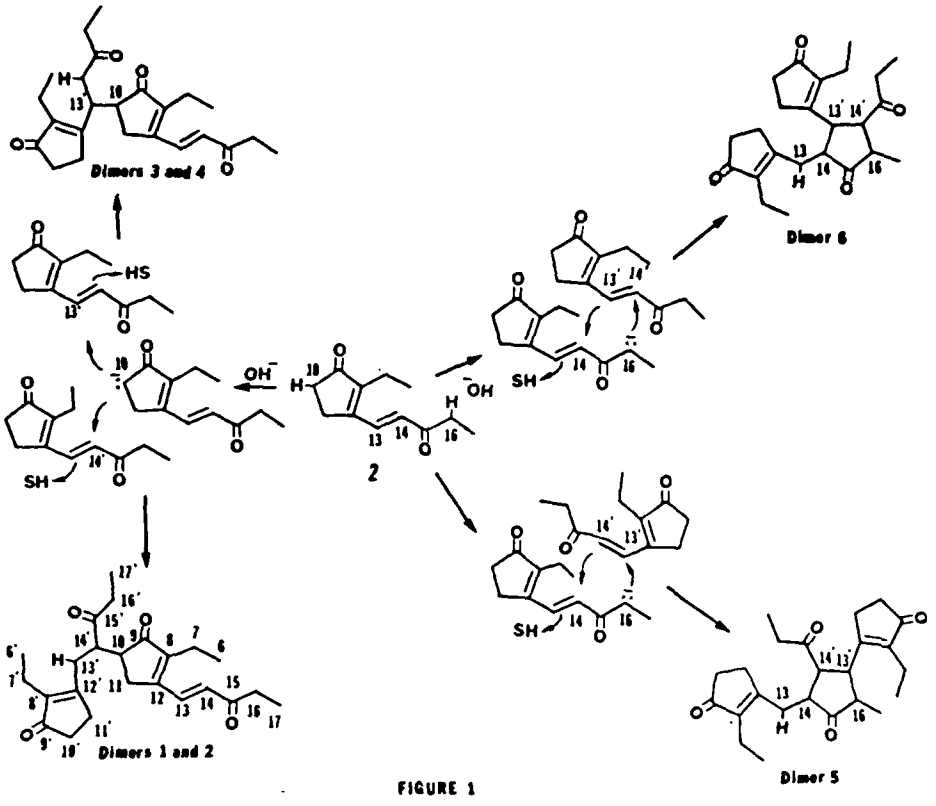



Figure 1. Reaction Scheme for Dimer Formation in the Oligomerization of Analog 2 with 0.005 M Ethanolic Potassium Hydroxide.

results in the formation of a complicated mixture of structural isomers further complicated by the presence of closely related stereoisomers. Dimers 1 and 2, a diastereomeric pair, are formed by the addition of the C-10 enolate of 2 to C-14' of a second unit of 2. Dimers 3 and 4, a second diastereomeric pair, arise from the addition of the C-10 enolate to C-13' of a second unit of analog 2. Dimers 5 and 6, the double addition dimers, result from the addition of the C-16 enolate of 2 to C-13' and C-14', respectively, of a second unit of analog 2 to form a new enolate which internally cyclizes to form a cyclopentanone ring by addition to C-14 of the original unit. Dimers 1-4 retain a residual 13,14-unsaturation through which further oligomerization can proceed in a similar manner. Dimers 5 and 6, which lack the residual 13,14-unsaturation, do not appear to undergo further oligomerization.

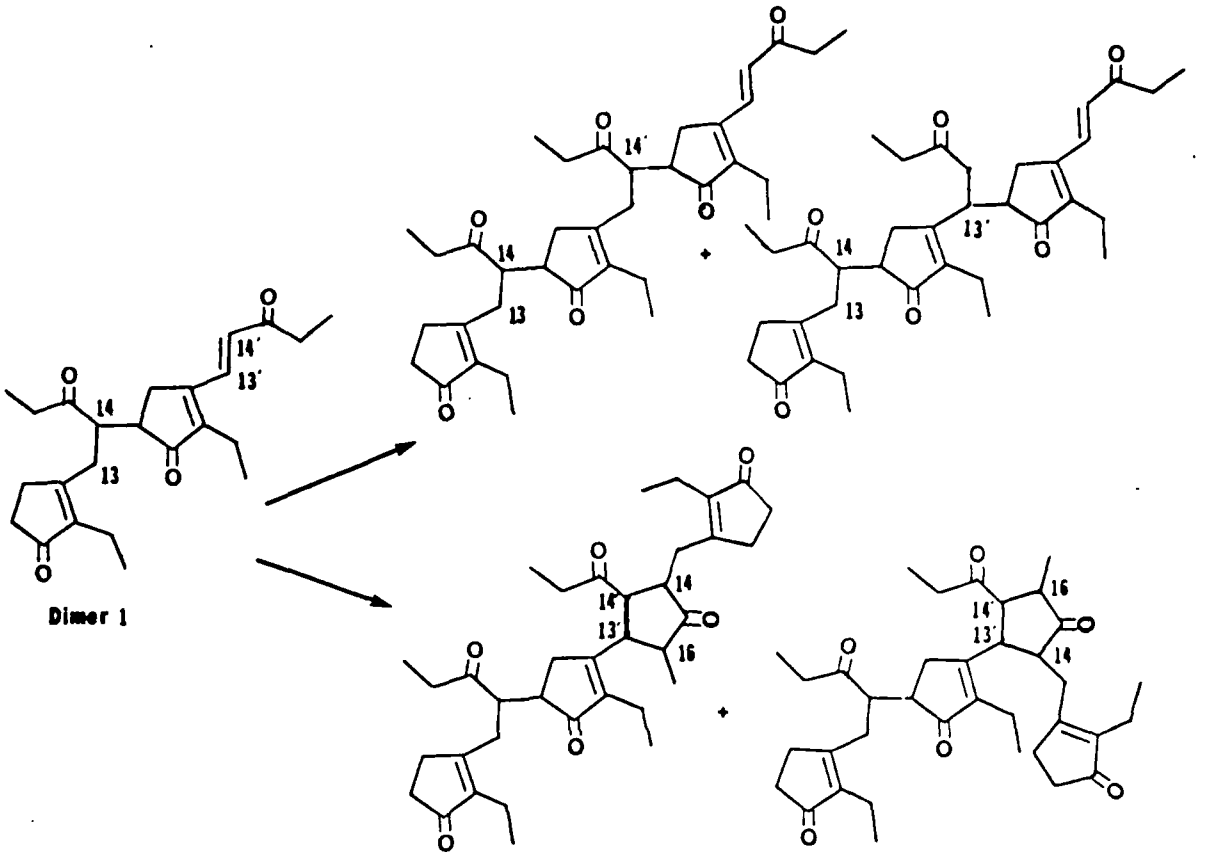



Figure 2. Reaction Scheme for Trimer Formation in the Oligomerization of Analog 2 with 0.005 M Ethanolic Potassium Hydroxide.

The reaction pathway leading to oligomer chain formation, as derived from the dimer studies of analog 2, has major implications concerning the structural complexity of PGBx. Providing the next higher oligomer arises from the addition of the C-10 enolate of 2 to the residual 13,14-unsaturation, the oligomeric mixtures that are formed rapidly become very complex. In the case of trimer formation, each of the four dimers (Dimers 1-4) could give rise to two pairs of diastereomeric trimers so that a total of 16 closely related trimers retaining a 13,14-unsaturation are possible as illustrated in Figure 3. This occurs since the addition of the third C-10 enolate unit could take place at either the C-13' or C-14' carbon of the residual dimer unsaturation. As in dimer formation, this results in the formation of two pairs of diastereomers so that from all four dimers, a total of 16 closely related trimers, each retaining a residual 13,14-unsaturation, could be formed. To further add to the complexity of the oligomeric mixture, the

addition of the C-16 enolate to either C-13' or C-14' of each of the four dimers i. e. Dimers 1-4, would lead to the formation of a total of eight of the double addition type trimers considering only the formation of the most stable all trans cyclopentanone diastereomer as was the case in the formation of the double addition dimers (Figure 2). If extended to the formation of higher oligomers and considering only those oligomers formed by the addition of the C-10 enolate to a residual 13,14-unsaturation, the 16 trimers having a residual unsaturation could give rise to 64 tetramers, the 64 tetramers to 256 pentamers, the 256 pentamers to 1056 hexamers, etc.; with the 13,14-unsaturation required for further chain growth being retained in each case. The actual oligomeric mixtures formed would be expected to be even more complex due to the double addition type products derived from addition of the C-16 enolate. However, the lack of a residual 13,14-unsaturation in this type of product would preclude conversion to a higher oligomer.

Based on such a projection, the direct structural elucidation of PGBx, for which the most active fraction has been estimated to be in the hexamer-octamer range,[8] would not appear feasible. The marked lack of success over the last several years by groups attempting direct isolation and structural elucidation of individual components of the PGBx mixture supports this conclusion. It is probable that all the predicted isomers would not be formed in significant amounts since Dimers 1-4 are formed in unequal amounts. But if even a fraction of the predicted number of hexamer - octamer oligomers are formed, a direct structural elucidation based on the isolation of individual components would still appear to be unfeasible. If further complicating reactions subsequent to the initial oligomer formation take place, which remains a distinct possibility, the direct isolation and structure elucidation would prove even more difficult. A realization of the probable complexity of the PGBx mixture has prompted our development of less complicated oligomeric mixtures through the application of very mild oligomerization conditions and the development of structural modifications designed to reduce the number of reaction sites.

The ultimate validity of the projections developed from the chemistry of the analog 2 is dependent on the operation of a similar reaction pathway in the oligomerization of the prostaglandin 15-dehydro-PGE1. We have now established that 15-dehydro-PGB1 follows a similar reaction pathway to that of analog 2 when oligomerized under the mild conditions developed for 2 thus providing further support for analog 2 as an appropriate model. Futhermore, spectral data obtained in the investigation of higher oligomer components such as trimers and tetramers is consistent with the proposed oligomerization pathway.

Trimers

The trimer component derived from the oligomerization of 2 was isolated by Sephadex LH-20 chromatography and was determined by normal phase HPLC analysis to be very complex. The major percentage of the trimer component retained a residual 13,14-unsaturation and, as illustrated in Figure 3, would arise from the addition of the C-10 enolate of a third unit of analog 2 to Dimers 1-4. The remaining trimer component lacked a residual 13,14-unsaturation and would be formed from the C-16 enolate addition of a third unit of analog 2 to Dimers 1-4 via the double addition pathway.

A preliminary HPLC separation of the trimer component was carried out. A general separation of trimers retaining a 13,14-unsaturation from those lacking the residual unsaturation was possible. Analysis of individual trimer components by 360 MHz $^1H$ NMR proved difficult because of the numerous overlapping signals. As an example, each trimer has six overlapping $-CH_3$ multiplets with quite similar chemical shifts. In view of the difficulty in interpretation in the case of mixtures, several structurally modified analogs were prepared that were more suitable as spectroscopic models for use in the trimer structural elucidation. Details related to the design and preparation of the modified analogs are provided in Section C.

Tetramers

The tetramer component could be obtained directly from the oligomeric mixture by Sephadex LH-20 chromatography using recycle techniques. HPLC analysis of the tetramer component indicated a very complicated mixture as was anticipated from projections derived from the dimer studies. Spectroscopic studies suggested, as in the case of the trimers, that both single addition products retaining a 13,14-unsaturation and double addition products lacking the residual 13,14-unsaturation were present. The direct isolation of the individual pure tetramers from this complex mixture did not appear feasible. An alternative route for the characterization of individual tetramers was developed and is discussed in Section B.

## B. Oligomerization of 15-dehydro-PGB1

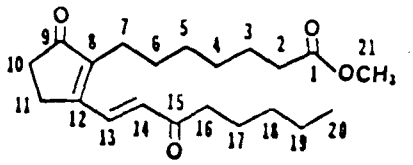
### 1. Oligomerization of the Methyl Ester

#### a) Treatment with 1 M KOH

It was previously established that the treatment of the analog 2 with 1 M ethanolic KOH in the presence of oxygen results in the formation of a bicarbonate soluble oligomeric mixture.[9] The unexpected bicarbonate solubility, which was attributed to the formation of the $-CO_2H$ functionality, represents a major structural transformation other than the simple chain formation taking place by Michael addition to the 13,14-unsaturation. The structural similarities of the prostaglandin 1 and the analog 2 led us to suspect a similar transformation in the oligomerization of 1 under similar reaction conditions even though the consequences of the oxygen mediated formation of the $-CO_2H$ functionality would not be as readily evident. In the oligomerization of 15-dehydro-PGB1 methyl ester, the formation of the $-CO_2H$ functionality by the oxygen mediated reaction would be effectively obscured by the expected presence of the $-CO_2H$ groups generated by simple ester hydrolysis.

A series of reactions were carried out in the presence of oxygen as well as under oxygen-free conditions under which the prostaglandin 1 and the analog 2 were oligomerized under identical reaction conditions. Under the published conditions for PGBx formation (1 M KOH for 4 hours at 80° C)[1] and in the presence of oxygen, the oxygen mediated reaction takes place in the oligomerization of 15-dehydro-PGB1 methyl ester. The extent to which this reaction occurs under

the less vigorous conditions presently used for PGBx formation is less certain. The oxygen mediated reaction does not appear to take place to any appreciable extent under the very mild reaction conditions developed for the oligomerization of the analog 2. The relationship of this oxygen mediated reaction to the degree of mitochondrial activity or the level of inhibition is not yet certain but the presence of this reaction would certainly add an increased level of complexity to the PGBx structural problem.



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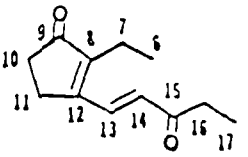

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b) Treatment with 0.005 M KOH

The investigation of the initial oligomerization step was extended to 15-dehydro-$PGB_1$ methyl ester (1) using the mild reaction conditions developed for analog 2. Treatment of the prostaglandin 1 with 0.005 M ethanolic KOH produced spectral changes similar to those observed in the oligomerization of the analog 2. Although the oligomerization reaction proceeded somewhat more slowly for 1, presumably due to the increased steric hinderance at the 13,14-unsaturation, a similar conversion to that obtained for the analog 2 was obtained by monitoring the course of the reacton by UV spectroscopy. The oligomeric mixture obtained under these conditions was in the ester form since the rate of hydrolysis of the methyl ester to the free acid ($-CO_2H$) was not competitive with the rate of oligomer formation.

The reaction mixture was separated by Sephadex LH-20 chromatography into the various oligomer components in the manner described for analog 2. In the subsequent HPLC separation and 360 MHz $^1H$ NMR analysis of the dimer component, the individual dimers were obtained as mixed methyl and ethyl esters. The mixed esters were formed as a result of a trans-esterfication reaction with ethanol that was rate competitive with the oligomerization reaction. The separation and spectroscopic analysis of the individual oligomers was severely complicated by the formation of the mixed esters and, as a result, further investigation of the initial oligomerization of the 15-dehydro-$PGB_1$ methyl ester was discontinued.

The various components of the oligomeric mixture derived from 15-dehydro-$PGB_1$ methyl ester upon treatment with 0.005 M ethanolic KOH were evaluated by Dr. T. Devlin of Hahnemann Medical College for the protection of oxidative phosphorylation in the mitochondrial assay. All the oligomer fractions showed very little, if any, activity in this assay. This result was not unexpected since it has been previously demonstrated that the conversion of PGBx to methylated PGBx by treatment with diazomethane results in the loss of the protective effect. Our results provide additional support for the requirement of a free $-CO_2H$ in the oligomer as being necessary for the successful operation

of the mitochondrial protective effect. The complications resulting from the formation of the oligomeric mixtures in the ester form were avoided in subsequent work by the oligomerization of 15-dehydro-PGB$_1$ as the free acid.

2. Oligomerization of 15-Dehydro-PGB$_1$ (Free Acid)

Treatment with 0.005 M KOH

Although the oligomerization of 15-dehydro-PGB$_1$ methyl ester (1) with 0.005 M ethanolic KOH appeared to follow the same pathway as the analog 2, the additional complexity arising from the competitive trans-esterfication reaction made the separation and characterization of individual components very difficult. Futhermore, the evaluation of the various oligomeric components for mitochondrial activity was not possible in the ester form. These complications were avoided by the oligomerization of 15-dehydro-PGB$_1$ in the free acid form.

The synthesis of 15-dehydro-PGB$_1$ as the free acid was accomplished by minor modification of the synthetic sequence previously developed in our laboratories for the preparation of 15-dehydro-PGB$_1$ methyl ester. The modification of the synthetic route required the development of new HPLC purifications that were based primarily on reverse phase chromatography. The initial synthesis provided amounts of 15-dehydro-PGB$_1$ free acid that were sufficient for evaluation of this material as a precursor to oligomeric mixtures formed under very mild conditions.

Treatment of 15-dehydro-PGB$_1$ free acid with an effective concentration of 0.005 M ethanolic KOH at room temperature resulted in a gradual conversion with a new 238 nm UVmax replacing the original 296 nm absorption. A percent conversion similar to that obtained for the analog 2 was obtained by monitoring the changes in the UV spectrum. The separation of the oligomeric mixture into dimer, trimer, tetramer, and higher components was accomplished by size exclusion chromatography on Sephadex LH-20.

The various oligomer components were evaluated by Dr. T. Devlin of Hahnemann Medical College for protection of oxidative phosphorylation in the mitochondrial assay. The dimer component exhibited very little, if any, protective effect but the trimer, tetramer, and higher components exhibited activities on the same order or higher than standard PGBx with an apparently somewhat lower inhibition effect.

Dimers

Following the separation of the dimer component by Sepadex LH-20 chromatography, the initial separation of the individual dimers was carried out using reverse phase chromatography. The fractions obtained from the reverse phase chromatographic separation were then methylated with diazomethane and subjected to normal phase chromatography. The dimers obtained in this manner were analyzed by 360 MHz $^1$H NMR spectroscopy by reference to the earlier analog studies and were found to correspond to Dimers 1-6 derived from analog 2. The similarity in dimer formation provides further validation of our contention that similar chemical pathways are functioning in the oligomerization of both the analog 2 and 15-dehydro-PGB$_1$.

Trimers

The preliminary HPLC analysis of the trimer component revealed a very complex mixture that was consistent with the projection derived from the earlier analog investigation. The trimer component could be readily separated into two general fractions by reverse phase chromatography; the major fraction exhibited both 238 and 296 nm UVmax while the minor fraction showed only a 238 nm absorption. The UV and NMR data indicated the retention of a 13,14-unsaturation in the major fraction which would be formed from the addition of the C-10 enolate of a third unit of 15-dehydro-$PGB_1$ to dimers 1-4 in the manner illustrated in Figure 2. The minor fraction, which lacks a residual 13,14-unsaturation, would arise as the result of the initial C-16 enolate addition of a third unit of 13,14-dehydro-$PGE_1$ to dimers of the type 1-4 via the double addition pathway. In the initial mitochondrial assays, both fractions appeared to have similar levels of activity in the protection of oxidative phosphorylation which were on the same order or higher than that exhibited by standard PGBx.

Some preliminary studies of the separation of the trimeric component have been carried out and the separation of the mixture into individual components, although very difficult, appears to be feasible. After separation, the structural assignment of the individual isomers will be assisted by comparison to model system trimers where the use of the structurally modified analogs (cf. Section C) should prove of considerable benefit. The separation and characterization of the individual trimer components would provide for the first time a series of individual structurally defined oligomers that exhibit activity in the mitochondrial assay. In turn, the availability of structurally defined active components should permit the establishment of structure-activity relationships essential to the determination of the mode of activity characteristic of these oligomeric mixtures. It should be emphasized that the oligomeric mixtures which are obtained by treatment with 0.005 M ethanolic KOH are different from the complicated mixture termed PGBx. The treatment of 15-dehydro-$PGB_1$ free acid under such mild conditions results in the formation of a lower molecular weight oligomeric mixture much less structurally complicated than PGBx and from which individual oligomer components, such as dimers, trimers, tetramers..., can be readily isolated. This program for the isolation and characterization of individual active trimers will be the major focus of our 1982 investigation.

Tetramers

The tetramer component of the oligomeric mixtures derived from the treatment of 15-dehydro-$PGB_1$ free acid with 0.005 M ethanolic KOH has been separated relatively free of trimer and pentamer contamination directly from the oligomeric mixture by Sephadex LH-20 chromatography by the application of recycle techniques to enhance the separation. The tetramer fraction exhibited activity in the protection of oxidative phosphorylation on the same order or greater than that of standard PGBx. The initial HPLC analysis of the tetramer component revealed a very complicated mixture as was anticipated from the projections derived from the analog dimer studies discussed in Section A. The direct separation of this complex poorly resolved mixture did not appear to be a feasible approach.

As an alternative, a method was developed to convert pre-formed dimer into a mixture that was primarily tetramer along with a smaller amount of hexamer. The separation of pure tetramer from this mixture was readily accomplished in the absence of trimer and pentamer components. The tetramer component prepared in this manner exhibited the same level of activity as that isolated directly from the oligomeric mixture, i.e. on the same order or higher than standard PGBx.

If the structural elucidation of individual tetramers is undertaken in the future, the formation of a limited number of tetramers from a dimer of known structure would be the method of choice for the preparation of individual tetramers of known structure.

C. Modified Prostaglandin Analogs

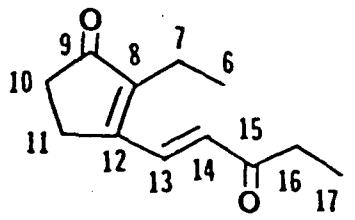
The structural modifications incorporated into the various analogs discussed in this Section, relative to the previously studied analog 2, were designed to eliminate or modify the reactivity of the multiple reaction sites available to 15-dehydro-$PGB_1$ and improve the spectroscopic characteristics useful for the analysis of trimers and higher oligomers.

We have now established that the formation of dimers from both the analog 2 and 15-dehydro-$PGB_1$ proceeds by Michael addition through two distinct nucleophilic sites (C-10 and C-16) and two acceptor (C-13 and C-14) sites. The structural complexity observed in the oligomeric mixtures derived from 1 and 2 originates in the simultaneous operation of these multiple reaction centers. Having defined the problem source, it has become feasible to design structural modifications in order to eliminate or modify the reactivity of some of the reaction centers in 1 and 2 which would result in the formation of less complicated oligomeric mixtures. A series of analogs have been prepared which incorporate structural modifications that: i) eliminate the double addition products resulting from C-16 enolate addition, ii) eliminate or substantially favor addition to one of the two acceptor sites, and iii) sharply reduce the number of closely related stereoisomers. The structural modifications were also designed with a view towards reasonable ease of incorporation into the prostaglandin skeleton.

The structural elucidation of a series of higher oligomers that are closely related structurally and stereochemically poses a particularly formidable problem. Our general approach has been to separate and characterize individual oligomer components derived from a structurally simpler analog precursor and use these as models for the structural assignment of the more complex oligomers derived from the prostaglandin 1. The application of this approach to the characterization of the dimer component derived from the analog 2, which was then used as a model for characterization of the dimer derived from the prostaglandin 1, proved very successful. The much greater complexity of the trimer and higher components places an even greater restriction on the selection of a suitable analog model system. For example, the $^1H$ NMR analysis of the trimers derived from analog 2 becomes extremely difficult unless each component can be obtained in a very pure state. The presence of a large number of structural isomers accompanied by closely related diastereomers with

very similar chromatographic properties make such clean separations very difficult. To alleviate some of the problems associated with the spectral analysis of impure compounds, it is desirable to provide a greater spectroscopic differentiation of the two side chains while retaining the structural features that serve as effective spectroscopic markers.

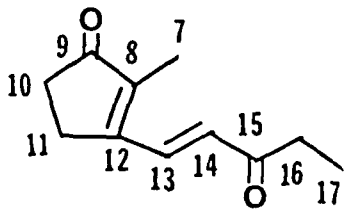
Analog 2




2

Analog 2, 3-(trans-3-keto-1-pentenyl)-2-ethyl-2-cyclopentenone, provided an excellent model for the initial studies of the pathway by which 15-dehydro-PGB1 was converted into complex oligomeric mixtures by treatment with ethanolic KOH. In the structural elucidation of the six dimers, the methyl ($-CH_3$) groups at C-6 and C-17 served as excellent spectroscopic markers of particular value in the analysis of the $^1H$ NMR spectra. The similar chemical shifts of the C-6 and C-17 multiplets make a similar spectroscopic analysis of the structurally more complex trimers and tetramers with six and eight methyls, respectively, considerably more difficult. In the structurally modified analogs described below, the methyl groups, which serve as spectroscopic markers, are substantially differentiated with respect to chemical shift and, in several cases, multiplicity. Also incorporated in analogs 4-7 are structural features designed to reduce the number of available reaction centers which should result in the formation of less complicated oligomeric mixtures.

Analog 3

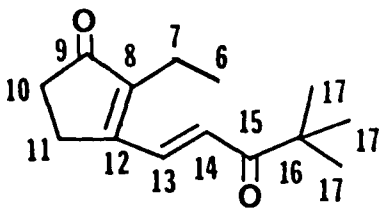



3

Analog 3, 3-(trans-3-keto-1-pentenyl)-2-methyl-2-cyclopentenone, differs structurally from analog 2 only in a modification of the alpha side chain. The differentiation in chemical shift of ca. 1 ppm and multiplicity (C-7 singlet and C-17 triplet), which are the result of this structural change, alleviates

many of the difficulties in the assignment of these resonances in the 360 MHz $^1H$ NMR spectra of individual trimers derived from analog 3 as compared to analog 2.

Analog 3 retains the same nucleophilic sites (C-10 and C-16) and acceptor sites (C-13 and C-14) of analog 2 and 15-dehydro-PGB1 so that both single addition and double addition type oligomers would be formed. The oligomerization of analog 3 under mild conditions has been carried out and determined to follow a reaction pathway similar to that of analog 2. The dimer component was separated and six dimers, corresponding to Dimers 1-6 from analog 2, were isolated and characterized. The trimer component as isolated was a complex mixture of single and double addition type oligomers. The complex trimer mixture could be further separated quite readily into single and double addition fractions. At present, analog 3 is viewed as the analog of choice to serve as the model for the structural elucidation of the double addition type trimer.

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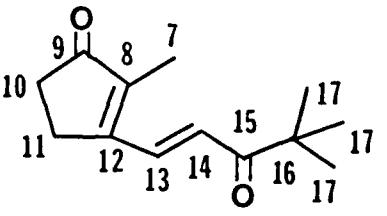


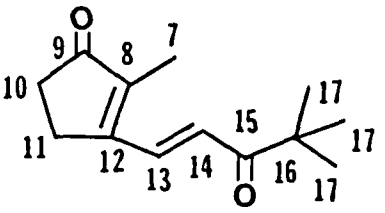

5

Analog 4

Analog 4, 3-(trans-3-keto-4,4-dimethyl-1-pentenyl)-2-ethyl-2-cyclopentenone, differs structurally from analog 2 by the replacement of the two C-16 hydrogens of 2 by methyl ($-CH_3$) groups. As a consequence the C-16 enolate can no longer be formed and all oligomeric product derived via the C-16 enolate would be absent in the oligomeric mixture. Analysis of the initial oligomerization reaction confirmed this expectation but the similarity in chemical shift of the C-7 and C-17 methyl resonances made the assignment of the less intense C-7 triplet quite difficult in the $^1H$ NMR spectra of the individual trimers. Because of this problem, analog 5 which has a greater difference in chemical shift between the C-7 and C-17 methyl resonances proved to be more suitable for spectroscopic studies.

Analog 5

Analog 5, 3-(trans-3-keto-4,4-dimethyl-1-pentenyl)-2-methyl-2-cyclopentenone, differs structurally from the previously discussed analog 4 by a simple modification of the alpha chain. As a result the C-7 and C-17 methyl groups in 5 are well separated in chemical shift in the $^1H$ NMR spectrum and the singlet multiplicity of both methyls further simplifies analysis in higher oligomers of these "marker" groups. As in analog 4, the substitution of two additional methyl groups at the C-16 position prevents the formation of the C-16 enolate effectively blocking all double addition pathways. Based on a consideration of molecular models, it also appeared likely that the increased steric hinderance experienced by C-14 in analog 5 might result in preferential addition to C-13.

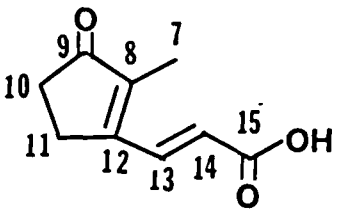



5

The oligomerization of the analog 5 was carried out using 0.005 M ethanolic KOH to give an oligomeric mixture that was readily separable into dimer, trimer, tetramer, and higher components by size exclusion chromatography on Sephadex LH-20. The HPLC analysis of the dimer component revealed three major and one minor (< 5%) components. The dimer fraction was separated by normal phase chromatography and the four components were readily characterized and corresponded to the previously characterized Dimers 1-4 derived from the analog 2 and 15-dehydro-PGB1. The absence of dimers corresponding to Dimers 5 and 6, the double addition dimers, results from the structural modification in analog 5 that eliminates the formation of the C-16 enolate. The difference in the product ratios of Dimers 1-4 derived from analog 2 (38% 1, 26% 2, 19% 3, and 16% 4) as compared to analog 5 (25% 1, < 5% 2, 35% 3, and 35% 4) results from the increased steric hinderance at C-14 due to the addition of the two new methyl groups at C-16. These results indicate the attainment of two of the projected goals of our modified analog program; the elimination of the double addition products arising from C-16 enolate addition and the selective enhancement of the reactivity of one of the two acceptor sites.

Two additional major benefits also were the result of the structural modifications that were incorporated into the analog 5. The elimination of the C-7 and C-16 methylene ($-CH_2-$) groups that are present in analog 2 resulted in a less complex $^1H$ NMR spectrum for the dimers derived from analog 5. As a result, a complete assignment of chemical shifts and coupling constants for the dimers derived from analog 5 has been possible. This data will provide the basis for the spectroscopic assignment of trimer stereochemistry. The structural modifications incorporated into the analog 5 also resulted in increased crystallinity and the dimers derived from the analog 5 have been obtained in crystalline form. The determination of the crystal structures by X-ray diffraction, which is now in progress, will provide unequivocal structures, including stereochemistry, for the diastereomeric dimers that are the immediate precursors to the trimers exhibiting mitochondrial activity. Since our previous work has generally indicated an increasing tendency for solid-state character with increasing oligomer size, a possibility exists that the trimers derived from the analog 5 will be crystalline and thus amenableto a complete structural determination by X-ray diffraction.

The analog 5 is presently our precursor of choice for the structural elucidation of the single addition type trimers in which a residual 13,14-unsaturation is retained.

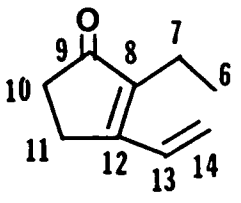



6

Analog 6

The structural modifications that were introduced into the analog 6, 3-(trans-2-carboxy-1-ethenyl)-2-ethyl-2-cyclopentenone, were intended to: i) eliminate the C-16 enolate pathway, ii) effect a difference in reactivity between the C-13 and C-14 acceptor sites, and iii) introduce the free carboxylic acid ($-CO_2H$) functionality into a structurally simpler oligomer. The absence of a C-16 carbon in analog 6 of course precludes the formation of a C-16 enolate and any product derived from that pathway. The anticipated difference in reactivity between the C-13 and C-14 acceptor sites of 6 derives from the difference in stability of the conjugated enolates that would be formed upon the initial addition of a C-10 nucleophile to either C-13 or C-14. The attack at C-14 of 6 would result in the formation of a stabilized enolate similar to that formed by C-10 enolate addition to C-14 of analog 2. In contrast, the addition of a C-10 nucleophile to C-13 of analog 6 gives an enolate, i.e. $-\dot{C}H-\ddot{C}H-CO_2^-$, that would be expected to be significantly less stable than the enolate derived from the addition to C-14. The combined effect of the elimination of the C-16 enolate pathway and the creation of a significant reactivity differential between the C-13 and C-14 acceptor sites should result in the formation of less complicated oligomeric mixtures. The inclusion of the $-CO_2H$ functionality in the analog precursor would insure the presence of this functionality in the oligomer, the presence of which appears to be a requirement for the operation of the mitochondrial protective effect.

The structural modifications introduced into the analog 6 resulted in a sharply reduced activity, relative to the analog 2, in the KOH promoted oligomerization. The oligomerization reaction was negligible using 0.005 M KOH but did proceed slowly at a 1 M KOH concentration. The oligomeric mixture obtained by treatment with 1 M ethanolic KOH was separated by Sephadex LH-20 chromatography in the usual manner. The initial $^1H$ NMR analysis of the dimer component was consistent with the expected reduction in the complexity of the oligomeric mixture. The results of the initial assay of the various oligomeric components for the mitochondrial protective effect were inconsistent and will require future reevaluation. Larger amounts of the analog 6 will be available in the near future for a more detailed evaluation of 6 as a potential precursor to a structurally less complicated but active oligomeric mixture.

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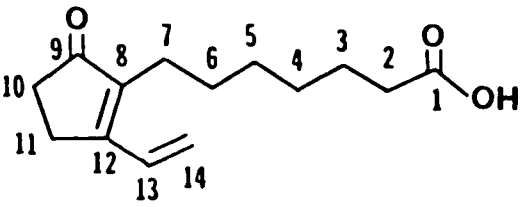


8

Analog 7

Potentially all three of the goals sought in an improved precursor to a structurally and stereochemically less complicated oligomeric mixture could be realized in the structurally simple analog 7, 3-vinyl-2-ethyl-2-cyclopentenone. The total absence of a C-16 carbon precludes any formation of a C-16 enolate and any oligomeric product derived from it. Nucleophilic attack at the C-14 acceptor site is vastly favored over that of C-13 due to the major difference in stability of the resultant anions formed; i.e. attack at C-13 leads to an unstable primary carbanion while C-14 attack would lead to the usual delocalized enolate. Since the attack of a C-10 enolate at the favored C-14 acceptor site would result in the formation of only one chiral center, the problems associated with the formation of closely related diastereomers, which have very similar chromatographic and spectroscopic properties, would be eliminated. Analog 7 itself is unlikely to be of value as a precursor to an oligomeric mixture having activity in the protection of oxidative phosphorylation because of the solubility properties and lack of the $-CO_2H$ functionality in the oligomeric mixtures derived from 7. The precursors with better potential will likely incorporate the structural advantages of analog 7 into the prostaglandin skeleton as is illustrated by analog 8.

The analog 7 has been prepared but the evaluation of its suitability as a potential precursor has not been completed. As was also the case for analog 6, analog 7 appears to require treatment with 1 M ethanolic KOH to effect reasonable rates of oligomerization.

## VI. EXPERIMENTAL

### A. Synthesis

The syntheses of analog 2 and 15-dehydro-PGB1 methyl ester (1) have been described previously.[1,9-11] The synthesis of 15-dehydro-PGB1 (free acid) was accomplished by minor modification of the synthetic sequence developed in our laboratories for the preparation of the 15-dehydro-PGE1 methyl ester.[1] The modification of the synthetic route required the development of new preparative HPLC separations that were primarily based on reverse phase chromatography. The syntheses of the modified prostaglandin analogs, i.e. analogs 3-7, were accomplished by modification of the synthetic route previously developed for the preparation of analog 2.[9-11] Purifications were carried out by normal phase preparative HPLC for analogs 3-5 and 7 while reverse phase chromatography was used in the case of analog 6.

### B. Oligomerizations

The general reaction conditions used for the oligomerization of analog 2 using both 1 M and 0.005 M ethanolic KOH have previously been described.[9] The crude oligomeric mixtures were chromatographed on Sephadex LH-20 using methanol as the carrier solvent.[9] In the case of the lower molecular weight oligomeric mixtures obtained from treatment with 0.005 M KOH, the direct separation into dimer, trimer, tetramer, and higher components was possible. Within an oligomeric fraction, e.g. the dimer fraction, the individual isomers were isolated by further preparative HPLC using either normal or reverse phase columns.

The individual isomers were characterized by 360 $^{1}H$ and $^{13}C$ NMR spectroscopy, high resolution mass spectrometry as well as IR and UV spectroscopy. The unequivocal $^{1}H$ and $^{13}C$ NMR assignments for the various analogs and 15-dehydro-PGB1 that were previously established in our laboratory were particularly useful in the structural elucidation of the individual oligomers.

### C. Mitochondrial Assays

Evaluation of the degree of protection and inhibition in the restoration of oxidative phosphorylation in isolated mitochondria was carried out by Dr. T. Devlin of Hahnemann Medical College, Philadelphia, PA.

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## VIII. ACKNOWLEDGEMENTS

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I would also like to gratefully acknowledge the generous assistance we have received in obtaining specialized spectroscopic data. The field desorption mass spectra of the lower molecular weight oligomeric mixtures were determined by R. Cotter of the Middle Atlantic Mass Spectrometry Laboratory, the Johns Hopkins University School of Medicine, Baltimore, MD. The high resolution mass spectrometric measurements were determined by D. T. Terwilliger of the Mass Spectrometry Laboratory, the University of Pennsylvania, Philadelphia, PA. The $^{13}C$ NMR spectra were recorded by M. Mutter, McNeil Pharmaceutical, Spring House, PA. The 360 MHz $^{1}H$ NMR spectra were determined by George McDonald, the Middle Atlantic NMR Facility, the University of Pennsylvania, Philadelphia, PA. The X-ray crystallographic structure determination of Dimer 6 was made by G. T. DeTitta, the Medical Foundation of Buffalo, Buffalo, NY.

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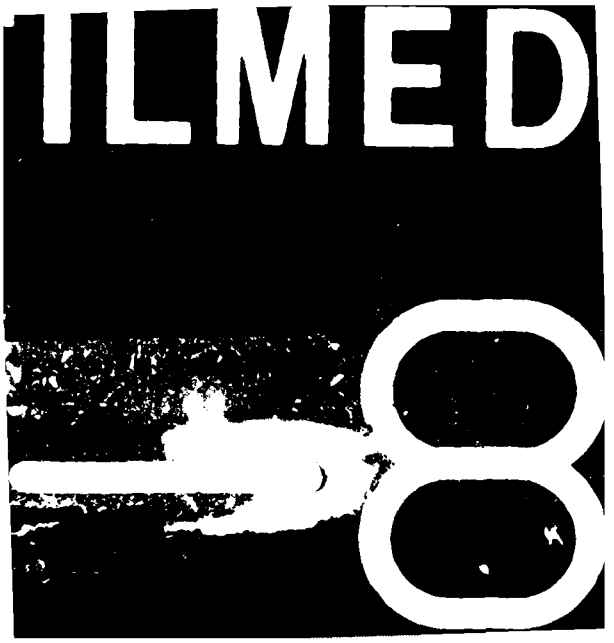
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