AD-A063 907 NAVAL AIR DEVELOPMENT CENTER WARMINSTER PA AIRCRAFT --ETC F/G 6/1
STUDIES ON PGBX A POLYMERIC DERIVATIVE OF PROSTAGLANDIN B1. I. --ETC(U)
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REPORT NO. NADC-78235-60

STUDIES ON PGB_x
A POLYMERIC DERIVATIVE OF PROSTAGLANDIN B_1:
I - SYNTHESIS AND PURIFICATION OF PGB_x

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30 OCTOBER 1978

PHASE REPORT
CONTRACT NO. N00014-78-WR800-19

Prepared for
OFFICE OF NAVAL RESEARCH
Department of the Navy
Arlington, Virginia 22217

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: NADC-78235-60
2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle): Studies on PGB_x A Polymeric Derivative of Prostaglandin B_1. I. Synthesis and Purification of PGB_x.

5. TYPE OF REPORT & PERIOD COVERED: Phase Report
6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s): B. David Polis, Sara F. Kwong, Edith Polis, George Nelson and Herman W. Shmukler

8. CONTRACT OR GRANT NUMBER(s): N00014-78-WR800-19

9. PERFORMING ORGANIZATION NAME AND ADDRESS:
Aircraft & Crew Systems Technology Directorate
Naval Air Development Center
Warminster, PA 18974

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

11. CONTROLLING OFFICE NAME AND ADDRESS:
Office of Naval Research
Department of the Navy
Arlington, VA 22217

12. REPORT DATE: 30 Oct 1978
13. NUMBER OF PAGES: 19

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report): UNCLASSIFIED
15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

PGB_x — PGB_1
Synthesis — Azelaic Acid
Purification

PGB sub x — PGB sub 1

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

PGB_x, a new polymeric derivative of PGB_1, previously was shown to restore oxidative phosphorylation to degraded isolated rat liver mitochondria *in vitro*, and to reverse the effects of cardiogenic ischemia in monkeys and cerebral ischemia in rabbits. This report describes in detail the synthesis and purification of PGB_x via PGB_1, starting with azelaic acid. In addition, details of the *in vitro* mitochondrial assay are reported. Purified PGB_x exhibiting maximal reactivation of mitochondrial phosphorylation has a mean →

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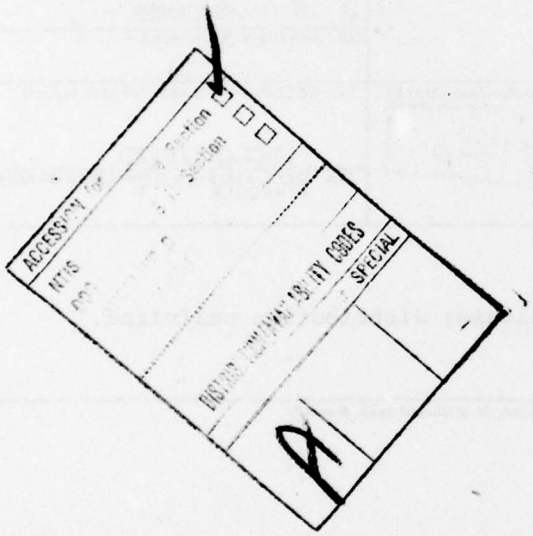
20. (Continued) molecular weight of 2350. The yield of PGB_x based on azelaic acid was 4% and based on PGB_1 was 25%.



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INTRODUCTION

A polymeric derivative of PGB_1, called PGB_x, was first synthesized by the late Dr. B. David Polis at NAVAIRDEVCEN and shown by him to have unique properties of restoring the in vitro phosphorylating ability of degraded rat liver mitochondria*[1]. He later showed this PGB_x to have unique properties of reversing the degenerative effects of experimentally induced myocardial ischemia in monkeys[2] and brain ischemia in rabbits[3].

The synthesis of PGB_x carried out by the late Dr. B. David Polis consisted of two main stages; (a) the synthesis of starting material (15-keto PGB_1 methyl ester) and (b) synthesis of PGB_x and its purification. The first stage was carried out in collaboration with Dr. G. Nelson at St. Joseph's College, Philadelphia, PA, who performed the synthetic steps while the purification of intermediates by large scale chromatography was devised and carried out solely at NAVAIRDEVCEN. The complete synthesis of PGB_x described here is therefore a compilation of the collaborative efforts of the two laboratories.

METHODS

Molecular Weights: Determined by vapor pressure osmometry at 60° using a Wescan Molecular Weight Apparatus.

Chromatography: A Waters Prep/LC Model 500 was used for large scale separations of PGB_x intermediates on silica gel Prep/pak 500. For molecular exclusion chromatography on Sephadex LH20 and PVP columns, a modular chromatograph was used which consisted of a Waters Co. pump, Chromatronix column, Waters Co. RI detector, Laboratory Data Control, 10 mv recorder and Buchler Fractomette fraction collector.

Isolation of Rat Liver Mitochondria: Mitochondria were isolated by a modification of the Hogeboom and Schneider method[4]. The procedure used in this study is described below. Rats were decapitated, and their livers excised as rapidly as possible and washed with 0.3 M sucrose (enzyme grade) containing 5×10^{-4} M EDTA pH 7.35. The livers were homogenized (glass barrel, teflon pestle) in the same solution and the mitochondria separated by differential centrifugation. The nuclei were sedimented at 1000g for 15 minutes. The yield of mitochondria was increased by rehomogenizing the nuclei in 3 volumes of sucrose-EDTA and centrifuging at 1000g. The 1000g supernatant layers were combined and centrifuged at 10,000g for 15 minutes to sediment the mitochondria. The mitochondrial pellet was homogenized in sucrose-EDTA and centrifuged at 6000g, rehomogenized in fresh sucrose-EDTA and centrifuged at

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*Raised arabic numberals indicate reference to bibliography except where raised numerals are obviously a superscript.

Note: Abbreviations used in this report are: PGB_1, prostaglandin B_1; EDTA, Ethylene diamine tetraacetic acid; AMP, ADP, ATP, Adenosine mono-, di-, and triphosphate respectively; PVP, polyvinyl pyrrolidone.

4000g. The supernatant layers from both the 10,000g and 6000g centrifugations were removed by aspiration while the 4000g supernatant layer was "poured hard" to remove the "fluffy layer." To increase the yield of mitochondria, the "fluffy layer" was homogenized with 2 volumes of sucrose-EDTA and centrifuged at 6000g. The 6000g supernatant layer was "poured hard" and the pellets from both the 4000g and 6000g centrifugations were homogenized in sucrose-EDTA and centrifuged at 600g to separate any cellular debris or nuclei that might still remain. After determining the protein content by the Biuret method[5], the mitochondrial suspension was diluted with cold-sucrose-EDTA to make a final concentration of 100mg protein per ml. Usually 1.3g of mitochondria were isolated from a 100g of rat liver. The mitochondria were then stored at 4° until used.

Assay of PGB_x Effect: The PGB_x activation of oxidative phosphorylation of degraded mitochondria was first reported by Polis et al[1] in 1973. In this report the Warburg technique was used and methodology employed was described very briefly. The current methodology used in the ACSTD laboratory to demonstrate the PGB_x effect on aged mitochondria is described below, in detail. The PGB_x activation of mitochondrial oxidative phosphorylation was demonstrated by first aging the mitochondria at 4° for at least 3 days and then degrading further by incubation at 28° in the absence of phosphate acceptor (ADP). Since the degree of degradation required for the PGB_x effect varied with each mitochondrial preparation as well as the time of storage, preliminary incubations of varying times were run to determine the optimum degree of degradation. For this purpose an aliquot of aged mitochondria (usually the amount needed for one day's use) was diluted with cold distilled water and centrifuged at 6000g. The supernatant was removed and an equivalent volume of 0.15 M sucrose + 2.5×10^{-4} M EDTA was added and the mitochondria suspended by gentle mechanical mixing. The optimal degradation time was determined by adding 4 mg of mitochondria each into 4 beakers (10 ml) containing 0.1 ml of 0.1 M phosphate buffer pH 7.35, 0.15 ml of 0.2 M α-ketoglutarate pH 7.35, 0.1 ml of 0.1 M $MgSO_4$ and water to a total volume of 2.01 ml. The beakers were covered and shaken at 28° for 5, 10, 15 and 20 minutes. At the end of each time period 0.15 ml of a mixture containing 0.0333 M ADP, 0.0333 M AMP and 0.66 M KCl was added followed immediately with 0.04 ml of 3.75 percent bovine serum albumin to give a final volume of 2.2 ml. (The order of addition and the composition of the reactants are summarized in table I.) The shaking was then continued for 20 minutes at which time the reaction was terminated by the addition of 0.5 ml of 31 percent $HClO_4$. The inorganic phosphate concentration was then determined in the protein free filtrate, by the method of Dreisbach[6], which was carried out as follows: 0.5 ml aliquot of protein free filtrate was added to 3.5 ml of H_2O, 1 ml of 10 percent ammonium molybdate in 5 N H_2SO_4 and 5 ml isobutanol. The mixture was shaken thoroughly and the phases allowed to separate. 0.5 ml of the isobutanol layer was diluted to 5.0 ml with 3.2 percent H_2SO_4 in ethanol and the absorbance measured at 310 nm. The phosphate disappearance (or phosphate esterified) was calculated by difference from the phosphate concentration found in each beaker at the end of the reaction time period to that in which no reaction had taken place; i.e., a reaction beaker in which perchloric acid was added prior to the addition of the mitochondria. The degradation time used to show the PGB_x effect was chosen as the minimum time required to reduce the level of phosphorylation to less than 5 percent of the level found with nondegraded mitochondria (figure 1). Thus having established the condi-

tion for mitochondrial degeneration to near zero phosphorylation, the PGB_X effect was shown by adding varying amounts of PGB_X, e.g., 2, 4, 6, 8μg during the predetermined incubation period and prior to the addition of phosphate acceptor (figure 2).

TABLE I

The Composition of the Medium for the Demonstration of the PGB_X Effect on Mitochondrial Oxidative Phosphorylation

Order of Addition	Mitochondrial Degrading Medium	Reaction Mixture
Water	1.55 ml	1.55 ml
Phosphate Buffer pH 7.35	4.98 mM	4.55 mM
α-Ketoglutarate pH 7.35	14.93 mM	13.64 mM
$MgSO_4$	4.98 mM	4.55 mM
Aged Mitochondria	1.99 mg/ml	1.82 mg/ml
Sucrose*	5.97 mM	5.45 mM
EDTA*	0.010 mM	0.009 mM
AMP	-----	2.27 mM
ADP	-----	2.27 mM
KCl	-----	45.45 mM
Bovine Serum Albumin	-----	0.68 mg/ml

Total Volume: 2.20 ml Degradation Time: 5-20 minutes

Temperature: 28° Reaction Time: 20 minutes

*Added with mitochondria

Quantification of PGB_X Activity: As seen in figure 2, in the absence of PGB_X the ability of degraded mitochondria to carry out oxidative phosphorylation is markedly inhibited. When PGB_X was added in small increments, a recovery of the phosphorylation ability was noted after a short induction period (0-2μg). At the level of 2-4μg PGB_X, a sharp increase in phosphorylation was found — which then leveled off above 4μg of PGB_X. This "S" shaped curve imposed difficulties for simple quantification of the PGB_X effect. To overcome these difficulties and to introduce a measure of quantitation, a unit of PGB_X was arbitrarily defined as the ratio of the slopes obtained for the plot of

$$\log_{10} \frac{\text{μmoles phosphate esterified}}{\text{μmoles maximum phosphate esterified}}$$

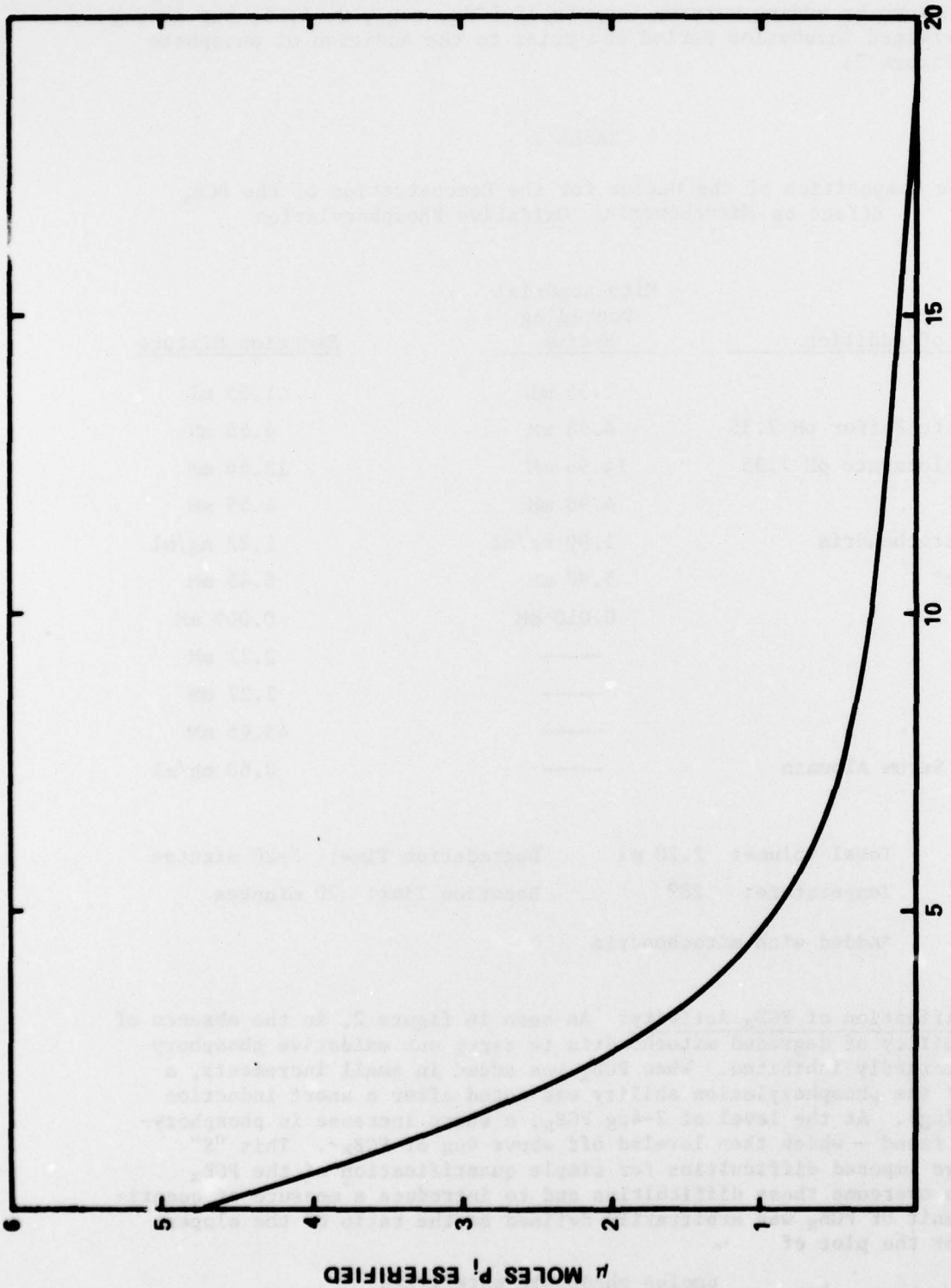


FIGURE 1 - Decrease in Oxidative-Phosphorylation of Aged Mitochondria Preincubated at 28° in Absence of Inorganic Phosphate and Phosphate Acceptor.

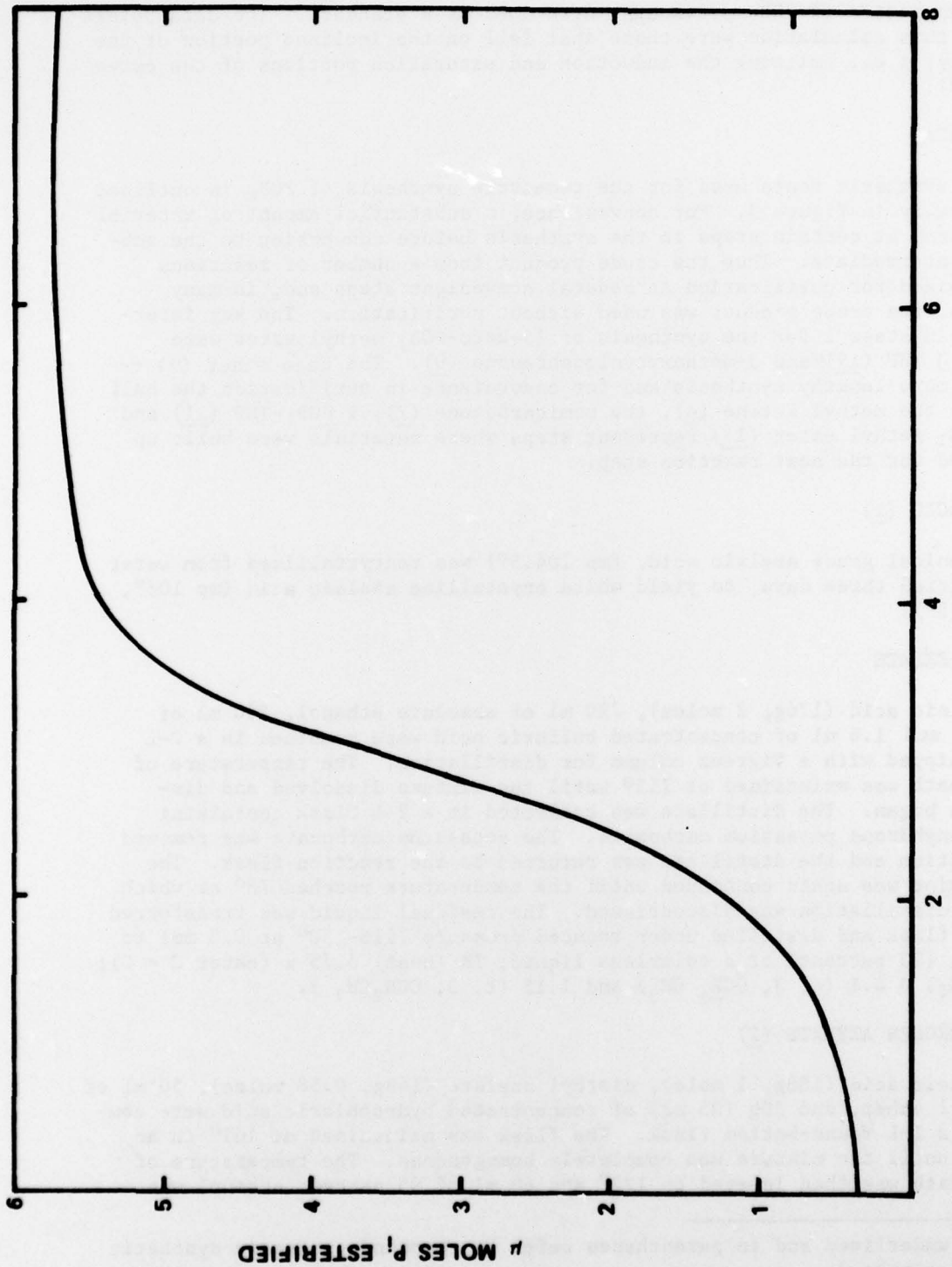


FIGURE 2 - Conservation of Oxidative Phosphorylation in Aged Degraded Rat Liver Mitochondria by PGB_x.

as a function of μg PGB_x used for the assay of unknown PGB_x, to that obtained for a preparation of PGB_x previously described as a standard. The data points used for this calculation were those that fell on the inclined portion of the curve only; i.e., omitting the induction and saturation portions of the curve (figure 2).

EXPERIMENTAL

The synthetic route used for the two-stage synthesis of PGB_x is outlined schematically in figure 3. For convenience, a substantial amount of material was prepared at certain steps in the synthesis before conversion to the subsequent intermediate. Thus the crude product from a number of reactions were combined for purification at several convenient steps and, in many instances, the crude product was used without purification. The key intermediates in stage 1 for the synthesis of 15-keto-PGB_1 methyl ester were octyn-3-ol THP (19)* and 3-methoxycyclopentenone (9). The keto ether (9) requires a more lengthy synthesis and for convenience in purification the half acid (2), the methyl ketone (4), the semicarbazone (7), ≡ PGB_1-THP (11) and trans-PGB_1 methyl ester (15) represent steps where materials were built up and stored for the next reaction step.

AZELAIC ACID (1)

Technical grade azelaic acid, (mp 104.5°) was recrystallized from water and air dried three days, to yield white crystalline azelaic acid (mp 106°, *lit.* 106.5°).

DIETHYL AZELATE

Azelaic acid (176g, 2 moles), 720 ml of absolute ethanol, 360 ml of toluene, and 1.6 ml of concentrated sulfuric acid were combined in a 2-L flask equipped with a Vigreux column for distillation. The temperature of the oil bath was maintained at 115° until the mixture dissolved and distillation began. The distillate was collected in a 2-L flask containing 300g of anyhdrous potassium carbonate. The potassium carbonate was removed by filtration and the distillate was returned to the reaction flask. The distillation was again continued until the temperature reached 78° at which time the distillation was discontinued. The residual liquid was transferred to a 1-L flask and distilled under reduced pressure (116-130° at 0.3 mm) to give 463g (93 percent) of a colorless liquid; IR (neat) 5.75 μ (ester C = O); NMR ($CDCl_3$) δ 4.1 (q, 3, OCH_2 CH_3) and 1.15 (t, 3, OCH_2CH_3).

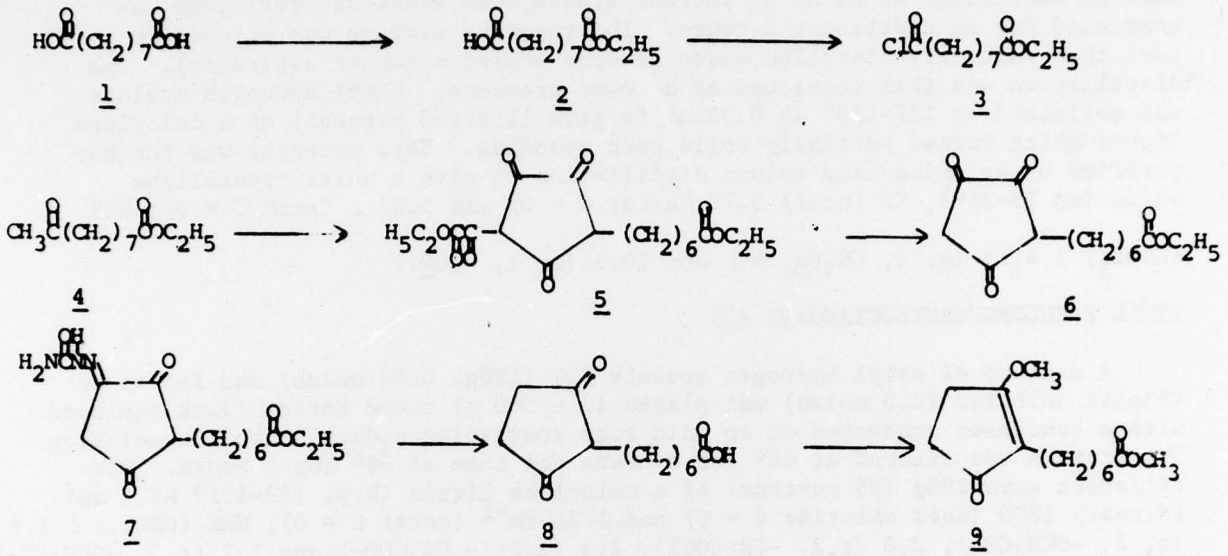
ETHYL HYDROGEN AZELATE (2)

Azelaic acid (188g, 1 mole), diethyl azelate (143g, 0.58 moles), 50 ml of di-n-butyl ether, and 30g (25 ml) of concentrated hydrochloric acid were combined in a 1-L round-bottom flask. The flask was maintained at 165° in an oil bath until the mixture was completely homogeneous. The temperature of the oil bath was then lowered to 120° and 60 ml of 95 percent ethanol was

*Numbers underlined and in parentheses refer to compound number in synthetic scheme in figure 3.

Schematic Preparation of PGB_x

PART I



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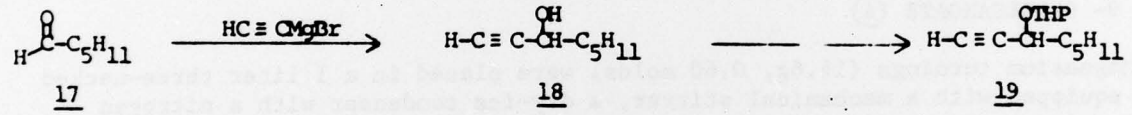

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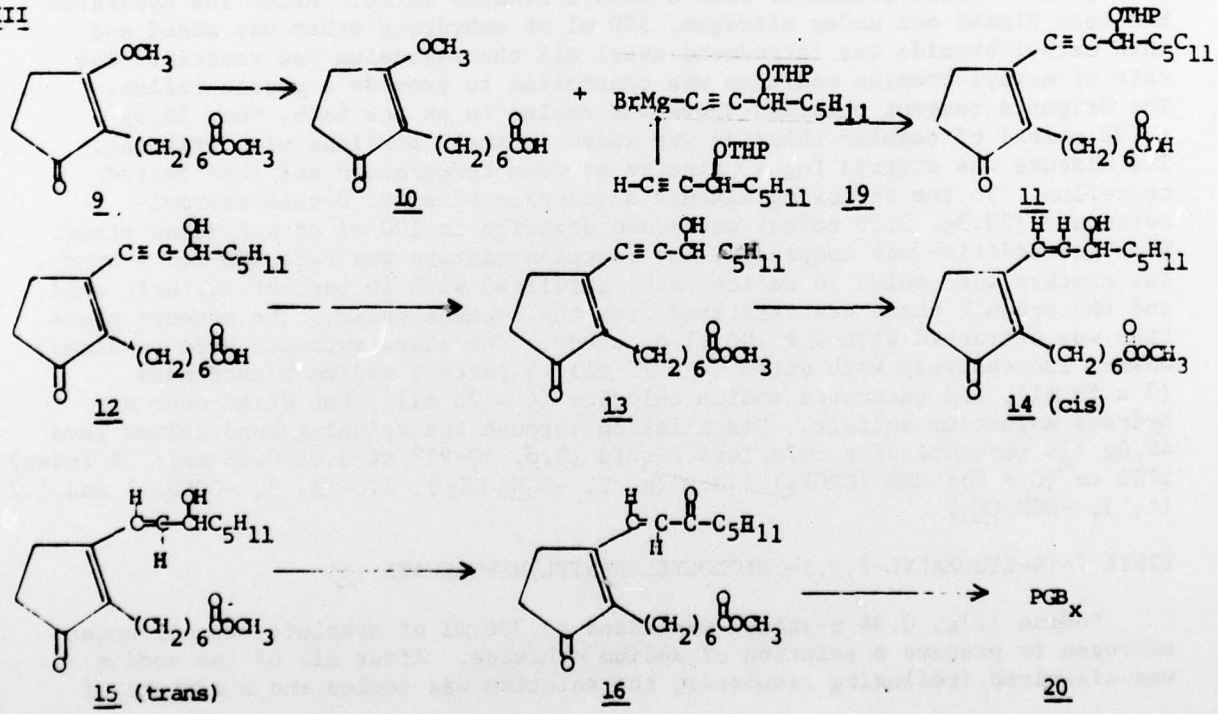

FIGURE 3 - Schematic Diagram of Synthesis of PGB_x from Azelaic Acid.

added through the condenser. The mixture was allowed to reflux for 3 hours, then an additional 20 ml of 95 percent ethanol was added and refluxing was continued for an additional 2 hours. The reaction mixture was allowed to cool then initially distilled under reduced pressure (water aspirator). The distillation was then continued at a lower pressure. Ethyl hydrogen azelate was collected at 157-159° at 0.35 mm to give 115g (53 percent) of a colorless liquid which turned partially solid upon standing. This material was further purified by spinning-band column distillation to give a white crystalline solid (mp 25-26°); IR (neat) 5.75 (ester C = O) and 5.87 μ (acid C = O); NMR ($CDCl_3$) δ 4.15 (q, 2, $CH_3\underline{CH_2}$ O-) and 10.6 (s, 1, $-\overset{O}{\overset{\|}{C}}O\underline{H}$.)

ETHYL 8-CHLOROFORMYLOCTANOATE (3)

A mixture of ethyl hydrogen azelate (2) (180g, 0.84 moles) and 145 ml of thionyl chloride (2.0 moles) was placed in a 500 ml round bottom flask equipped with a condenser connected to an acid trap containing sodium hydroxide solution. The mixture was stirred at 60° for 4 hours and then at 90° for 4 hours. Distillation gave 198g (85 percent) of a colorless liquid (b.p. 153-155° at 1 mm): IR(neat) 1800 (acid chloride C = O) and 1730 cm^{-1} (ester C = O); NMR ($CDCl_3$) δ 3.9 (q, 2, $-O\underline{CH_2}CH_3$), 2,8 (t,2, $-\underline{CH_2}COCl$), 2,3 (t,2, $-\underline{CH_2}COO-$) and 1.2 (t,3, $-OCH_2\underline{CH_3}$).

ETHYL 9- OXODECANOATE (4)

Magnesium turnings (14.6g, 0.60 moles) were placed in a 1 liter three-necked flask equipped with a mechanical stirrer, a dry-ice condenser with a nitrogen inlet, and a water condenser with a methyl bromide inlet. After the apparatus had been flamed out under nitrogen, 300 ml of anhydrous ether was added and then methyl bromide was introduced until all the magnesium had reacted. The rate of methyl bromide addition was controlled to provide a gentle reflux. The Grignard reagent obtained above was cooled in an ice bath, then 58.5g (0.32 moles) of cadmium chloride was added in small portions with stirring. The mixture was stirred for 10 minutes at room temperature and then heated to reflux. To the refluxing mixture a solution of ethyl 8-chloroformyl-octanoate (70.3g, 0.30 moles) was added dropwise in 100 ml of anhydrous ether. When the addition was completed, the reaction mixture was refluxed for 4 hours. The mixture was cooled in an ice bath, acidified with 10 percent sulfuric acid, and the organic phase was separated from the aqueous phase. The aqueous phase then was extracted with 4 x 100 ml of ether. The ether extracts were combined; washed successively with water (4 x 75 ml), 5 percent sodium bicarbonate (3 x 50 ml), and saturated sodium chloride (4 x 75 ml); then dried over anhydrous magnesium sulfate. Distillation through the spinning-band column gave 48.0g (75 percent) of a colorless liquid (b.p. 90-92° at 0.05-0.06 mm); IR (neat) 1720 cm^{-1} (C = O); NMR ($CDCl_3$) δ 3.9 (q, 2, $-O\underline{CH_2}\ CH_3$), 2.0 (s, 3, $-COCH_3$) and 1.2 (t, 3, $-OCH_2\underline{CH_3}$).

ETHYL 7-(4-ETHOXALYL-2,3,5-TRIOXOCYCLOPENTYL)HEPTANOATE (5)

Sodium (21g, 0.94 g-atoms) was added to 300 ml of absolute ethanol under nitrogen to prepare a solution of sodium ethoxide. After all of the sodium was dissolved (refluxing required), the solution was cooled and a mixture of

ethyl 9-oxodecanoate (4) (59g, 0.27 moles) and diethyl oxalate (92g, 0.65 moles) was added dropwise under a nitrogen atmosphere. After the addition was complete, the reaction mixture was stirred at room temperature for 1 hour and then at reflux for 1 hour. The reaction mixture was cooled and cautiously acidified with ca. 50 ml of 15.6 N sulfuric acid. The solution was filtered through a layer of Super-Cel (Johns-Manville) to remove the sodium sulfate precipitate which resulted from acidification. The filtrate was concentrated under vacuum and the residue dissolved in 300 ml of ether and extracted with a 5 percent sodium bicarbonate (2 x 200 ml and 5 x 100 ml). The combined aqueous extracts were acidified with 10 percent sulfuric acid and extracted with ether (5 x 200 ml). The ether extracts were dried over anhydrous magnesium sulfate and the solvent removed under vacuum to yield 96g (96 percent) of a reddish viscous product; UV (95 percent C_2H_5OH) 252 and 322 nm; IR (neat) 3300 (enol OH), 1730 and 1670 cm^{-1} (C = O); NMR ($CDCl_3$) δ 10.6 (s, 2, enol OH) and 4.1 (m, 4, $-\overset{O}{\overset{\|}{C}}\overset{O}{\overset{\|}{C}}OCH_2\ CH_3$ and $\overset{O}{\overset{\|}{C}}OCH_2\ CH_3$ and $COCH_2\ CH_3$). This material was used in the following step without further purification.

ETHYL 7-(2,3,5-TRIOXYOCYCLOPENTYL)HEPTANOATE (6)

A mixture of crude ethyl 7-(4-ethoxalyl-2,3,5-trioxocyclopentyl)heptanoate (5) (96g), 200 ml of 95 percent ethanol and 200 ml of 6 N hydrochloric acid was heated and stirred at reflux (bath temperature 100°) for 5 hours. The reaction mixture was then concentrated under vacuum and the residue was extracted with 3 x 150 ml of ether. The combined ether extracts were washed with 2 x 75 ml of water, dried over anhydrous magnesium sulfate and concentrated under vacuum to give a reddish oily product (82g, 115 percent): UV maximum (95 percent ethanol) 266 and 323 nm; IR (neat) 3200 (enol OH), 1730, 1680, and 1650 cm^{-1} (C = O); NMR ($CDCl_3$) δ 10.1 (s, 2, enol OH), 4.2 (q, 2, $-OCH_2\ CH_3$) and 3.0 (s, 2, methylene at 4 position).

ETHYL 7-(2,5-DIOXO-3-SEMICARBAZONOCYCLOPENTYL)HEPTANOATE (7)

To a solution of crude trione (6) (82g) in 300 ml of 95 percent ethanol, a solution of semicarbazide hydrochloride (42g, 0.38 moles) and sodium acetate (59g, 0.72 mole) in 445 ml of water was added with stirring. The reaction mixture was then stirred for 1 hour and diluted with 100 ml of water and allowed to stand overnight. The filtered precipitate was washed with water, then with 3 x 100 ml of methanol and dried under vacuum to yield 61g (68 percent based on 5) of a powdery yellow product (m.p. 246-248°); IR (nujol) 3350 (NH), 1760, 1710, 1660 and 1605 cm^{-1} (C = O).

7-(2,5-DIOXOCYCLOPENTYL)HEPTANOIC ACID (8)

The semicarbazone (7) (61g) from the above procedure was added to a 1 liter round-bottom flask containing a solution of potassium hydroxide (94g, 1.7 moles) in 366 ml of ethylene glycol. The mixture was gradually heated with stirring to 160° over a 1 hour period and was then refluxed for an additional 6 hours at 192°. The solvent was removed under vacuum and the residue dissolved in 150 ml of water. The resulting solution was refluxed for 45 minutes. After the solution was cooled, it was acidified with hydrochloric acid (ca. 150 ml)

and then filtered. The precipitate was washed with cold water and dried under vacuum to give 38g (86 percent) of a powdery light brown product (m.p. 120-125°): IR (KBr) 3200-2300 (enol and acid OH), 1700 (carboxy C = O), 1610 (ketonic C = O) and 1550 cm^{-1} β-diketone (enolic) C = O.

METHYL 7-(2-METHOXY-5-OXOCYCLOPENTENYL)HEPTANOATE (9)

(a) Crude 7-(2,5-dioxocyclopentenyl)heptanoic acid (8), 282g from the previous step, was combined with 600 ml of methanol, 600 ml of chloroform and 8 ml of concentrated sulfuric acid and refluxed ca. 48 hours. The reaction product, after cooling, was poured into a mixture of 300g cracked ice and 1.2 L water. The aqueous layer was separated and washed with two additional portions of chloroform. The combined organic extracts were then washed with water. After removal of the chloroform under vacuum, 275g (94 percent based on simple esterification) was obtained. This material was used in the following procedure without purification.

(b) The crude ester from the above procedure was treated in 30g portions with ethereal diazomethane generated from 50g of Diazald (Aldrich Chemical Co., Milwaukee, Wisc.). After removal of the solvent ca. 32g of crude (9) was obtained.

The crude dark oily (9) was distilled in 100g amounts (b.p. 160-165° at 0.1 mm) to give 78-80 percent of a light yellow oil which crystallized upon standing in the freezer: UV max (95 percent ethanol) 251 nm; IR (neat) 1730 (ester C = O), 1680 (cyclopentenone C = O) and 1630 (enolic double bond), 1370 and 1270 cm^{-1} (enol ether); NMR ($CDCl_3$) δ 3.7 (s, 3, $-OCH_3$) and 3.4 (s, 3, $-COOCH_3$).

OCT-1-YN-3-OL (18)

Magnesium turnings (30g, 1.23 moles) were placed in a three-necked round-bottom flask, equipped with a mechanical stirrer, reflux condenser and dropping funnel. The flask was flamed out under nitrogen and to it was added 200 ml of dry tetrahydrofuran. Ethyl bromide (120g, 1.1 moles) was dissolved in 400 ml of dry tetrahydrofuran and this solution was added dropwise to the magnesium turnings. After completion of dropwise ethyl bromide addition, the reaction mixture was heated on the steam bath for 2 hours. In the meantime, a 2-liter round-bottom flask equipped with a stirring bar, inlet tube, and dropping funnel was flamed out under nitrogen, and to it was added 600 ml of dry tetrahydrofuran. After the reaction flask was cooled, acetylene was passed through a dry-ice acetone trap and bubbled into the reaction vessel. The solution of ethyl magnesium bromide which had been previously prepared was cooled and rapidly transferred to a 500 ml dropping funnel. The ethyl magnesium bromide was slowly added dropwise to the acetylene solution over a period of 2 hours. After the addition of the ethyl magnesium bromide was complete, 100g (1.0 mole) of hexanal (17) in 100 ml of dry tetrahydrofuran was added dropwise with stirring to the above mixture over a period of 1 hour. The addition of acetylene was stopped and the reaction mixture was cooled to room temperature and then stirred overnight. A solution of 200g of ammonium chloride in 100 ml of water was then added gradually to the reaction mixture. The tetrahydrofuran layer was separated and the solvent was removed under vacuum. The water layer was extracted with 4 x 100 ml of chloroform and the

combined extracts were added to the crude alcohol. The combined material was washed with water until neutral and then dried over anhydrous $MgSO_4$. After removal of the solvent, 134g of crude alcohol was obtained. On distillation of crude material, 103.3g (82 percent) of a colorless liquid (35° at 0.2 mm) was obtained: IR (neat) 3.0 (hydroxyl and acetylene C-H) and 4.7 μ (acetylene stretch); NMR ($CDCl_3$) δ 4.15 (d of t, 1, $-C\equiv C-\underset{OH}{\underline{C}}H$), 2.2 (d, 1, J=3, $\underline{H}-C\equiv C-\underset{OH}{\underline{C}}H-$) and 0.95 (t,3, $-CH_2\underline{CH_3}$).

2-OCT-1-YN-3-YLOXY) TETRAHYDROPYRAN (19)

Concentrated hydrochloric acid (0.7 ml) was added dropwise with stirring to a mixture of 126g (1 mole) of oct-1-yn-3-ol (18) and 92g of dihydropyran and allowed to stand at room temperature overnight. The reaction mixture was then diluted with 150 ml of ether, washed with 10 percent sodium bicarbonate and dried over anhydrous $MgSO_4$. Removal of drying agent by filtration and the solvent under vacuum, yielded 219g crude THP derivative (19). On distillation of the crude material, 208.1g (99 percent, 72° at 1 mm) of colorless liquid was obtained: IR (neat) 3.0 (sharp $-C\equiv C-\underline{H}$) and 4.7 μ (weak C=C stretch); NMR ($CDCl_3$) δ 3.2-5.0 (complex absorption envelope, 4, $-C\equiv C-\underline{CH}-O$, $-\underline{CH}-O$, and $-\underline{CH_2}-O$), 2.2 (t, 1, $\underline{H}-C\equiv C-\underset{H}{\underline{C}}-$, diastereomeric) and 0.9 (t, 3, $-CH_2\underline{CH_3}$).

7-(2-METHOXY-5-OXOCYCLOPENTENYL)HEPTANOIC ACID (10)

A mixture containing 76.3g (0.3 moles) of methyl 7-(2-methoxy-5-oxocyclopentenyl)heptanoate (9), 1.5 L of 95 percent ethanol and 3.2 L of 0.1 N sodium hydroxide (0.32 moles) was stored at room temperature for two days. Ethanol was removed under vacuum and the aqueous residue was washed with ether. The aqueous solution was then acidified with 0.5 N hydrochloric acid to pH 5, and extracted with ethyl acetate (2 x 150 ml). The aqueous solution was further acidified to pH 3 and again extracted with ethyl acetate (3 x 100 ml). The combined ethyl acetate was removed under vacuum. A reddish oily product (70g, 97 percent) was obtained: UV max (95 percent ethanol) 251 nm; IR (neat) 3300-2600 (acid OH), 1720 (carbonyl C=O), 1603 (enolic double bond, 1370 and 1270 cm^{-1} (enol ether); NMR ($CDCl_3$) δ 10.9 (s, 1, $-COO\underline{H}$) and 3.8 δ (s, 3, $-O\underline{CH_3}$).

7-(2-(3-(2-TETRAHYDROPYRANOXY)-1-OCTYNYL)-5-OXOCYCLOPENTENYL)HEPTANOIC ACID (11)

To a solution of 3-(2'-tetrahydropyranoxy)-1-octyne (19) (315g, 1.5 moles) in 500 ml of dry tetrahydrofuran was added dropwise 500 ml of 3.0 M ethereal ethyl magnesium bromide (1.5 moles) under nitrogen. The mixture was refluxed for 2 hours, then cooled in an ice bath. To the Grignard reagent prepared above was added dropwise, a solution of 7-(2-methoxy-5-oxocyclopentenyl)heptanoic acid (10) (70g, ∿ 0.3 moles) in 250 ml of dry tetrahydrofuran under nitrogen. The resulting reaction mixture was stirred overnight at room temperature. The reaction mixture was then poured into 1 liter of cold water, acidified with 2 N hydrochloric acid, and then extracted with ether (4 x 150 ml). The combined ether layers were washed with 1 N sodium hydroxide (4 x 150 ml). The aqueous layers were washed with ether (2 x 150 ml), acidified with 2 N hydrochloric acid, and extracted with 4 x 150 ml of ether. The combined ether extracts were washed with 5 x 100 ml of water and dried over anhydrous magnesium sulfate. The solvent was removed under vacuum. The crude dark reddish oily product (100g ca. 90 percent) was used directly in the following procedure. UV max (95 percent

ethanol) 269 nm; NMR ($CDCl_3$) δ 4.8-3.3 (complex absorption, 4, $C{\equiv}C\underline{C}\overset{O^-}{\underline{H}}-$, $-O\underline{C}\underline{H}O-$, $-\underline{C}\underline{H}_2O-$) and 0.9 (t, 3, $-CH_2\underline{C}\underline{H}_3$).

7-(2-(3-HYDROXY-1-OCTYNYL)-5-OXOCYCLOPENTENYL)HEPTANOIC ACID (12)

A mixture containing 110g of the THP derivative (11), 200 ml of tetrahydrofuran, 600 ml of glacial acetic acid and 200 ml of water was stirred overnight at 50°. The reaction mixture was added to 2.5 L of cold water and then extracted with a total of 500 ml of chloroform. The combined chloroform extracts were washed with a total of 2 liters of water. Removal of the chloroform under vacuum gave 90g of crude (12).

In a preliminary purification to remove polymeric contaminants, 90g of crude (12) was slurried with 75g of PVP and extracted with 1,2 dichloroethane in a continuous extractor. The solvent was removed by flash evaporation and the residue chromatographed on preparative columns of silica gel using a stepwise gradient of ethyl acetate in cyclohexane. Purified (12) eluted between 15 and 20 percent ethyl acetate. The solvent was removed by flash evaporation and the purified product (12) was stored for later conversion to the methyl ester. UV max (95 percent ethanol) 269 nm; IR (neat) 3400 (alcohol OH), 3300-2600 (broad, acid OH), 2200 (weak, C≡C), 1710 (C=O) and 1610 cm^{-1} (conjugated double bond; NMR ($CDCl_3$) δ 6.45 (s, 2, acid and alcohol OH), 4.75 (t, 1, $\equiv C\underline{C}\overset{OH}{\underline{H}}CH_2-$) and 0.85 (t, 3, $-CH_2\underline{C}\underline{H}_3$).

METHYL 7-(2-(3-HYDROXY-1-OCTYNYL)-5-OXOCYCLOPENTENYL)HEPTANOATE (13)

A solution of ethereal diazomethane (prepared from 50g of Diazald) was added dropwise to a cooled solution of 50g of ≡ PGB_1 (12) in 100 ml of ethyl ether. After completion of the addition, acetic acid was added to destroy excess diazomethane. The ethereal solution was extracted with a total of 150 ml of 5 percent $NaHCO_3$ followed by 100 ml of water. The ether was removed under vacuum with the residual water removed by the addition of chloroform. In this manner, ca. 52g of the methyl ester (13) was obtained. UV max (95 percent ethanol) 269 nm; IR (neat) 3300 (alcohol OH), 2200 (weak, C≡C), 1750 (ester C=O), 1710 (C=O) and 1610 cm^{-1} (conjugated double bond); NMR ($CDCl_3$) δ 4.6 (t, 1, $\equiv C\underline{C}\overset{OH}{\underline{H}}CH_2-$), 3.6 (s, 3, ester $O\underline{C}\underline{H}_3$) 3.9 (s, 1, $-CH_2\underline{C}\underline{H}_3$). This material was hydrogenated without purification.

METHYL 7-(2-CIS-3-HYDROXY-1-OCTENYL)-5-OXOCYCLOPENTENYL)HEPTANOATE (CIS-PGB_1 METHYL ESTER) (14)

Palladium on barium sulfate (5 percent, 2.5g), 0.75 ml of quinoline and 50 ml of methanol were combined in a 500 ml hydrogenation vessel (Joshel apparatus) and equilibrated overnight. A solution of 50g of (13) in 40 ml of methanol was introduced and was hydrogenated to 90 percent of theoretical hydrogen uptake. The catalyst was removed by filtration and the methanol removed under vacuum. The resulting residue was dissolved in 200 ml of ethyl ether, washed with 0.1 N hydrochloric acid (3 x 30 ml) and water (3 x 50 ml). The ether was removed under reduced pressure with chloroform added to remove residual water to give ca. 50 g of residue. This material was combined with

the product of similar hydrogenations and chromatographed on PVP and silica gel as described in the previous step. UV max (95 percent ethanol) 276 nm; IR (neat) 3300 (alcohol OH), 1750 (ester C=O), 1700 (conjugated C=O), 1620 and 1570 cm^{-1} (C=C stretch); NMR ($CDCl_3$) δ 6.4 (d, 1, J=12 Hz, -CH=CH-CH(OH)-), 5.8 (d of d, 1, J=12 and 9 Hz, -CH=CH(OH)-CH-), 4.6 (m, 1, -CH=CH-CH(OH)-), 4.0 (s, 1, OH) 3.6 (s, 3, ester $-OCH_3$) and 0.85 (t, 3, $-CH_2CH_3$).

METHYL 7-(2-(TRANS-3-HYDROXY-1-OCTENYL)-5-OXOCYCLOPENTENYL)HEPTANOATE (TRANS-PGB_1 METHYL ESTER) (15)

One hundred grams of cis-PGB_1 methyl ester (14) was dissolved in 400 ml of cyclohexane and 20 ml of chloroform to give a clear solution. To this solution was added 100 ml of 0.5 percent (w/v) solution of iodine in cyclohexane/chloroform (20:1, v/v) and the reaction mixture was stored in the dark. The progress of the isomerization was periodically monitored by NMR and if the isomerization was not complete after 7 hours, a second 100 ml aliquot of the iodine solution was added and the reaction mixture stored in the dark overnight. When NMR analysis indicated complete isomerization, the reaction mixture was extracted with a 5 percent sodium thiosulfate solution (3 x 100 ml) followed by water (3 x 100 ml). The solvent was removed under reduced pressure with the addition of chloroform to remove the residual water. In this manner, 100g of a colored crystalline material was obtained. This material was purified by recrystallization and chromatography on silica gel: mp = 66-67°, UV max (95 percent ethanol) 276 nm; IR (neat) 3300 (alcohol OH), 1750 (ester C=O), 1700 (conjugated C=O), 1640 and 1590 (C=C stretch) and 970 cm^{-1} (trans C=C); NMR ($CDCl_3$) δ 6.7 (d, 1, J=15 Hz, -CH=CH-CH(OH)-), 6.1 (d of d, 1, J=15 and 5 Hz, -CH=CH(OH)-CH-), 4.15 (m, 1, -CH=CH-CH(OH)-CH_2-), 3.65 (s, 3, ester $-OCH_3$) and 0.85 (t, 3, $-CH_2CH_3$). The trans PGB_1 methyl ester served as a convenient storage material and was converted to 15-keto PGB_1 methyl ester (16) shortly before conversion to PGB_x.

METHYL 7-(2-(TRANS-3-KETO-1-OCTENYL)-5-OXOCYCLOPENTENYL)-HEPTANOATE (15-KETO-PGB_1) (16)

A solution of 25g (0.071 moles) of trans-PGB_1 (15) in 200 ml of acetone was added to a 500 ml three-neck round-bottom flask equipped with mechanical stirrer and low temperature thermometer. The solution was initially cooled to between -10° to -15° in an ice-methanol bath and the dropwise addition of the Jones reagent[7] was controlled to maintain the reaction temperature between -5° to -10°. Since the UV absorption maximum for (15) is at λ278 nm and for (16) at λ296 nm, the progress of the oxidation could be monitored by UV spectroscopy. When the reaction was judged complete, 10 ml of isopropyl alcohol was added to destroy excess Jones reagent. The reaction mixture was then diluted with water and extracted with 3 x 100 ml of ethyl ether. The combined ether extracts were washed sequentially with 1 x 100 ml of water, 100 and 50 ml of 5 percent $NaHCO_3$ and finally with 2 x 100 ml of water. The ether was removed at reduced pressure and chloroform was added to remove the water. Typically 80-90 percent crude product was obtained. The crude product was chromatographed on silica gel with heptane: ethyl acetate (8:2) solvent system to yield pure (16) and fractions containing trans-PGB_1 (15). These fractions were recovered and recycled in a subsequent oxidation reaction to increase the overall yield: UV (95% ethanol) 396 nm; IR (neat) 1750 (ester C=O) and 1700 cm^{-1} (conjugated C=O); NMR ($CDCl_3$) δ 7.4 (d, 1, J=15 Hz, -CH=CH-C(=O)-), 6.5 (d, 1, J=15 Hz, -CH=CH-C(=O)-), 3.5 (s, 3, ester $-OCH_3$) and 0.85 (t, 3, $-CH_2CH_3$).

PGB_X (20)

15-keto PGB_1 (5g), (16) was dissolved in 100 ml ethanol and 100 ml of 1.0 N KOH was added. The solution was heated at 80° for 4 hours. The course of the reaction was followed by measuring the disappearance of the characteristic 15-keto PGB_1 (16) absorption maximum at λ296 nm and the appearance of the λ243 nm absorption maximum of PGB_X. When the reaction was complete the mixture was cooled to room temperature and equal volumes of water and isobutanol were added. The mixture was then acidified to pH 3.0 with 2.3 N perchloric acid. The isobutanol layer containing active PGB_X was separated and washed twice with 100 ml of water and then the PGB_X was extracted into 100 ml 0.1 M $NaHCO_3$. The $NaHCO_3$ layer was separated, acidified to pH 3.0 and shaken with an equal volume of isobutanol. The isobutanol layer containing PGB_X was evaporated under reduced pressure to yield a crude product of PGB_X (4g, 80 percent yield).

Studies were undertaken to further purify PGB_X by preparative thin layer and preparative column chromatography. Of the many adsorbents and solvent systems examined, Sephadex LH20 with methanol as the carrier solvent gave the best results from the standpoint of increased purification and recovery of PGB_X activity. The use of silica gel in acid media appeared especially deleterious since only a portion of the PGB_X activity could be recovered. PGB_X (20 percent in methanol) was injected on a Sephadex LH20 column (95 x 5 cm) previously swollen according to the manufacturer's directions. Because of the low capacity of Sephadex LH20 only 2g charges of crude product could be chromatographed for each loading. The flow rate was 20 ml per minute and fractions were collected in 1 minute intervals. The course of the chromatographic separation was monitored with UV (λ280 nm) or refractive index detectors. Since Sephadex LH20 adsorbent separates solutes according to molecular size, the resultant chromatogram did not show distinct peaks; consequently, the fractions obtained were not homogeneous. In addition, because of this low resolution, the chromatographic runs differed slightly from each other so that combinations of the same fraction from different runs varied slightly in molecular weight range of products; consequently, combinations of these fractions had to be rechromatographed to isolate PGB_X with a narrower range of molecular weight products, and with an activity per mg equivalent to the standard PGB_X. Figure 4 illustrates a typical chromatogram of PGB_X on Sephadex LH20. The typical chromatogram shows a leading shoulder (fraction 1) on the main peak (fraction 2) followed by tailing shoulder (fractions 3 and 4), and finally two smaller peaks (fractions 5 and 6). The arrows shown at the top of the figure define each fraction. The contents of the tubes from each fraction were combined and evaporated to dryness and then assayed for *in vitro* mitochondrial PGB_X activity. Each combination was stored at 4° as a stockpile to which similar fractions from subsequent chromatography were added. Attempts have been made to increase the yield of purified PGB_X by rechromatography of combined fractions 3 and reheating the lower molecular weight fractions in alkaline media as described in the first part of the preparation of PGB_X. These attempts have resulted in recovering some additional purified PGB_X. Because of the difficulty in keeping an inventory for such a complicated separation, data are not available to describe these recoveries adequately. However, the purification of PGB_X resulting from one chromatography on Sephadex LH20 is shown in table II. As expected, the separation proceeds with higher molecular weight species being less retentive and the smaller molecular weight

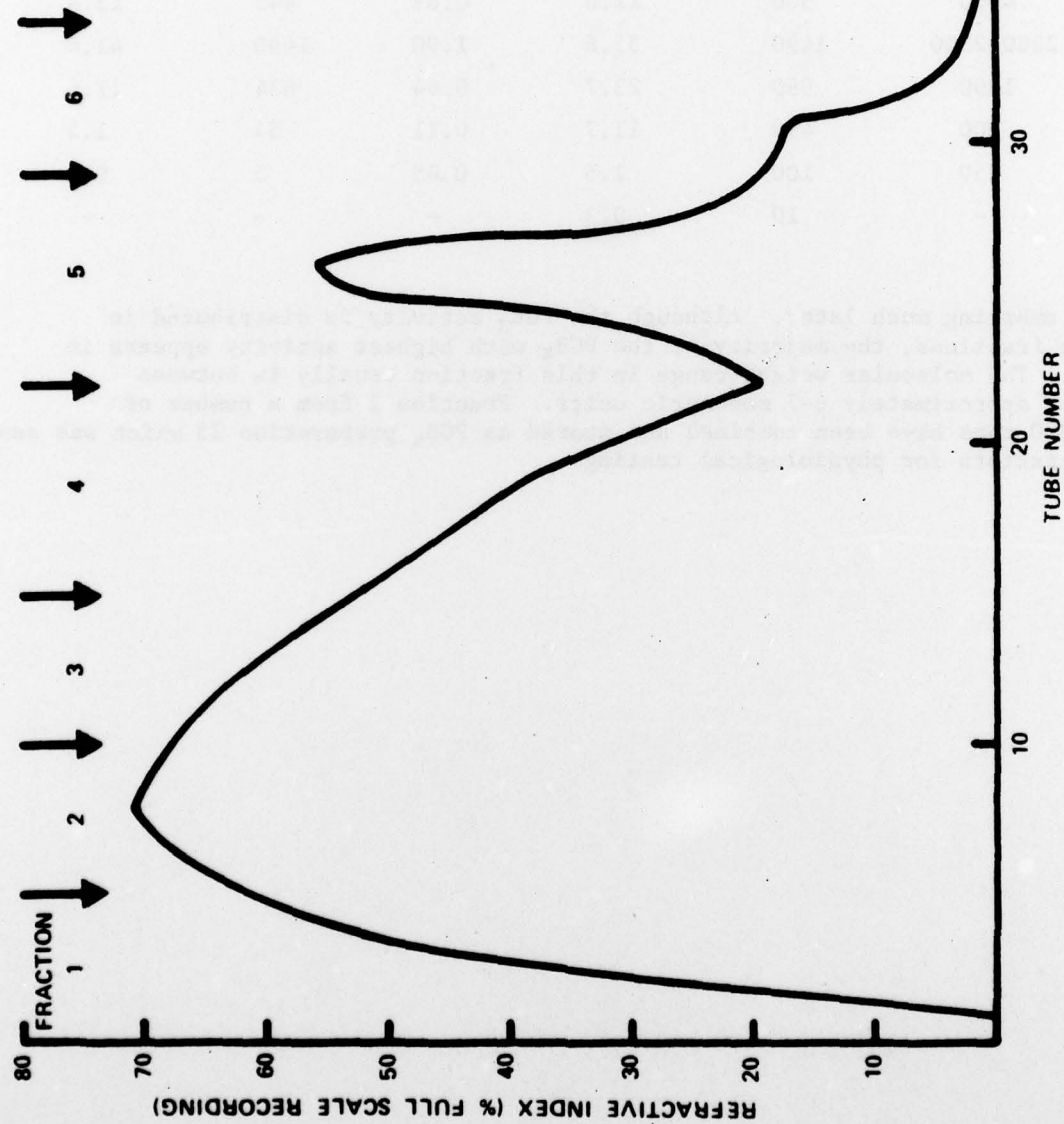

FIGURE 4 - Sephadex LH-20 Column Chromatographic Separation of PGB_x. Column Size, 95 x 5 cm; Carrier Solvent, Methanol; Flow-Rate 20 ml/min; Fraction Collector, 1 min. Intervals; Detector, Refractive Index Monitor.

TABLE II

Sephadex LH20 Chromatography of PGB_x

Fraction	Molecular Weight	Weight		PGB_x Activity		
		Mg/Fraction	% Recovery	Units/Mg	Total Per Fraction	% Recovery
Starting		4190		0.86	3603	
1	4300	500	12.0	0.89	445	12.4
2	2200-2500	1490	35.6	1.00	1490	41.4
3	1300	990	23.7	0.64	634	17.6
4	800	490	11.7	0.11	54	1.5
5	350	100	2.5	0.05	5	0.1
6	-	10	0.3	-	-	-

substances emerging much later. Although the PGB_x activity is distributed in most of the fractions, the majority of the PGB_x with highest activity appears in fraction 2. The molecular weight range in this fraction usually is between 2200-2500 or approximately 6-7 monomeric units. Fraction 2 from a number of Sephadex LH20 runs have been combined and stored as PGB_x preparation 25 which was sent to ONR contractors for physiological testing.

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REPORT NO. NADC-78235-60

CONTRACT NO. N00014-78-WR800-19

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