

PROJECT ADMINISTRATION DATA SHEET

ORIGINAL

REVISION NO.

Project No. G33-Q03

DATE 5-20-82

Project Director: L. H. Zalkow

School/Dept Chemistry

Sponsor: DHHS/PHS/NIH - National Eye Institute  
Bethesda, Md 20014

Type Agreement: Grant No. 5-RO1-EY03342-03

Award Period: From 4-1-82 To 3-31-83 (Performance) 6-30-83 (Reports)

Sponsor Amount: \$ 85,025 Contracted through:

Cost Sharing: \$ 4475 (G33-316) GTW/GIT

Title: Anti-glaucoma Compounds From Cannabis Sativa

ADMINISTRATIVE DATA

OCA Contact

Don Harty

1) Sponsor Technical Contact:

Ms Anita A. Suran, Ph.D.  
Extramural Program Director  
Glaucoma Program  
National Eye Institute  
Bethesda, Md 20014

2) Sponsor Admin/Contractual Matters:

Ms Carolyn E. Grimes  
Grants Management Office  
Extramural Services Branch  
National Eye Institute  
Bethesda, Md 20014

phone (301) 496-5884

Defense Priority Rating: N/A

Security Classification: N/A

RESTRICTIONS

See Attached N/A Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval - Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of \$500 or 125% of approved proposal budget category.

Equipment: Title vests with N/A - none proposed

COMMENTS:

Follow on to G33-Q02/zalkow

COPIES TO:

- Admin Network
- Administrative Coordinator
- Research Property Management
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- Project File
- Other



SPONSORED PROJECT TERMINATION SHEET

SR-189

Date 8-26-83

Project Title: Antiglaucoma Compounds From Cannabis Sativa

Project No: G-33-Q03

Project Director: L. H. Zalkow

Sponsor: DHHS/PHS/NIH- National Eye Institute

Effective Termination Date: 3-31-83

Clearance of Accounting Charges: 3-31-83

Grant/Contract Closeout Actions Remaining:

NONE

- Final Invoice and Closing Documents
- Final Fiscal Report
- Final Report of Inventions
- Govt. Property Inventory & Related Certificate
- Classified Material Certificate
- Other \_\_\_\_\_

NOTE: Follow-on to project G-33-Q02  
 New project is G-33-Q04  
 Annual report of expenditures prepared 8-9-83

Assigned to: Chemistry (School/Laboratory)

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EES Public Relations (2)  
 Computer Input  
 Project File  
 Other L.H. Zalkow

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1		GRANT NUMBER	
<b>SECTION IV—SUMMARY PROGRESS REPORT</b>		EY-03442-04	
		PERIOD COVERED BY THIS REPORT	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)		FROM	THROUGH
ZALKOW, LEON H.		April 1, 1983	March 31, 1984
NAME OF ORGANIZATION			
Georgia Institute of Technology			
TITLE (Repeat title shown in Item 1 on first page)			
<u>Antiglaucoma Compounds from <i>Cannabis sativa</i></u>			

- List all publications, not previously reported, resulting from work supported by this grant (author(s), title, page numbers, year, journal or book). List manuscripts separately as submitted for publication or accepted for publication.
- Provide two reprints of publications not previously submitted to the awarding unit.
- Progress Report. (See instructions)

### 1. Publications:

K. Green, C. M. Symonds, R. D. Elijah, L. H. Zalkow, H. M. Deutsch, K. A. Bowman and T. R. Morgan, "Water Soluble Marijuana-Derived Material: Pharmacological Action in Rabbit and Primate", *Current Eye Research*, 1, 599 (1982).

K. Green, "Marijuana and the Eye - A Review", *J. Toxicol. Cut. & Ocular Toxicol.*, 1, 3 (1982).

### Presentations:

H. M. Deutsch, L. C. Hodges, L. H. Zalkow and K. Green, "Isolation and Characterization of Ocular Hypotensive Agents from *Cannabis Sativa*", 66th Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, Louisiana, April 15-23, 1982.

K. Green, H. M. Deutsch, L. C. Hodges, C. M. Symonds, R. D. Elijah and L. H. Zalkow, "Characterization of Water-Soluble Ocular Hypotensive Agents from *Cannabis Sativa*", 54th Annual ARVO Spring Meeting, Sarasota, Fla., May 3-7, 1982.

H. M. Deutsch, L. C. Hodges, L. H. Zalkow, and K. Green, "Isolation and Characterization of Ocular Hypotensive Agents from *Cannabis Sativa*", Southeast Regional Meeting of the American Chemical Society, Birmingham, Alabama, November 3-5, 1982.

### 3. Progress Report

#### 1. Objectives:

##### a. Total Project.

(1) Isolate and characterize water soluble compound(s) from *Cannabis sativa* which on the basis of screening results have been shown to lower intraocular pressure in test animals.

(2) Based on the results above, synthesize and/or modify the basic structural type to develop structural activity data and more effective compounds.

(3) Synthesize and screen water soluble derivatives of naturally occurring cannabinoids.

(4) Select and screen other plants as possible sources of compounds with IOP lowering activity.

##### b. Current Year.

(1) Continue established separation methods, with special emphasis on ion exchange and affinity chromatography, gel electrophoresis and isoelectric focusing, until a pure active material has been isolated.

(2) Most of our effort will be geared towards the physical and chemical characterization of the above active fraction. Special emphasis will be placed on characterization of the carbohydrate portion.

(3) Selected chemical and biological transformations of the active material will be attempted in order to understand which portions of the molecule are necessary for IOP lowering activity, and to study its mechanism of action.

(4) Screening of other plants will not be emphasized.

(5) At least one water soluble derivative of a natural cannabinoid will be made and screened for activity.

## 2. Studies During Current Year:

### A. Isolation and Purification

The active component(s) from aqueous extracts of Cannabis sativa have been successfully fractionated into several high molecular weight species by gel filtration chromatography on Fractogel TSK HW 75F. The chromatography was performed in 0.05M sodium phosphate, 0.2M LiBr, pH 6.5, conditions which should disrupt aggregation. This size exclusion media has a molecular weight range of  $1 \times 10^5$  to  $10 \times 10^6$  for polysaccharides. Samples of extracts partially purified by ion exchange chromatography on DEAE cellulose when applied to this material fractionate into three peaks as determined by the phenolsulfuric acid test for carbohydrates. The lowest molecular weight material elutes last from the column, is highly colored and has no intraocular pressure (IOP) lowering activity. The first two fractions from this column contain the active material with IOP lowering activity between -30 and -50% at  $1 \mu\text{g}/\text{animal}$ . A sample of a dextran with known molecular weights was used to calibrate the column. The active fractions had molecular weights between  $1.5 \times 10^6$  and  $5 \times 10^5$  by this technique. Other DEAE-cellulose fractions when passed through this column show similar elution patterns but somewhat different composition of the fractions. These differences may be due to incomplete separation of the three fractions. The high molecular weight of these samples makes tests for homogeneity difficult. Conventional methods such as polyacrylamide electrophoresis are not useful since such high molecular weight material cannot enter the gel matrix. Currently we are exploring the use of agarose as an electrophoretic medium since it has essentially no molecular weight exclusion limit.

In addition to gel filtration techniques for separation, a new ion exchange material, DEAE Trisacryl M which is noncellulose-based has been tried. Earlier separations of crude, dialyzed extracts had been complicated by nonspecific adsorption phenomena. The Trisacryl M support matrix eliminates nonspecific interactions and has a high adsorption capacity. An active, crude dialyzed extract applied to this column at low ionic strength (0.05M Tris·HCl, pH 8.6) separated into three fractions as monitored by UV absorption at 228 nm. Elution with buffer containing 0.25M NaCl gave a large UV absorbing fraction which contained the only IOP lowering activity. Further work with this ion exchange material will include quantifying recovery on a larger sample, rechromatography of active components to enhance separation, and gradient elution.

Problems have been encountered with extraction of the active material from the marijuana samples. The material may not be present in all varieties of the plant, or it may only be released under specific conditions of ionic strength, pH, or maceration. Studies had been done on varying conditions for extraction, including acidic and basic solvents, warm (50-60°C) or cold water, one or two extractions. Warm distilled water after two extractions appeared to release most of the active material. However, two different varieties of marijuana did not release active material under these same conditions.

A study was again made on the effect of different solvents on the release of active material from dried plants. Samples of marijuana were extracted twice with one of four different solvent systems all at 55°C: distilled water, 10 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100. For the first extraction material was macerated for four minutes in the Waring blender. The maceration was not repeated for the second extraction. No activity was detected in any of these samples. When a sample of one of these extracts was eluted from DEAE-cellulose with buffer containing 0.25M NaCl, and then fractionated on Fractogel TSK HW75F, no high molecular weight material

was detected by phenol-sulfuric acid assay for carbohydrate. This result supported our prior conclusion that a high molecular weight component is associated with IOP lowering activity.

The effect of maceration on release of active material was then examined. Four samples from a variety of marijuana previously shown to have activity were each extracted twice with warm water (55-60°C). Samples were macerated in the first extraction for 0, 1, 2 or 4 minutes in a Waring blender. In all cases the second extraction did not include maceration. Only the second extraction on the nonmacerated material (0 time) was active. These studies will be extended to include more gentle extraction and maceration conditions.

### B. Analytical Results

The first two fractions (Samples A and B) from DEAE-cellulose chromatography of a crude, dialyzed extract eluted in 0.05M Tris-HCl, 0.10M NaCl, pH 6.7, were further fractionated by gel filtration on Fractogel TSK HW75F. The carbohydrate and amino acid composition of the resulting fractions are given in the tables below. Carbohydrate composition was determined by GLC of the alditol acetates essentially using the method of Albersheim, et al. (Carbohydr. Res. 1967, 5, 340). Amino acid analysis was performed using an HPLC method modified in our laboratory. (V. T. Wiedmeier, et al, J. Chromatogr. 1982, 231, 410 and B. L. Karger, et al., Anal. Biochem. 1981, 115, 123). Samples are hydrolyzed in acid under standard conditions for protein, then dansylated, and the dansyl derivatives are separated and quantitated by reverse phase HPLC using a gradient elution system.

In addition to carbohydrate and amino acid composition, the tables below indicate the percent of the total weight of the ion exchange sample represented in each fraction from the gel filtration column. Also the IOP activity in terms of an ED<sub>40</sub> is given. The ED<sub>40</sub> is the amount of material required to bring about a 40% drop in intraocular pressure.

Sugar	Sample	% Composition							
		Gel Filtration Fractions				Sample	Gel Filtration Fractions		
		A	1a	1	2		3	B	1
Rhamnose	9	15	20	10	6	14	21	16	15
Arabinose	37	28	24	36	39	29	25	30	30
Xylose	4	5	7	6	4	11	10	11	11
Mannose	5	4	3	4	5	4	3	3	5
Galactose	35	16	15	37	40	32	16	32	32
Glucose	10	24	22	8	6	11	18	6	7
Glucosamine	trace	9	9	-	1	trace	7	2	1

Amino Acid	% Composition								
	Sample	Gel Filtration Fractions				Sample	Gel Filtration Fractions		
		A	1a	1	2		3	B	1
Hydroxyproline	6	-	-	9	10	3	-	6	5
Serine	10	12	19	11	12	10	8	11	11
Aspartic Acid	11	7	7	7	9	10	10	11	13
Glutamic Acid	10	9	14	8	9	11	15	11	12
Threonine	14	32	15	13	12	8	22	12	10
Glycine	8	10	13	6	7	8	12	8	9
Alanine	12	7	8	10	11	10	8	11	10
Proline	5	3	3	4	7	7	5	7	9
Valine	6	4	4	6	6	6	5	7	7
Leucine	6	4	3	4	6	6	7	6	6
% by Weight of Fraction	-	5	13	18	64	-	5	17	78
ED <sub>40</sub> (µg)	0.6	2	3	0.6	>10	12	0.8	10	>10

The high molecular weight fractions purified by gel filtration are not completely soluble in water. This factor has caused problems in quantifying analytical samples and in further purification steps. Other solvent systems were examined for their ability to solubilize the samples completely. A solution of 50% pyridine and water appears successful in solubilizing most of the material, and samples treated in this fashion retain IOP lowering activity. Also gel filtration was performed in the presence of the denaturing agent 4M guanidine-HCl to disrupt noncovalently bound aggregates and promote solubility. The elution pattern was the same as that obtained when sodium phosphate - LiBr was the solvent system employed. Yields from this method are currently being compared.

### C. Chemical Modifications

Since the large molecular weight of the active fractions poses unique problems, several methods were attempted to reduce the size of the material and yet retain activity. A sample of an active DEAE fraction was digested extensively with pronase, a mixture of nonspecific proteases which digest peptides to free amino acids. The digested sample was applied to the Fractogel gel filtration column, and the elution profile was compared to that of an undigested sample. Data indicated that the high molecular weight peaks had shifted slightly toward lower molecular weight, and a broader peak appeared in the region of the lowest molecular weight material. Activity was retained in the high molecular weight fractions. The active material was not of significantly lower molecular weight to make the pronase digestion useful.

An attempt was then made to reduce the size of the carbohydrate portion of the molecule. Since certain O-glycosidic linkages are susceptible to cleavage with alkali, an active sample obtained by gel filtration after DEAE chromatography was treated with 0.05M NaOH at 50°C in the presence of 1M NaBH<sub>4</sub> (to prevent unwanted side reactions) for varying lengths of time. The 15 hour sample was polydisperse in

molecular weight when reapplied to the Fractogel column. However, none of the samples were active after this treatment including the control (0 time). Incomplete solubility of some of the fractions may be a problem in these results, and the experiment should be repeated.

#### D. Screening of Other Plants

Not much emphasis was placed on other plants during this period. A pronase digest of dried Erigeron philadelphicus as well as a water extract from fresh plant seemed to show some activity at high doses, but no other extraction conditions employed so far (water extract on dried plant, 10 mM EDTA extract) resulted in active material.

#### E. Water Soluble Cannabinoids

No effort was made on this problem during this period.

### 3. Significance to Health Problems

In view of the fact that glaucoma is the second leading cause of blindness in the United States, the importance of developing new and useful drugs in this area is obvious. The water soluble compounds isolated from marijuana have a more profound effect (Lower IOP by 60%) than any other known compound at any dose level. Therefore, the potential for benefit is indeed very great. If an easy to deliver, safe, effective drug can be developed from this work, it would be a very significant advance in glaucoma therapy. In any case, due to the potency of action, both in regards to the large decreases in IOP and the very small dose levels, this material is of fundamental interest to scientists interested in glaucoma and the mechanism of pressure regulation.

### 4. Research Goals During Coming Year

1. Establish with certainty the best methodology for isolation of active components from Cannabis sativa.
2. Continue established separation methods such as gel filtration, ion exchange and affinity chromatography, agarose electrophoresis and isoelectric focusing until a pure active material has been isolated.
3. We will continue physical and chemical characterization of the above active fraction.
4. Increased emphasis will be given to the area of chemical and physical transformations of the active material to determine which portions of the molecule are necessary for IOP lowering activity.
5. Screening of other plants will be intensified.
6. Water soluble derivatives of natural cannabinoids will be made and screened for activity.