BIOCHEMISTRY DEPARTMENT
AGRICULTURAL DIVISION
CIBA-GEIGY CORPORATION
GREENSBORO, N. C.

CHARACTERIZATION OF POLAR METABOLITES OF ϕ-^{14}C-CGA-48988 IN GREENHOUSE GROWN BRIGHT TOBACCO

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A B S T R A C T

Greenhouse bright tobacco was treated 0.5 lb. a.i./acre with ϕ-^{14}C-CGA-48988 (transplant water). At maturity bright tobacco leaves contained at least 23 radioactive polar metabolites. A portion of these polar metabolites was shown to be sugar type conjugates of ϕ-^{14}C-CGA-48988 metabolites. These conjugates composed 14% of the total leaf radioactivity at 3 weeks following treatment and 31% at 12 weeks (mature), indicating increasing conjugation of metabolites of ϕ-^{14}C-CGA-48988 with age.

Cellulase hydrolysis of the polar conjugates in uncured tobacco released 9 aglycones, 6 of which corresponded to 6 unconjugated metabolites. Four of these 9 aglycones are key substances in the metabolism of CGA-48988 because they are present in higher amounts than other aglycones, their amounts increase with plant age, and they appear to be present as unconjugated metabolites. Three are neutral and one is acidic.

The acidic one, unconjugated and conjugated, amounted to 15% of the total ^{14}C in a 12-week cured bright tobacco. TLC quantitation showed that curing tobacco has a minimal affect on changing the number and amounts of these aglycones. Age, though, increases the amounts of these aglycones in tobacco. The four key metabolites represent about 20-30% of the radioactivity in the uncured and cured tobacco 12 weeks after treatment.

β-D-glucosidase released only 1/2 the radioactivity that cellulase did. The acidic aglycone was not released. These data indicate that it is covalently linked to sugars by a different type of bonding than are the other aglycones.

Acid hydrolysis of the polar metabolites of CGA-48988 in uncured tobacco resulted in the same amount of hydrolysis and the same TLC pattern as did enzyme treatment except that two neutral aglycones were not found. It is possible that 1N HCl further degrades these aglycones after release from sugar.

A speculative metabolic pathway for CGA-48988 is proposed and is based on TLC, mass spectroscopy, electrophoresis and ion exchange chromatography data of the four key aglycones.

INTRODUCTION

The experimental compound CGA-48988, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alanine methyl ester*, is a fungicide proposed for control of Black Shank on tobacco. Buckets of soil, each containing one bright tobacco plant, were treated with [U-ring-^{14}C]-N-(2,6-dimethylphenyl)-N-(methoxyacetyl) alanine methyl ester** at a rate of 0.5 lb. a.i./A using a transplant water technique. Since burley tobacco treated at 6.3 lb. a.i./A ppi showed the same qualitative and quantitative pattern of uptake and metabolism as did the bright tobacco, the latter could be used for characterizing polar metabolites of CGA-48988 for both types of tobacco (1). The objective of this work was to characterize, specifically, aglycones released by enzymes from polar metabolites.

EXPERIMENTAL

Treatment and Radioactive Dose: Coker 319 bright tobacco was treated by a transplant water procedure at 0.50 lb. a.i./A with ϕ-^{14}C-CGA-48988. Details regarding planting, treatment, sampling and curing of the tobacco are in ABR-78036 (1) and Biological Report 78003 (2). Tobacco leaves taken 3 and 12 weeks after treatment provided the polar metabolites.

Sample Preparation and Analysis: Uncured or cured tobacco leaves were homogenized with dry ice in a Wiley mill (3). Samples of 100-200 mg were combusted in a Harvey oxidizer (4).

Radioactivity Measurements: Radioassays were done in a Beckman LS-255 or Mark III liquid scintillation counter. Efficiencies were obtained by external standardization. Limits of detection and quantitation were determined in accordance with AG-276 (5).

Fractionation Scheme: The flow diagram in Figure 3 outlines the steps used for isolating conjugates of ϕ-^{14}C-CGA-48988 metabolites from ϕ-^{14}C-CGA-48988 and polar unconjugated acids and neutrals. Ground tobacco, 50 g of uncured or 10 g of cured leaves, was extracted with 200 ml of methanol and water - 80/20 (v/v). The extract was filtered, concentrated to 100 ml, and then partitioned against 150 ml methylene dichloride (MDC) to give organic solubles ORG 1 and an aqueous phase AQ1. The AQ1 was adjusted to pH 1 with 6N HCl and partitioned twice against ethyl acetate to give ORG 2 + 3, which contained polar unconjugated acids and/or neutrals, and AQ3, which contained ^{14}C-conjugates and other very polar ^{14}C-material.

*Chemical names and structures are given in Figure 1.
**Hereafter referred to as ϕ-^{14}C-CGA-48988.

Enzyme Hydrolysis of AQ3: The following were combined and incubated in a stoppered flask for 48 hours at 37°C: 0.5 ml AQ3, pH = 7; 4.3 ml sodium acetate buffer, pH = 4.6; and 10 mg of enzyme. A control flask contained only buffer and AQ3. After incubation aliquots were analyzed by TLC.

HCl Hydrolysis of AQ3: The following were combined and refluxed 1 hour: 2 ml of AQ3 and 2 ml of 2N HCl. The solution was then partitioned against 5 ml ethyl acetate and aliquots of the organic phase analyzed by TLC.

Thin Layer Chromatography of Extracts of Tobacco: Aliquots of the AQ1 or aglycone fractions from tobacco were spotted on Analtech QF1 TLC plates (250 microns). TLC plates were developed in two dimensions using the following saturated solvent systems:

A. Two Dimensions:

1st ethyl acetate/2-propanol/H_2O/HCOOH (65/25/10/2)

2nd chloroform/methanol/HCOOH/H_2O (75/20/4/2)

See Figure 2.

B. Two Dimensions:

1st ethyl acetate

2nd ethyl acetate/acetic acid (90/10)

See Figure 4A and 4B.

Selected standards (Figure 1) were cochromatographed with the extracts. Quantitation was by locating the radioactive zones with Kodak No-screen (NS-2T) x-ray film, scraping them off and eluting radioactivity with 2 ml methanol. Aquasol® was added prior to radioassay.

Electrophoresis of Aglycones from Cellulose Treatment of Polar Metabolites: Electrophoresis of aglycones I', II', III', IV', VI' was carried out according to AG-300 (6).

Ion Exchange Chromatography of Aglycones: Ion exchange chromatography of aglycones was carried out by applying a mixture of the aglycones to a 1 x 10 cm Sephadex A-25 (Pharmacia Fine Chemicals) column and eluting the neutrals with water. KBr (0.1N) was used to elute VI'.

Gas Liquid Chromatography and Chemical Ionization Mass Spectroscopy of II', III', IV': Aglycones II', III' and IV' were purified by silica gel chromatography (solvent ethyl acetate) followed by preparative TLC (solvent = 50% ethyl acetate/heptane). A final preparative TLC (solvent ethyl acetate) was necessary prior to GC-MS analysis. A Finnegan 3200D mass spectrometer equipped with a GLC was used. A 6' DC-200 (10%) column was used with an oven temperature of 210°C and injection port at 230°C. The flow of methane was adjusted to maintain a pressure of 1000 microns in the source.

RESULTS AND DISCUSSION

In preliminary metabolism data for ϕ-^{14}C-CGA-48988 in tobacco (1), two dimensional TLC (ABR-78036 - Figure 2B) of the aqueous soluble polar fraction showed a multiplicity of polar metabolites of ϕ-^{14}C-CGA-48988 for 12-week tobacco that has been cured. Since these metabolites represented 50% of radioactivity in the leaf and CGA-48988 only 27%, the characterization of these polar metabolites becomes important.

For easier characterization of these metabolites, the radioactivity in leaves was fractionated into organic soluble, polar unconjugated acids or neutrals, conjugates, and nonhydrolyzable polar metabolites and nonextractables (Figure 3). Table I shows the radioactive balance of this fractionation. The radioactivity in AQ3 could be made organic soluble by incubation with cellulase, thus this radioactivity must represent conjugates. As the data in Table I show for uncured tobacco at 3 and 12 weeks, the organic soluble radioactivity (mainly CGA-48988) decreased from 76% to 44%, the radioactive polar unconjugated acids or neutrals increased from 7% to 16%, and the conjugates increased from 14% to 31%. The metabolism of CGA-48988 progressed to polar unconjugated metabolites which became conjugated with increasing age of the plant.

Cellulase or hesperidinase released 9 aglycones from the conjugate fraction (AQ3) of the uncured bright tobacco. Figure 4A shows a qualitative 2D-TLC autoradiogram of the aglycones

while Table I gives their quantitative distribution. I'a composed 0.3% of the total radioactivity in the uncured bright leaf; I', 1.2%; II', 4.5%; III', 5.1%; III'a, 0.4%; IV', 12.5%; V'a, 1.1%; V'b, 0.4%, and VI' = 11.3%. All aglycones increased with the age of the plant, especially II', III', IV' and VI. Bright tobacco contains 6 polar unconjugated acidic or neutral ^{14}C-metabolites (ORG 2 + 3 - Figure 3) which are equivalent by 2D-TLC to 6 of the aglycones released from the aqueous polar fraction of the uncured leaf by cellulase treatment. The insert in Figure 2 shows a two-dimensional TLC pattern of the aglycones developed in the same solvent systems as were metabolites in AQ1. Four aglycones, II', III', IV' and VI', are key substances in the metabolism of CGA-48988 because they are present in a higher amount than other aglycones, their amounts increase with plant age and they appear to be present also as unconjugated metabolites.

Six of the aglycones correspond to metabolites I, II, III, XI, XIV and XV. No corresponding metabolites were found for three of the aglycones. Unconjugated and conjugated VI' added together composed 15% of the total ^{14}C in cured bright tobacco, indicating VI' is a major metabolite of CGA-48988 in tobacco. Regarding all aglycones, TLC quantitation (Table I) shows very little differences between aglycones in uncured and cured tobacco. Curing tobacco has a minimal affect on changing the number and amounts of these aglycones.

Acid hydrolysis also releases aglycones. Hydrolysis with 1N HCl of the conjugates (AQ3) in 3-week tobacco resulted in the same amount of hydrolysis and the same qualitative TLC pattern (Table I) of aglycones as did cellulase digestion, except that III' and IV' (major aglycones at 3 weeks) were not released by HCl. Acid may have destroyed these metabolites after their release from a sugar conjugate.

Some differences were noted (Figure 4B) when aglycones were released with β-D-glucosidase instead of cellulase. Glucosidase released only 1/2 of the radioactivity that cellulose released. Glucosidase did not release VI'. Such a result suggests that different types of covalent bonds may be involved in the conjugation of III' relative to the other aglycones and that cellulase contains enough nonspecific enzymes in the mixture to release all of the aglycones. Further, by ion exchange chromatography and electrophoresis, VI' showed acidic properties, while II', III' and IV' show neutral (possibly these three are alcohols). β-D-glucosidase would not hydrolyze a sugar-ester linkage while it would hydrolyze a sugar-alcohol linkage, thus releasing II', III' and IV' but not VI'.

Other enzymes such as β-D-glucuronidase, protease, pepsin, and sulfatase did not release any radioactivity from polar metabolites, indicating the absence of glucuronides, protein and sulfate conjugates.

The above data indicate that the polar fraction of ϕ-^{14}C-CGA-48988 treated tobacco contains at least 9 conjugates which are hydrolyzable with cellulase. Six of the 9 aglycones which are released also occur as free unconjugated metabolites. TLC data using standards listed in Figure 1 do not cochromatograph with any of these aglycones. However, II' chromatographs adjacent and below CGA-48988 and VI' adjacent and below CGA-62826. Perhaps the reason is that a ring methyl group has become an alcohol. Preliminary GC-MS data for partially purified aglycones indicates such may occur. A possible metabolic pathway of CGA-48988 is given in Figure 5. Further mass spectral and NMR data as well as supportive synthesis of these compounds is necessary to prove this metabolic pathway.

SUMMARY

The data in ABR-78036 (1) and in this report indicate the following:

1) Mature bright tobacco treated at transplant at 0.5 lb. a.i./A with ϕ-^{14}C-CGA-48988 contains about 40% of the radioactivity in organic solubles which are composed mostly of CGA-48988 and 7 unknown metabolites.

2) The polar aqueous soluble fraction (50-60%) contains at least 23 polar metabolites, at least nine of which are sugar conjugates. Six of these conjugates contain aglycone moieties which are equivalent by two-dimensional TLC to 6 unconjugated polar aqueous soluble metabolites. Of the nine aglycones released by enzyme treatment 4 are usually greater than 3% of the radioactivity in leaf metabolites. One metabolite VI' composes a total of 15% of the total leaf radioactivity when the amounts of free and conjugated VI' are added.

3) The ^{14}C-nonextractables from the bright tobacco leaves are low.

4) The above results vary only slightly with the species of tobacco or with the rate and type of application of CGA-48988.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the technical assistance of Ms. 5.1.2.e Woo and Ms. 5.1.2.e Woo. We would also like to acknowledge Mr. 5.1.2.e Woo who prepared the ϕ-^{14}C-CGA-48988.

REFERENCES

1. [redacted] and [redacted], ABR-78036, "Uptake and Balance of ϕ-^{14}C-CGA-48988 and Its Metabolites in Greenhouse Grown Bright and Burley Tobacco."

2. [redacted], Biological Report No. 78003, "Biological Report for the Metabolism of ϕ-^{14}C-CGA-48988 in Greenhouse Grown Tobacco When Applied as a Drench to Bright Tobacco and Preplant Incorporated to Burley Tobacco."

3. [redacted]., AG-233, "Blending of Soils and Homogenization of Biological Materials for Radioassay and Extraction."

4. [redacted] AG-252, "Radioassay of ^{14}C in Biological Materials Using the Harvey Biological Material Oxidizer (BMO)."

5. [redacted], AG-276, "Statistical Methods in the Measurement of Radioactivity."

6. [redacted] and [redacted] AG-300, "High Voltage Electrophoresis for Characterization of Charged Radioactive Metabolites."

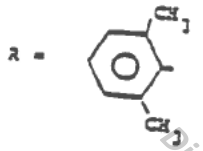
TABLE I: BALANCE AND CHARACTERIZATION OF POLAR AQUEOUS SOLUBLE METABOLITES OF φ-^{14}C-CGA-48988 IN BRIGHT (0.5 LB. AI/A)

THI (Weeks)	3		12	
Sample	Uncured		Uncured	Cured
	A	B	A	B
Balance	% of Total ^{14}C in Leaf			
(ORG1) Organic solubles (CGA-48988 mainly)	76	76	44	35
(ORG 2+3) Polar unconjugated acids or neutrals	7	7	16	21
(AQ3) Conjugates	10	14	31	34
(AQ4) Nonhydrolyzable polars	8	3	0	0
Nonextractables	-	-	2	3
Characterization	HCl	Cellulase	Cellulase	Cellulase
Aglycones from AQ3 I'a	NA*	NA	0.3	NA
I'	2.0	<0.01	1.2	0.5
II'	2.0	1.3	4.5	2.5
III'	NP**	2.0	5.1	3.0
III'a	NA	NA	0.4	NA
IV'	NP	7.4	12.5	6.4
V'a	NA	NA	1.1	NA
V'b	NA	NA	0.4	NA
VI'	4.0	1.9	11.3	10.5
Origin	0.5	2.1	2.9	10.9
Unconjugated acid metabolite VI'	NA	NA	NA	4.2

*NA = not analyzed for
**NP = not present

60

R =



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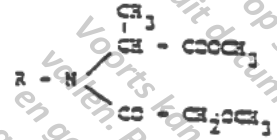
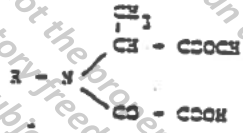
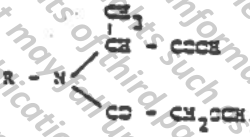
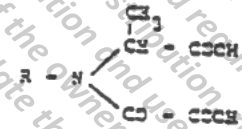
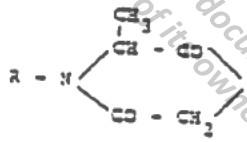
1		CGA-48988 · N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester
2		CGA-67869
3		CGA-79353
4		CGA-62826
5		CGA-78532
6		CGA-68125

FIGURE 1. CHEMICAL NAMES AND STRUCTURES

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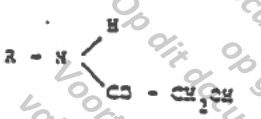
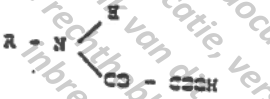
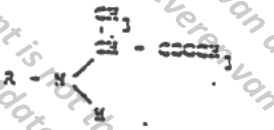
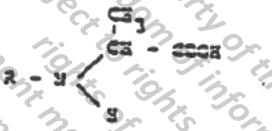
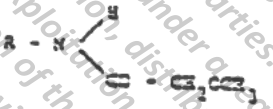
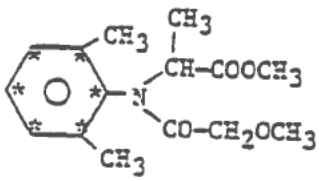
7		CGA-37734
8		CGA-68124
9		CGA-67866
10		CGA-67867
11		CGA-67868
12		CGA-72649

RADIOACTIVE COMPOUND



* = ^{14}C

ϕ-^{14}C-CGA-48988

[U-ring-^{14}C] N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester

FIGURE 1. CHEMICAL NAMES AND STRUCTURES (Continued)

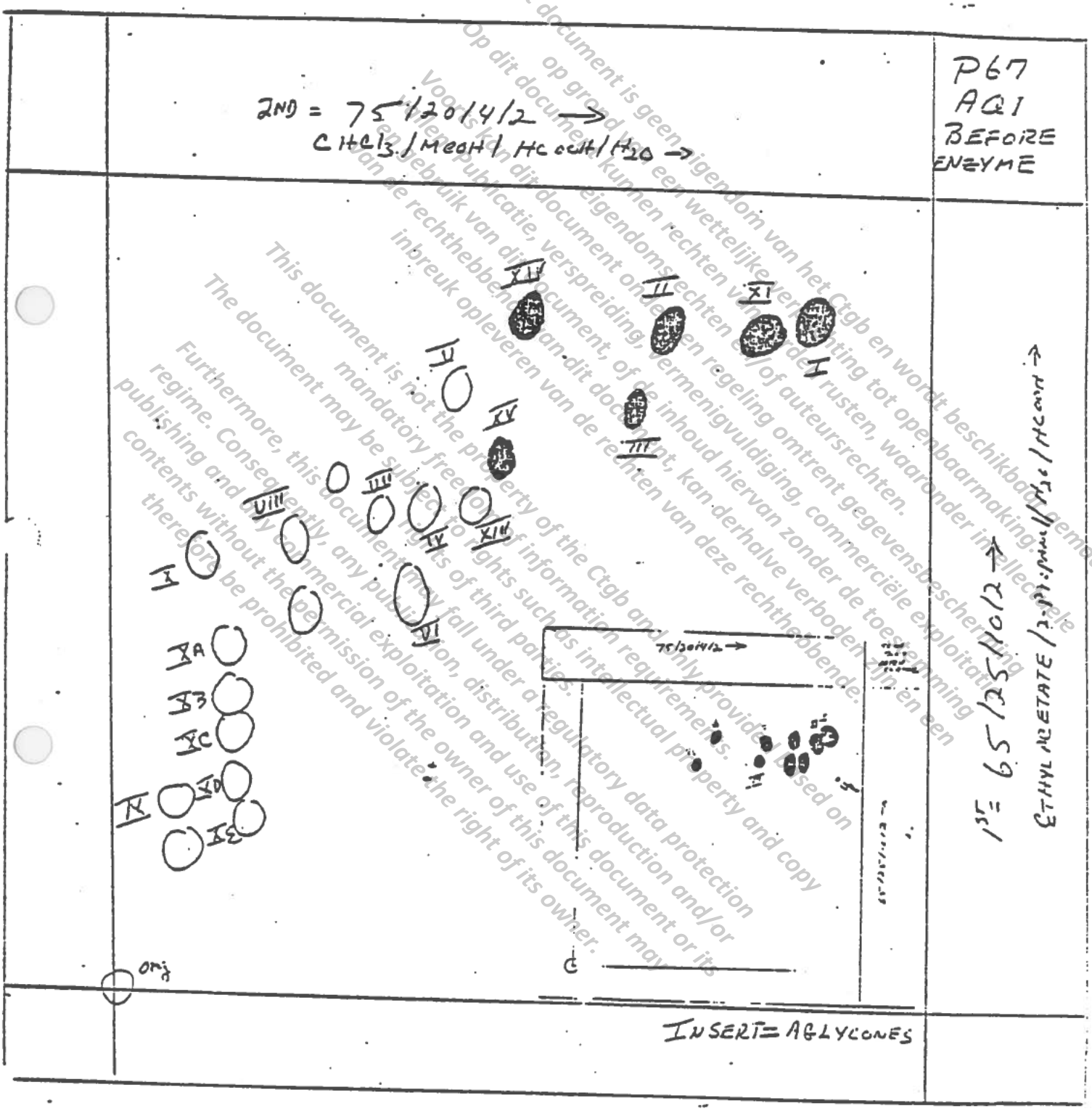


FIGURE 2. COMPARISON OF POLAR METABOLITES AND AGLYCONES

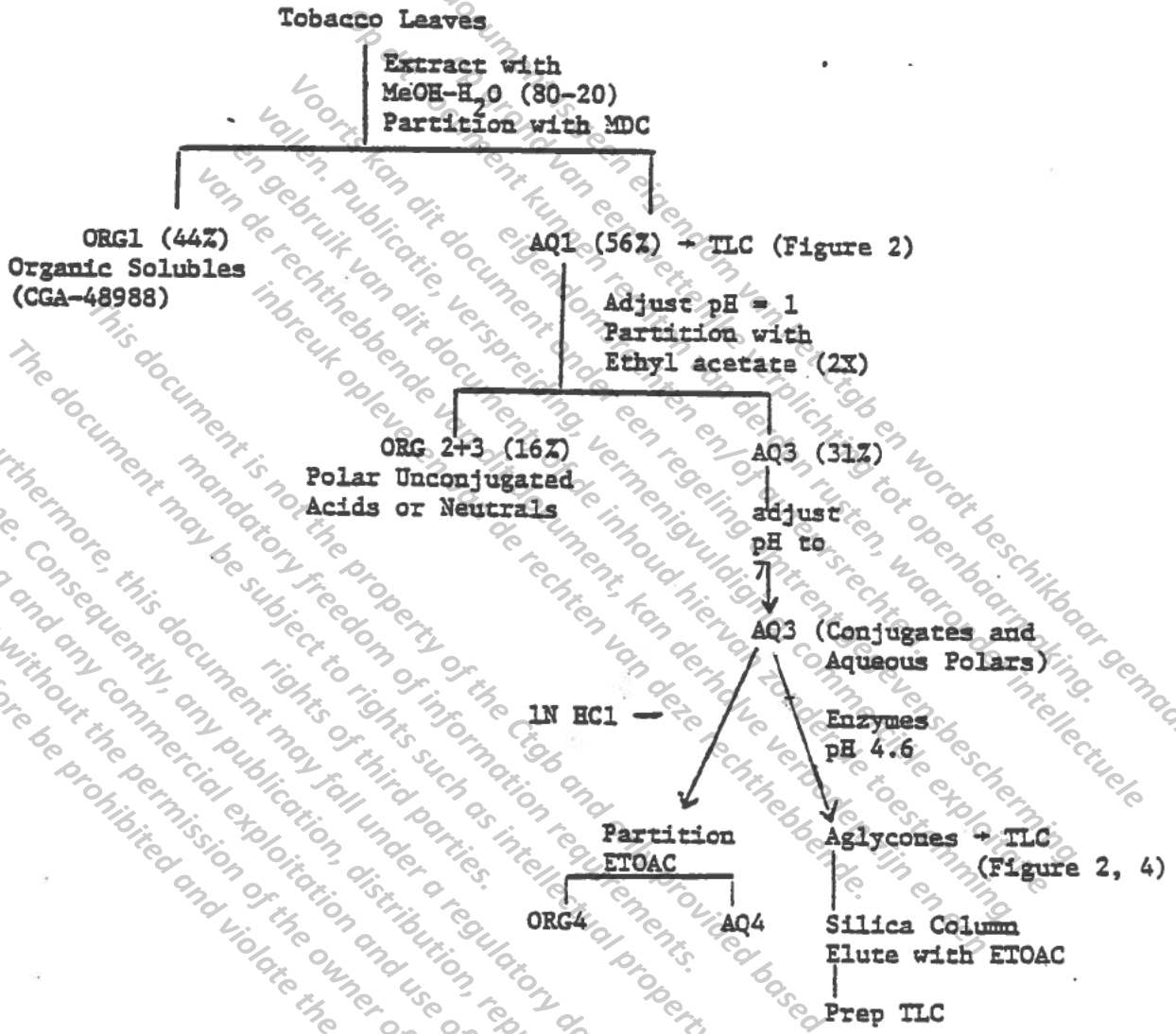
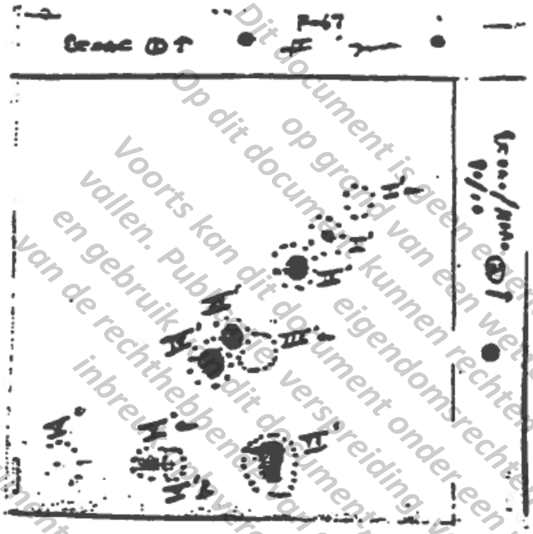
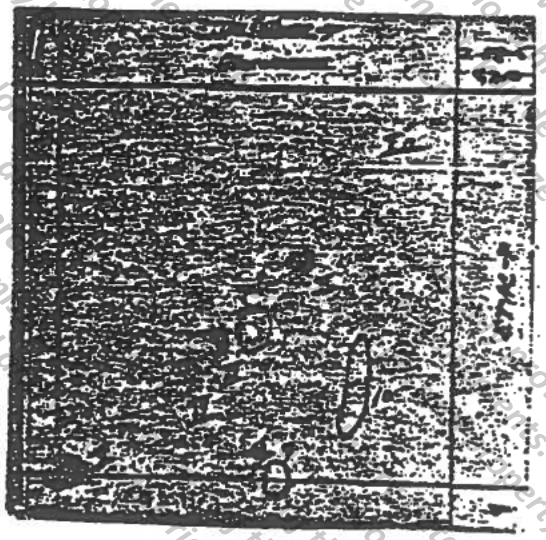


FIGURE 3: FRACTIONATION AND ANALYSIS OF ϕ-^{14}C-CGA-48988 AND POLAR METABOLITES IN 12-WEEK MATURE UNCURED BRIGHT TOBACCO (0.5 LB./A)

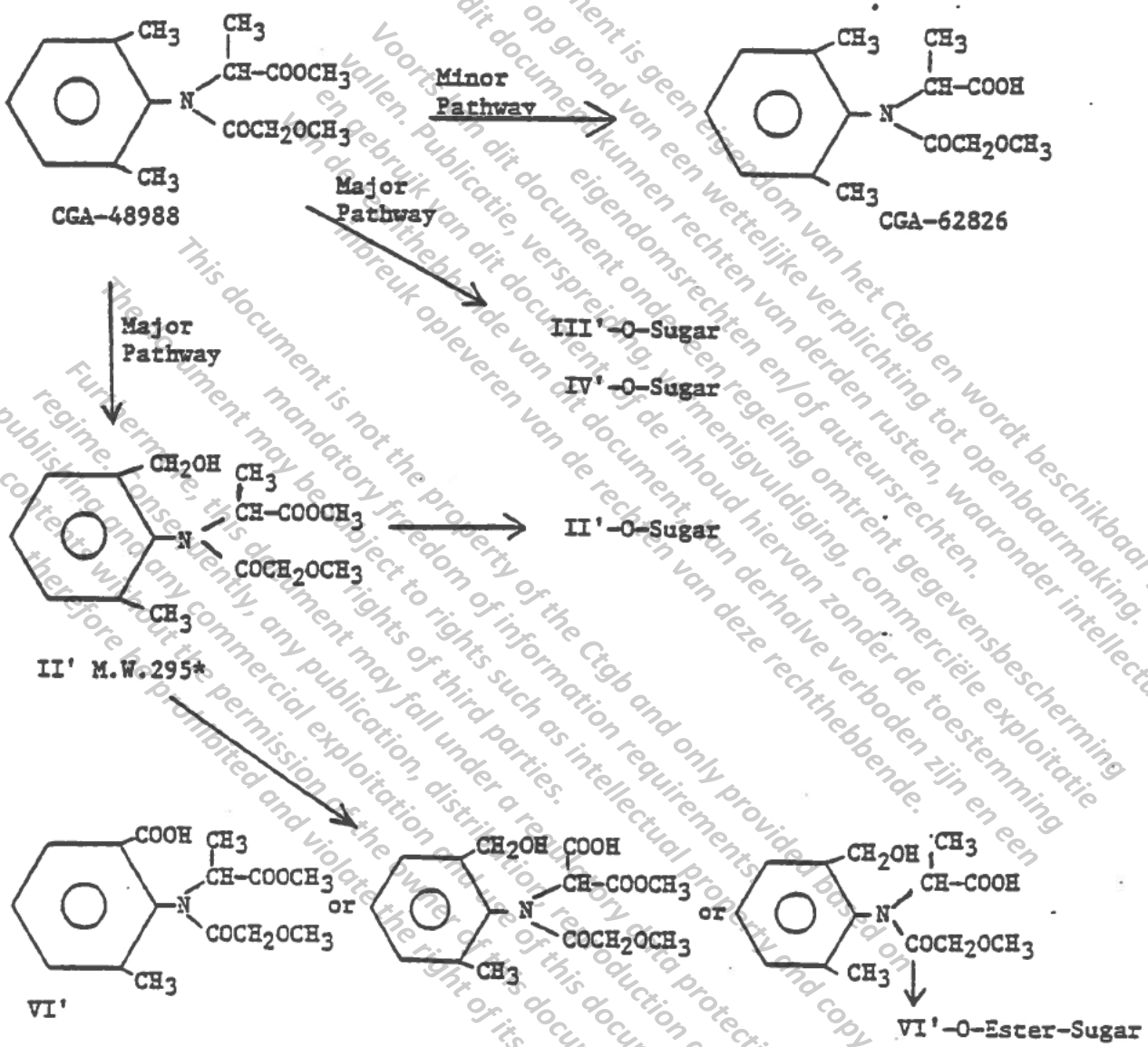


4A 2D-TLC CELLULASE TREATMENT
dotted circles = radioactive zones



4B 2D-TLC GLUCOSIDASE TREATMENT
solid circles are standards (see Figure 1)

FIGURE 4: <u>AUTORADIOGRAM OF TLC OF AGLYCONES RELEASED FROM POLAR METABOLITES WITH ENZYMES</u>



*Based on C.I. Mass Spectral Data

FIGURE 5: POSSIBLE METABOLISM PATHWAY OF CGA-48988 IN TOBACCO