



Isolation and identification of the toxic principle from *Tetradymia glabrata*
by Samuel Kenneth Reeder

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Chemistry

Montana State University

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Abstract:

Tetradymol, 3,4a(R),5(S)-trimethyl-8a(S)-hydroxy-4,4a,5,6,7,8,8a,9-octahydro-naphtho [2,3-b] furan (II), has been isolated from *Tetradymia glabrata*, a desert plant of the great Salt Lake Basin, which is the cause of extensive range losses of sheep. This compound was shown to be an active principle of the plant by sheep and mouse feeding experiments.

The structure of the compound was established on the basis of chemical and spectral data, and confirmed by an X-ray crystallographic structure of the 2-chloromercury derivative.

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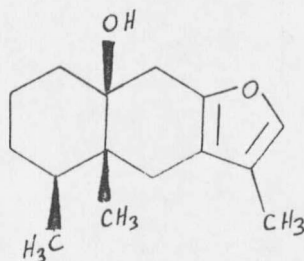
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ABSTRACT

Tetradymol, 3,4a(R),5(S)-trimethyl-8a(S)-hydroxy-4,4a5,6,7,8,8a,-9-octahydro-naphtho [2,3-b] furan (II), has been isolated from Tetradymia glabrata, a desert plant of the great Salt Lake Basin, which is the cause of extensive range losses of sheep. This compound was shown to be an active principle of the plant by sheep and mouse feeding experiments.

The structure of the compound was established on the basis of chemical and spectral data, and confirmed by an X-ray crystallographic structure of the 2-chloromercury derivative.



(II)

INTRODUCTION

The living organism is a very complex unit performing a great number of integrated functions, but it could be considered as an integrated chemical plant taking in raw materials and converting them into energy and products that it needs for building and other functions. However, as is the case in most chemical plants, the entering raw materials and the products must be purified of the nonusable materials and by-products, and these materials must be eliminated.

In mammals there are a number of organs that are responsible for the above type of purification and elimination; the lungs, intestines, kidneys, and liver to name a few. One of these, the liver, is important for purification of the raw materials coming from the digestive tract, removal of some deleterious materials of normal metabolism from the blood, and excretion of these materials or their conjugates by means of the bile duct. These materials, to be excreted, are of many different types and require diverse enzymatic processes for reaction and/or excretion. The liver, thus, plays a very important role in the function of the total system and because of this diversity of function can be injured in many ways.

A brief outline of hepatic function with respect to substances entering the body from the intestine is shown in Figure 1. Food substances and other molecules pass through the intestinal walls into the blood capillaries and are then transported into the portal vein.

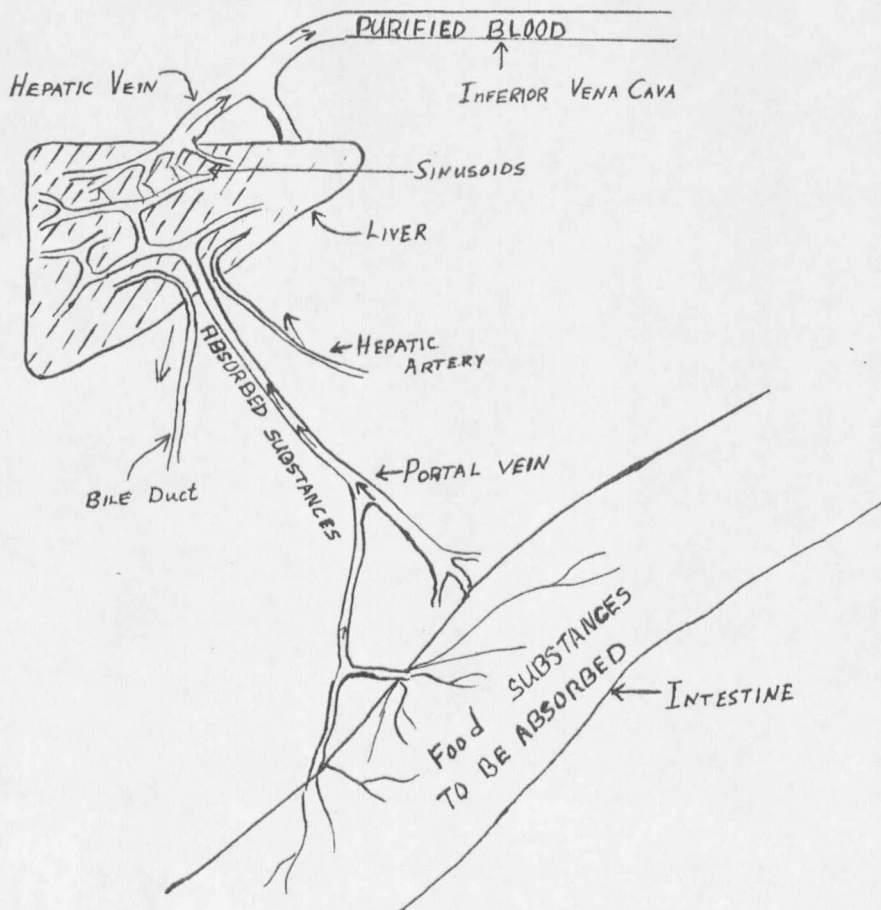


Figure 1. A Diagram of Hepatic Function

which transports them into the liver. Here the blood passes into smaller vessels until it reaches the sinusoids of the lobules. Classically these lobules are the small units of the liver bounded by planes drawn between the lines of the hepatic triad; hepatic artery, portal vein, and bile duct surrounded by Glisson's capsule; centered about the central vein as shown in Figure 2. The purification of incoming blood

The classic hepatic lobule is outlined with solid lines, the portal lobule with an interrupted line, and the liver acinus or functional unit with a dotted line. The branches of a portal vein and a hepatic artery (solid) from one portal area are shown at lower right. Portal areas are labeled "P", central veins "C".

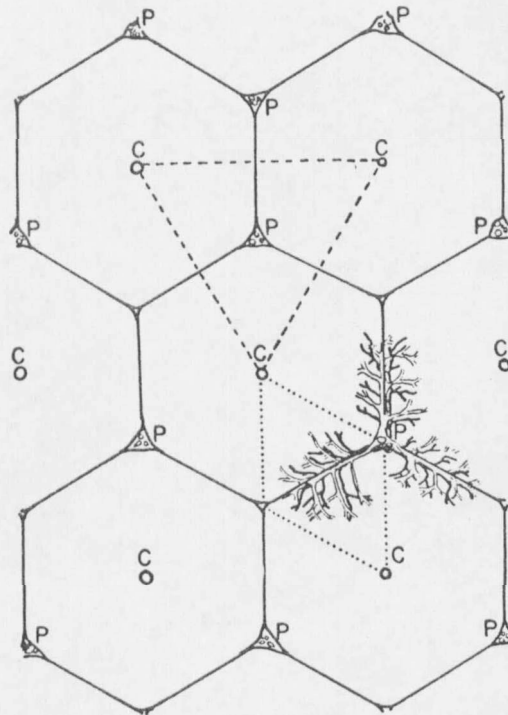


Figure 2. A Diagram of Hepatic Lobules¹

takes place in the sinusoids by means of the parenchymal and Kupffer cells with the materials to be excreted going into the bile ducts and the purified blood then passing into the ramifications of the hepatic venous system and finally into the inferior vena cava, a part of the more general circulatory system. In humans the total hepatic blood flow is approximately one and one half liters per minute with sixty to eighty per cent of this flow coming from the portal vein to the hepatic vein. The other twenty to forty per cent comes from the hepatic artery to the hepatic vein.² About twenty per cent of the total blood of the

human body is to be found in the liver and spleen.³

Harmful substances entering the liver from the portal vein affect the liver in two basic ways. They can sensitize the liver and lead to an allergic reaction, induced hepatitis, or they can demonstrate a true hepatotoxic activity, toxic hepatitis.⁴

"Hepatotoxins are a heterogeneous group of naturally occurring and synthetic chemical compounds"⁵ with diverse actions. They do, however, exhibit a number of common characteristics. They "(1) exhibit a distinctive histological pattern for any given hepatotoxin, (2) vary in severity in direct relation to the dose, (3) can be elicited in all individuals, (4) are reproducible in laboratory animals, and (5) appear after a predictable and usually brief latent period following exposure."⁶

Numerous tests of liver function have been devised to determine both the extent and the mode of attack of hepatotoxins. Among these are tests of the removal and excretion of injected dyes from the blood, and levels of particular enzymes or metabolites in the blood. In general, these tests measure the liver's ability to "sort" molecules and excrete them directly or in an altered form, and its ability to enzymatically perform chemical reactions.

Specifically one of the most widely used measures of liver function is the BSP (sulfobromophthalein) excretion test.⁷ Normally, this organic dye is injected intravenously and its disappearance from

the blood monitored by the use of a colorimeter. The test is an extremely sensitive measure of general liver function. The dye is known to be removed from the blood almost exclusively by the liver and to be excreted through the bile duct after conjugation with glutathion. Thus, this test gives the sum of the uptake, conjugative, and excretive abilities of the liver.

The three most common tests of specific enzyme activity related to the liver are the serum alkaline phosphatase (SAP), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) tests which are run on blood serum.⁸ These tests have been shown to be diagnostic for different types of hepatodysfunction. The SAP activity is greater with biliary obstruction than hepatocellular damage while the GOT and GPT activities are much greater in hepatocellular damage than biliary obstruction. Possible reasons for the changes in enzyme levels that have been suggested, but not proven, are an increased production of the enzymes or a decreased excretion of the enzymes by the liver. Though these possibilities are logical they are neither original nor definitive. Much work is left to be done in order to obtain an understanding of the importance of these enzymes to liver function.

Analysis for the amount of ammonia that can be freed from the blood upon the addition of base has also been used as a measure of liver function. The form in which this ammonia is carried in the blood

is not known but it obviously could not be free ammonia at physiological pH. Some authorities⁹ have suggested that this easily available ammonia is carried in the blood as simply ammonium hydroxide while others¹⁰ favor a protein bound form. Regardless of its form in the blood, this ammonia, which primarily comes from the gastro-intestinal tract, is normally converted into urea by the liver cells through the biochemical pathway known as the urea cycle (Figure 3). This urea is then allowed to enter the hepatic vein and to circulate throughout the body as a useful reduced form of nitrogen. Increased levels of blood ammonia have been attributed to the shunting of portal blood around the liver of the decreased ability of the parenchymal cells to synthesize urea. Abnormally high concentrations of blood ammonia can lead to pulmonary edema, which has been attributed to membrane irritation or increased venous pressure as a result of damage to the sympathetic nervous system.¹² Ammonia is also reported to lead to neural damage resulting in coma and ultimately death.¹³

The above tests and others, along with histological studies, are often used to give a picture of the gross changes in the liver caused by hepatotoxins. But these methods do not monitor functions that are specific enough to elucidate the actual mechanism of the attack of the hepatotoxin. In all instances they simply show that the liver is not functioning properly or has been damaged.

"Considering that hepatotoxins differ markedly in chemical

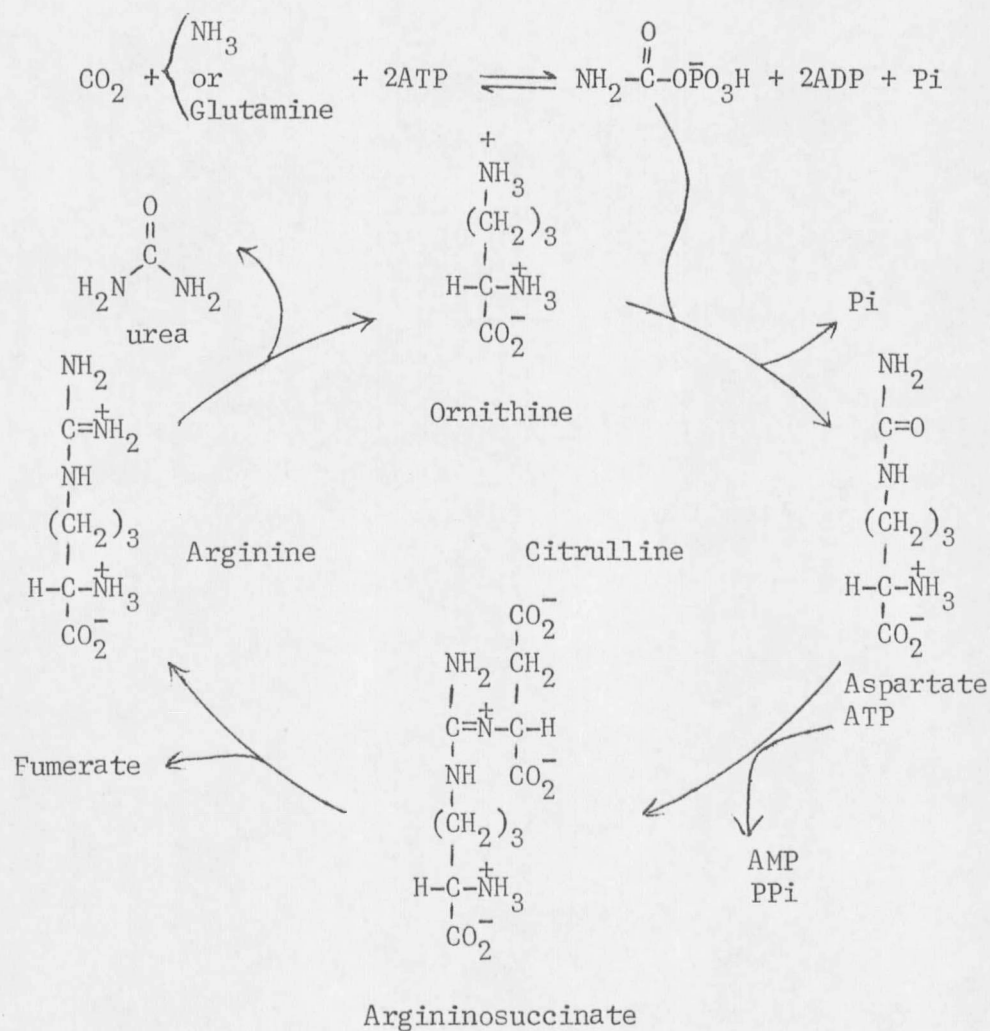


Figure 3. The Urea Cycle¹¹

structure, it is highly improbable that they all attack the liver in the same way. The uniformity of the biochemical finds in the liver after exposure to hepatotoxins of diverse character suggests that, although these agents probably initiate cellular injury in different ways, the subsequent chain of biochemical events leading to necrosis

follows a common pathway."¹⁴ The problem seems to be that only a few hepatotoxins have been studied intensively at the biochemical level. The most work seems to have been done on the halogenated hydrocarbons with numerous compounds being related to them rather than being individually studied.

A number of mechanisms for the initial attack of a hepatotoxin have been proposed. Some of these mechanisms have rather good experimental evidence while others are much more poorly founded. Among them are: "(1) impairment of hepatic blood flow,...; (2) alterations in lipid metabolism; and (3) disturbances in protein synthesis and energy production in the liver resulting from direct injury to hepatic cell membranes, or metabolic alterations in essential constituents of the hepatic cell."¹⁵

A brief examination of these mechanisms reveals that the most weakly supported one is that suggesting impairment of hepatic blood flow as a primary lesion. The evidence¹⁶ shows that there is intra-lobular vasoconstriction in carbon tetrachloride poisoning that can be blocked by cordotomy, a procedure that blocks sympathetic stimuli which control the sphincters in the blood vessels which in turn control the blood flow. This operation fully protects against hepatic necrosis and alterations in mitochondrial enzymatic activity usually seen following carbon tetrachloride poisoning. However, the evidence from the cordotomy is refuted by other workers¹⁷ who have found that the

operative procedure without actual cordotomy also protects. They suspect that hypothermia is the real factor here, not the neural change. There are neural changes in toxic hepatitis but these are usually attributed to ammonia toxicity or other factors after the initial attack on the liver. Regardless, the primary lesion here is in the sympathetic nervous system not in the liver blood flow, if the above mechanism is correct. The blood flow does obviously change in toxic hepatitis, but this does not necessarily point to the sympathetic nervous system nor indeed to blood flow changes as a primary lesion. It could well be that swelling in parenchymal cells due to some type of cellular damage is the cause of the change in blood flow.

Alterations in lipid metabolism and resultant fatty liver is certainly an important factor in liver disease but there seems to be little evidence that it is a primary lesion. Many toxins cause fatty liver but a few that do cause this symptom have been shown to decidedly cause other changes in the liver first. None mentioned in Klatskin's review¹⁸ have been shown to cause fatty infiltration first. It seems more likely that fatty infiltration is an evidence of some change in the liver not the change itself, i.e., something has happened to an enzyme system or a membrane that allows the fatty build-up.

The third suggested mechanism involves the more basic components of the cell's function--membrane permeability, energy utilization, and enzymatic catabolism and anabolism. Changes in liver membrane

permeability have been noted as the result of the attack of numerous toxins. But again, at least in carbon tetrachloride and ethionine poisoning, these changes appear to be a secondary effect.¹⁹ The suggestion that a non-polar solvent such as carbon tetrachloride dissolves a segment of the membrane is much less than pleasing as the concentrations that are toxic are of such a low level that it is difficult to conceive of this occurring in many cells of the liver. A more useful postulate would be that the particular toxic molecule interacted directly with a specific molecule in the membrane, some enzyme, or another specific molecule of importance in the cytoplasm. The best example of this latter interaction is the proven effect of ethionine. This molecule has been shown²⁰ to specifically form a compound with adenosine, S-adenosyl ethionine, that cannot be used by the cell. This bonding of adenosine obviously cuts off the supply of ATP, the main energy currency of the cell, and thus, sets off a sequence of events that cause the cell's destruction including in the process detachment of ribosomes from the endoplasmic reticulum and fatty infiltration. One has difficulty observing ATP supplies by either electron microscopy or light microscopy--in fact it is impossible to do so at present--so these means again are shown only to give gross changes, i.e., the results of minute biochemical change not the change itself.

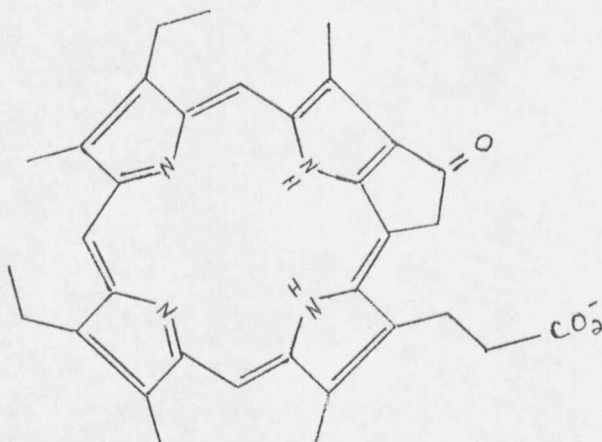
Perhaps the most intensively studied hepatotoxin is carbon tetrachloride which has been found to first attack the endoplasmic reticulum

dislocating the ribosomes and subsequently inhibiting protein synthesis and plasma lipoprotein synthesis.²¹ The means by which these changes are effected is not known, however. After the initial attack of a hepatotoxin there are sometimes rather drastic changes in membrane permeability leading to mitochondrial destruction and cellular swelling. This hypertension can cut off the cell's oxygen supply from the blood, as has already been noted. This can then lead to a stoppage of the anabolic processes coupled with the release of autolytic enzymes which result in the cell's destruction. With other types of hepatotoxins there seem to be little or no inflammation but rather simply acute necrosis. It is also important to note that necrosis can be localized within the lobule as centro-, midzonal, peripheral, or panlobular depending on both the toxin and its concentration.²² It is not known, however, whether the location of the lesion in the liver is due to the ratio of blood flow to cell mass, the localization of enzymatic activity or other factors. What is needed is studies of actual biochemical interactions on the molecular level with the specific toxin to find where and how it is interacting. When an understanding of this occurs on the molecular level it will be possible to begin to design molecules that will reverse the toxic effect or replace the bound metabolites.

One demonstration of liver dysfunction is photosensitization, type III of Clare.²³ In this syndrome the injured liver is unable to perform its normal function of removing the pigment phylloerythrin (I) from the

portal blood and excreting it in the bile. In this manner the pigment is able to reach the peripheral circulation and, thence, the skin tissue where photosensitization occurs. An outstanding example of this syndrome is found in the work of Rimington and Quinn²⁴ with geeldikkop, "big yellow-head" of sheep, in South Africa.

(I)



Clare²⁵ suggests that the "big head" problem of sheep in the intermountain area of the western United States of America is another example of type III photosensitization. His suggestion is based on two studies of the desert plant, Tetradymia glabrata, that were completed previous to 1940.

T. glabrata is a strongly scented shrub of the Compositae family, Senecio tribe, that grows over the rather broad region that is shown in Figure 4. The plant (figure 5) resembles sage brush and is found in the same general region as sage. It can be as much as three feet tall. The current year's growth (A), or new growth, has thin light colored leaves that are pointed and about one half inch long. The entire new growth

(leaves, stem, and flower buds) are tomentose (covered with fine white hairs). The older growth (B) is woody and highly branched with fewer leaves. These leaves are more rounded, shinier, smaller and thicker than those of the new growth. The flower heads that appear from April to mid-



Figure 4. Range of the Occurrence of Tetradymia²⁶

June are yellow but soon turn white from the silky hairs on the seeds.



Northwestern Utah Desert
where the plant grows

A typical range specimen
of T. glabrata



A- New Growth

B- Older
Growth

Mounted plant specimen
in the Montana State
University Herbarium

Figure 5. The Plant, Tetradyimia glabrata.

Fleming²⁷ first indicated that T. glabrata was responsible for not only the "big head" symptoms of facial and ear tissue swelling, but also the death of many sheep that occurred during these "big head" outbreaks on the ranges of Nevada. In the years up through 1922, Fleming and his colleagues²⁸ conducted many feeding experiments and a brief chemical study in an attempt to ascertain (1) the conditions required for poisoning, (2) the amount of the plant required for poisoning, and (3) the nature of the toxic principle. They succeeded in showing that a feeding of approximately two and one half per cent of the sheep's body weight of the green tops and buds was sufficient to kill the animal but there is no mention of the swelling of the head in their later paper. They attributed the deaths of the animals to hepatodysfunction and cardiac failure caused by a substance contained in the plant. Their work on chemical characterization was scanty but they did succeed in demonstrating the toxicity of the petroleum ether and acetone extracts of the green plant by a single rabbit feeding experiment with each extract.

Clawson and Huffman²⁹ working on the "big head" problem in Utah obtained much the same results as Fleming³⁰ had earlier reported. These later workers did not attempt any chemical characterization of the toxin. They did, however, pursue the problem of photosensitization as there was no external edema observed resulting from their early feeding experiments. They later reported³¹ nine sheep had developed "big head" when

experimentally fed Tetradymia glabrata while being herded on the desert range. Huffman then tried the co-feeding of T. glabrata with many other range plants to find if the photosensitization was due to the interaction of two plants. In his unpublished data³² is found that, of the approximately forty plants co-fed, only by feeding Artemisia tridentata or nova with T. glabrata or canescens could any "big head" swelling symptoms be noted. This doesn't follow from the Clare type III photosensitization as phylloerythrin (I) is the product of the metabolism of chlorophyll which can come from any higher plant source not just Artemisia. However, in all whole plant, Tetradymia, feeding experiments the most characteristic lesion was liver necrosis so the problem is still probably related to Clare type III photosensitization though the sensitizing pigment may be different.

The following investigation was undertaken to ascertain the chemical structure of the compound or compounds responsible for the toxicity of T. glabrata and to further study its effect on the living system.

DISCUSSION

This discussion is divided into four major sections to facilitate presentation and organize the materials. The sections deal with:

I. physiological activity, II. isolation of the toxin, III. chemical and spectral elucidation of structure, and IV. X-ray structural analysis.

I. Physiological Activity

In agreement with the findings of Clawson and Huffman,³³ the toxic principle of T. glabrata was localized in the new growth and flower buds of the plant. When the whole top of the plant; which included considerable older, dry, woody material; was fed approximately five times as much plant material was required to produce death (sheep S-553) as with just buds and flowers (sheep P-311) as is shown in Table 1. Since these two samples of plant material were picked at different times it might be argued that the lack of toxicity demonstrated by the whole plant was due to different growth stages of the plant. This does not appear to be true, however, since Tg I was picked earlier than Tg II and Fleming³⁴ and Huffman³⁵ indicate that toxicity decreased as the season progressed. It should also be noted that due to late rains in 1968 the plant was in a quite similar growth stage at both pickings. Thus, there appeared to be little toxin in the older growth. The hexane extract of the root stock of the plant showed no evidence of the active principle of T. glabrata when it was analyzed by tlc.

Table 1

Sheep Feeding Experiments Using the Whole Plant

Sheep number	Weight pounds	Age years	Days fed	Plant fed	Total pounds fed	% plant of body weight	NH ₄ ⁺ level [†]	BSP clearance**	Result
S-553	115	2	11	Tg I	16	13.8		8/11 10/16	death
S-536	108	2	7	Tg I	6	5.5			release
S-230	108	2	11	Tg I	16.6	15.4		5/35 7/70 9/26	death
S-563	110	2	9	Tg I	13.5	12.3		4/8 6/13 8/7	release
				At*	2.5	2.3			
P-380	114	2	3	Tg II	3.0	2.6			death
				At	3.0	2.6			
P-311	110	2	4	Tg II	4.0	3.6	4/24	4/26	death
H-641	102	1	3	Tg III	4.1	4.0	2/12 3/13	2/36	death
H-522	120	1	0				0/4		control
H-552	132	1	0				0/4		control
Ewe-1	87	1	0					0/3	control

* Artemisia tridentata picked while very lush near Bozeman, Montana

[†] Recorded as a fraction with the day of the feeding experiment when the NH₄⁺ level test was run in the numerator and the µg/ml of NH₄⁺ found in the serum recorded as the denominator.

** Recorded as above with the day in the numerator and T_{1/2} in minutes recorded as the denominator.

The results of the oral feeding experiments with T. glabrata, recorded in Table 1, give an idea of the mode of action of the toxic principle. The BSP test clearly revealed that the liver was undergoing severe damage. The normal sheep, as illustrated by Ewe-1, had a one-half time (time required for one half of the dye to be eliminated) of from three to seven minutes while a sheep receiving T. glabrata demonstrated a one-half time ($T_{1/2}$) of from sixteen minutes in sheep S-553 to as high as seventy minutes in sheep S-230.

Another test of liver function, blood ammonia, also revealed that there was severe hepatic dysfunction. The normal sheep, as illustrated by H-522 and H-552, had a blood ammonia level of about 4 $\mu\text{g}/\text{ml}$. When a sheep was fed T. glabrata this level rose to 13 $\mu\text{g}/\text{ml}$ in H-641 and 24 $\mu\text{g}/\text{ml}$ in P-311. Both these tests indicated that the liver was malfunctioning previous to the death of the sheep but did not show how the toxin attacked the liver nor did they reveal if the liver was attacked first.

Since Fleming³⁶ had stated that the acetone extract was possibly cardiac-active all sheep were monitored by EKG for possible heart damage but no significant change was noted.³⁷ An example of an EKG taken before any plant feeding and one taken after fifteen days had elapsed in the feeding of S-230 (Table 1) is shown in Figure 6. The only change noted is a slight slope in the S-T segment which indicated some damage but not of a marked nature.



Just prior to first oral feeding of T. glabrata
Lead 4



After fifteen days of the feeding experiment had elapsed,
two days before death
Lead 4

Figure 6. Electrocardiograph of Sheep S-230

Sheep S-536 was released after feeding it a sublethal dosage of T. glabrata to find if there might be a delayed activity of the toxin or if photosensitization might occur. The sheep was visually examined on a number of occasions up to two months after release and found to be normal. Sheep S-563 was released after the feeding of a sublethal dosage since co-feeding of A. tridentata had caused no photosensitization nor increase in toxicity of T. glabrata. This sheep was also observed after release and demonstrated no visible adverse effects from the feeding.

Autopsies were performed on all sheep that were sacrificed or died as a result of T. glabrata feedings. In all cases macroscopically severe liver necrosis was evident. The liver tissue was friable and from tan to clay in color. The gall bladder was extended and filled with watery green bile. Most, 5 out of 6, of the sheep demonstrated considerable lung congestion while one half demonstrated some macroscopic changes in the kidney including roughness of some of the cortical portion, a marbled appearance of the outside of the cortex, and a yellow discoloration (one example of each).

Samples of tissue were taken from the liver, lung, heart, kidney, adrenal gland, bronchial lymph node, pancreas, abomasum, spleen, and thyroid gland for microscopic examination. Dr. K. Anderson in the Diagnostic Laboratory located at Montana State University examined the tissue specimens and made the following observations, some of which are

summarized.

The liver tissue typically demonstrated "marked panlobular necrosis of liver parenchyma. Necrosis was centrilobular in location and bordered on more peripheral parts of the lobules in which fatty change was evident in degenerated, but still viable, cells adjacent to the portal triads. ...The lesions described are non-inflammatory. Necrosis tended to extend from one central vein to another and resulted in an appearance of pseudo-lobulation. Kupffer cells within necrotic areas appeared viable. In portal areas, there appeared to be an early, slight proliferation of bile ducts."³⁸

The kidney tissue showed hyperemia (increase in the amount of blood) especially in the medullary portion and some general congestion and swelling.

The lungs displayed all the way from slight to extreme congestion and from alveolar emphysema to intra-alveolar and intra-bronchiolar hemorrhage. Some bronchioles showed "pronounced exfoliation of lining epithelium suggestive of the presence of an irritant."³⁹

The cardiac tissue revealed some congestion and a few sub-epicardial hemorrhages but there was no great change from normal heart tissue. In the only sample of abomasum tissue examined hyperemia was evident and there was "a slight mononuclear inflammatory response in the deeper aspect of the mucosa and in some related areas of the submucosa."⁴⁰ The other tissues examined seemed to be quite normal

microscopically.

From these studies it was concluded that the active principle of T. glabrata was hepatotoxic to all animals tested. These findings indicated that any damage to other body tissues was minor and probably of a secondary nature. The cause of death was probably heart failure induced by acute toxemia.

That a problem existed in relating photosensitization to poisoning by T. glabrata was alluded to in the introduction. Huffman⁴¹ suggested that "big head" was an example of what Clare⁴² later calls type III. photosensitization. However, this was merely a surmise as he presented no experiments in his article to support the suggestion. Clare⁴³ also supported this view in his article but again without evidence. In the experiments described in this study no swelling of exterior tissue was observed in any animal. The sheep were on green pasture with much sunlight during the time of the experimental feedings so that if chlorophyll were required in the diet for the accumulation of phyllorethrin leading to a sunlight induced photosensitization the conditions should surely have been met, especially since even a diet of hay and chaff provides enough sensitizing pigment.⁴⁴ As the feedings were over varied lengths of time it does not follow that a small amount of liver damage, caused by T. glabrata, followed by green feed leads to photosensitization as was earlier suggested by Huffman.⁴⁵

Since the swelling of external tissue was not observed in the

present study it lends credence to Huffman's later observations⁴⁶ that have already been noted. It would thus seem very unlikely that "big head" is a type III syndrome but rather that it is a new type that requires very special conditions and perhaps a particular pigment from another plant to serve as sensitizer after liver destruction. If it was a simple relationship between A. tridentata and T. glabrata, photosensitization should have been observed in sheep P-380 but this was not the case. Dr. M. Madsen of Utah State University reported⁴⁷ that if a sheep was fasted then fed a sublethal dose of T. glabrata followed by water and A. tridentata photosensitization regularly occurred. The above finding has not been checked in this laboratory. Therefore, the way photosensitization occurs, if indeed it does from T. glabrata poisoning, is still uncertain.

When the hexane and acetone extracts of T. glabrata were separately given to sheep by means of capsules, the same toxicity demonstrated with the whole plant was exhibited as shown in Table 2. Since it was not certain that hexane or acetone would extract all the toxin from the plant material, the extract of a sample equivalent to twice the toxic dose was fed in each instance. The results of the BSP tests and the blood ammonia tests on H-636 and H-665 recorded in Table 2 indicated the same liver dysfunction as had been found previously. The findings of histopathological studies also supported this conclusion. When the plant that had been extracted with acetone and hexane was fed in a

quantity that had been sufficient to kill in previous experiments (with unextracted plant) no toxicity was observed (sheep H-641 and H-671). Thus, it was revealed that the toxic principle of T. glabrata had been quite effectively extracted into hexane and acetone.

The fresh plant was also extracted with ethanol. This ethanol extract was evaporated to a viscous oil then diluted slightly with aqueous ethanol. The extract was partitioned into two fractions by extraction with pentane. These two fractions, the pentane extract of the ethanol extract and the remaining part of the ethanol extract after pentane extraction, were evaporated and separately fed to sheep H-628 and H-634 respectively. Neither of the extracts demonstrated toxicity. The ethanol extracted plant was also fed to sheep H-653 and showed no toxicity. The reasons for the lack of toxicity demonstrated in these experiments with the ethanol extract was not certain since the extracts were not analyzed to determine what molecules were present except in the case of the pentane extract of the ethanol extract. This did show a high concentration of the compounds later shown to be toxic from T. glabrata. It could well be that not all the toxin was extracted from the aqueous ethanol layer or that ethanol was not as effective in extracting the toxin from the plant. Thus, the lack of toxicity could possibly be attributed to partition of the toxin into too many fractions reducing the actual dosage to a level below the effective dose. However, there are other possibilities including the destruction of the

Table 2

Sheep Feeding Experiments Using the Plant Extracts

Sheep number	Wt. lbs.	Age yrs.	Extract fed	Extract from pounds	% plant of body weight	NH ₄ ⁺ level*	BSP clearance†	Result
H-636	98	1	Acetone	4.4	4.5	3/12 5/18	3/36 5/79	Death
H-665	104	1	Hexane	5.0	4.8	3/14 5/13 7/14	5/44 7/39	V. ill†† sacrificed
H-634	103	1	Pentane of ethanol	2.5	2.5	3/6		Release
H-628	104	1	Ethanol remains	2.5	2.5	3/6		Release
			Plant extracted with	Pounds fed				
H-651	102	1	Acetone	2.2	2.2	3/5		Re-use
H-671	90	1	Hexane	2.5	2.8	3/8		Release
H-653	100	1	Ethanol	2.5	2.5	3/6		Release

* Recorded as a fraction with the day of the feeding experiment when the NH₄⁺ level test was run in the numerator and the µg/ml of NH₄⁺ found in the serum recorded as the denominator.

† Recorded as above with the day in the numerator and the T_{1/2} in minutes recorded as the denominator.

†† V. ill meaning very sick, actually down and on the verge of death.

toxin in the crude extract by the higher temperatures required in stripping off the ethanol or by some other factor.

The experiments with the hexane and acetone extracts, never the less, did confirm Fleming's finding⁴⁸ that the lipid extract was toxic.

Since sheep required such large quantities of toxin, due to their size, and were quite expensive as a laboratory animal it was decided to attempt using small animals for testing purposes. After trying five different animal species (Table 3) and finding that the acetone and hexane extracts were both hepato-toxic, in all but one case, mice were chosen as a test animal because they were inexpensive, were generally easier to handle, and didn't bite as hard.

Acetone extracted approximately 3.7 times as much total material from the fresh plant as did hexane. Thus, to feed the extract of the same weight of plant it was necessary to feed 3.7 times as much of the crude acetone extract as of the hexane extract. For this reason the percentages of body weight differ between the two extracts in Table 3. Each feeding represents the extract of fresh plant equivalent to approximately 5 per cent of the animal's body weight.

When these animals were opened after death a marked change had taken place in the liver. Instead of the normal dark mahogany color of the liver there were observed white patches on a light pink background. Microscopic examination revealed for the greater part the same type findings as were observed in sheep tissue. The lungs were congested,

Table 3

Small Animal Feeding Experiments Using the Plant Extract^{*}

Animal	Weight (g)	Extraction Solvent	% Extract of body wt.	Hours to death
Rabbit	3220	Hexane	0.25	14.5
Rabbit	2680	Acetone	0.95	20.5
Guinea pig	780	Hexane	0.25	6
Guinea pig	620	Acetone	0.95	12
Gerbil	77	Acetone	0.95	33
White rat	336	Hexane	0.25	3
Black rat	445	Acetone	0.95	not ill
Mouse	22	Hexane	0.25	3
Mouse	22	Hexane	0.25	5
Mouse	22	Acetone	0.95	4
Mouse	22	Acetone	0.95	4

* All extracts taken from an amount of plant equivalent to 5% of the small animal's body weight.

the heart and brain tissue appeared to be normal while the liver tissue displayed the most prominent changes.

Cloudy swelling of the parenchymal cells without necrosis was apparent in the gerbil, while the guinea pig showed scattered areas of blanching of the parenchymal cells suggestive of early degenerative change but without necrosis. Acute necrosis similar to that found in sheep was apparent in the rabbit with one obvious difference. In sheep the necrosis was centro-lobular while in the rabbit it was periportal in distribution. There was only a beginning of bile duct proliferation in the rabbit. Panlobular degeneration and necrosis of hepatic parenchymal

cells was apparent in the mouse. Necrosis affected numerous cells but was preceeded by degenerative changes and was not present in clearly defined foci as was noted in the livers of sheep.⁴⁹ There appeared to be no differences in the microscopic findings between any two animals of the same species fed the hexane or acetone extract indicating the toxic material was present in both extracts.

Fleming⁵⁰ indicated that the one rabbit that died as a result of the toxicity of the acetone extract had suffered heart failure. He then suggested that there were two different toxins in the plant, a cardiac-active and a hepato-active substance. Since the amount of plant extracted for feeding and the results of the necropsy findings were the same for both extracts in all animals of the same species tested in this laboratory, the postulation of different active principles in the two extracts seems to lack foundation. To this can be added that the results of blood ammonia and BSP tests performed on sheep H-636 and H-665 (Table 2) indicated the two extracts were also effecting the sheep in the same way.

A possible reason for these results differing from those of Fleming⁵¹ alluded to above, is found in his use of only one rabbit for testing purposes. He suggested heart failure as the cause of death but without sufficient experimental evidence to warrant this conclusion. From the paper⁵² it appears that the cause of death was not obvious and was therefore attributed to heart failure. The other possible causes of

death for this one rabbit could have been stress from the feedings, a trace contaminate in the acetone, or even some type of infection that was overlooked in the necropsy. Had Fleming used more experimental animals his results would probably have been quite different and in line with the findings recorded in this current study.

After the pure toxin, tetradymol (II, page 49), had been isolated it was important to establish an approximate LD_{50}^{53} for mice. As the toxin is a solid, it was necessary to use a carrier for the oral feedings. Propylene glycol, 50 per cent ethanol in n-hexane, and n-hexane were all tried as carrier. As is illustrated in Table 4 the toxicity of the extract was greatly lowered or removed when it was fed in propylene glycol (group 8) while the purified toxin dissolved in 50 per cent ethanol in n-hexane (groups 1-5) demonstrated the same toxicity as the carrier alone (groups 6 and 7). When the crude extract (groups 9 and 10) or purified compound (groups 13-20) was fed in n-hexane alone a much more consistent picture was revealed. Further it was shown that n-hexane alone (groups 11 and 12) was non-toxic when fed orally in doses about three times that required for it as a carrier.

Table 4 illustrates that the LD_{50} (mice) of tetradymol lies somewhere between 170 and 330 mg/kg (groups 15 and 16).

Autopsies were performed on a number of mice that were fed purified toxin. Two of the mice that did not die from group 16 (Table 4) were autopsied revealing severely necrotic livers. One liver was almost

Table 4

Mouse Feeding Experiments

Materials and/or carrier	mg/kg of toxin	ml/kg total volume	Number of animals	% dying in one week
1. Pure (II)* 50% ethanol in n-hexane	460	3.8	9	25
2. "	360	3.6	8	50
3. "	280	3.5	8	63
4. "	190	3.1	8	75
5. "	160	4.0	8	50
6. 50% ethanol in n-hexane	0.0	3.3	8	50
7. "	0.0	5.0	9	45
8. Crude extract in propylene glycol	2000-3000	3.0-4.5	5	00
9. Crude extract in n-hexane	2100-3300	4.4-5.8	12	100
10. "	1100-1200	1.9-2.0	4	25
11. N-hexane	0.0	4.0-4.9	8	00
12. "	0.0	7.0-8.6	7	00
13. Sublimed (II) in n-hexane	750-580	4.8-6.2	7	100
14. "	330	3.3	9	100
15. Pure (II) in n-hexane	330	3.3	9	100
16. "	170	3.3	11	45
17. "	282	3.9	5	40
18. "	200	3.9	5	00
19. "	140	3.9	5	00
20. "	100	3.9	5	00

* Pure (II) refers to sublimed, base washed tetradymol

totally white. Also three mice that did not die from each of the groups 17 through 20 (Table 4) were autopsied. Those in group 17 showed very marked lesions while the lesions were macroscopically modest in group 18. Groups 19 and 20 had no macroscopically visible lesions.

All of the observed lesions in the mice shown in Table 4 were quite similar to those seen in the mice fed the crude hexane extract (Table 3, page 28); but no histopathology studies have been made on this tissue. These gross findings do, however, support the conclusion that tetradymol is the active principle of T. glabrata. Further evidence for this conclusion is presented in the following section.

In general it could be concluded from all these physiological studies that the active principle of T. glabrata was a hepatotoxin of moderate toxicity. The effects on the liver in all instances were demonstrated as cellular damage but the method by which this damage was mediated was not determined.

One item of unusual interest was the difference in location of the lesion in rabbits and sheep, peripheral lobular and centro-lobular respectively. Dr. Anderson of Montana State University stated⁵⁴ that he knew of no other instance of this type of interspecies difference in the manifestation of the hepatotoxicity of a particular substance. This finding suggests, but does not prove, that some cells are attacked more readily than others in the liver by tetradymol. Since blood flow is similar in the livers of both sheep and rabbits, if the point of attack

of the hepatotoxin was in this flow, similar lesions would be expected. The author suggests, without proof, that tetradymol is really attacking a particular enzyme system that is localized in specific cells of the liver. That these cells are localized in different areas of the livers of different animals seems to be a logical postulate. The different locations of the lesions then logically follows.

II. Isolation of the Toxin

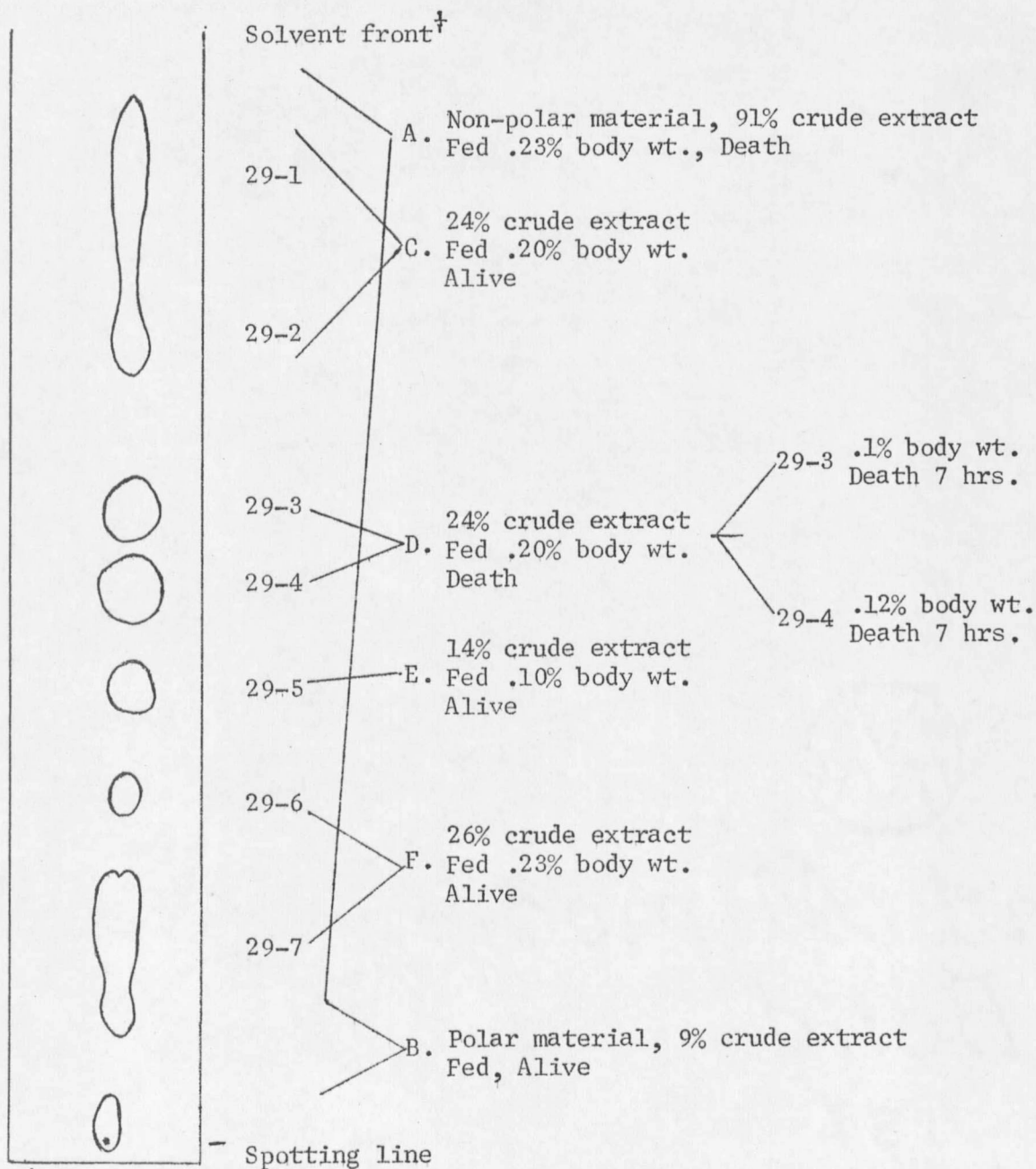
This section of the discussion is presented in three parts. The first part (A) will deal with the assignment of greatest toxicity to a particular molecule by a process of elimination utilizing mice as a test animal. The second part (B) will deal with the methods used in isolating sufficient purified compound for structural elucidation and partial determination of the LD₅₀ of the toxin for mice. The third part (C) will deal with some of the characteristics of tetradymol, the toxic principle of T. glabrata, excluding the chemical structure.

A. When either the crude n-hexane or acetone extract was spotted on Anasil S tlc plates and developed with 40 per cent n-hexane in diethyl ether, it separated into a number of spots which were made visible by spraying the plate with sulfuric-dichromate solution. These spots were given the numbers indicated in Scheme 1.

The total n-hexane extract was first divided by silica gel chromatography into a non-polar fraction (A), material eluted off the column with 50 per cent n-hexane in diethyl ether, and a polar fraction (B), material eluted off the column with 10 per cent methanol in diethyl ether. The materials eluted and quantities involved are shown in Scheme 1. The non-polar fraction demonstrated toxicity when administered to a mouse in the amount indicated which is relatively equivalent to the toxic dosage of the total extract.

Scheme 1

Isolation of the Toxin Followed by Toxicity*



* Mice were used as test animals for the above feeding experiments.

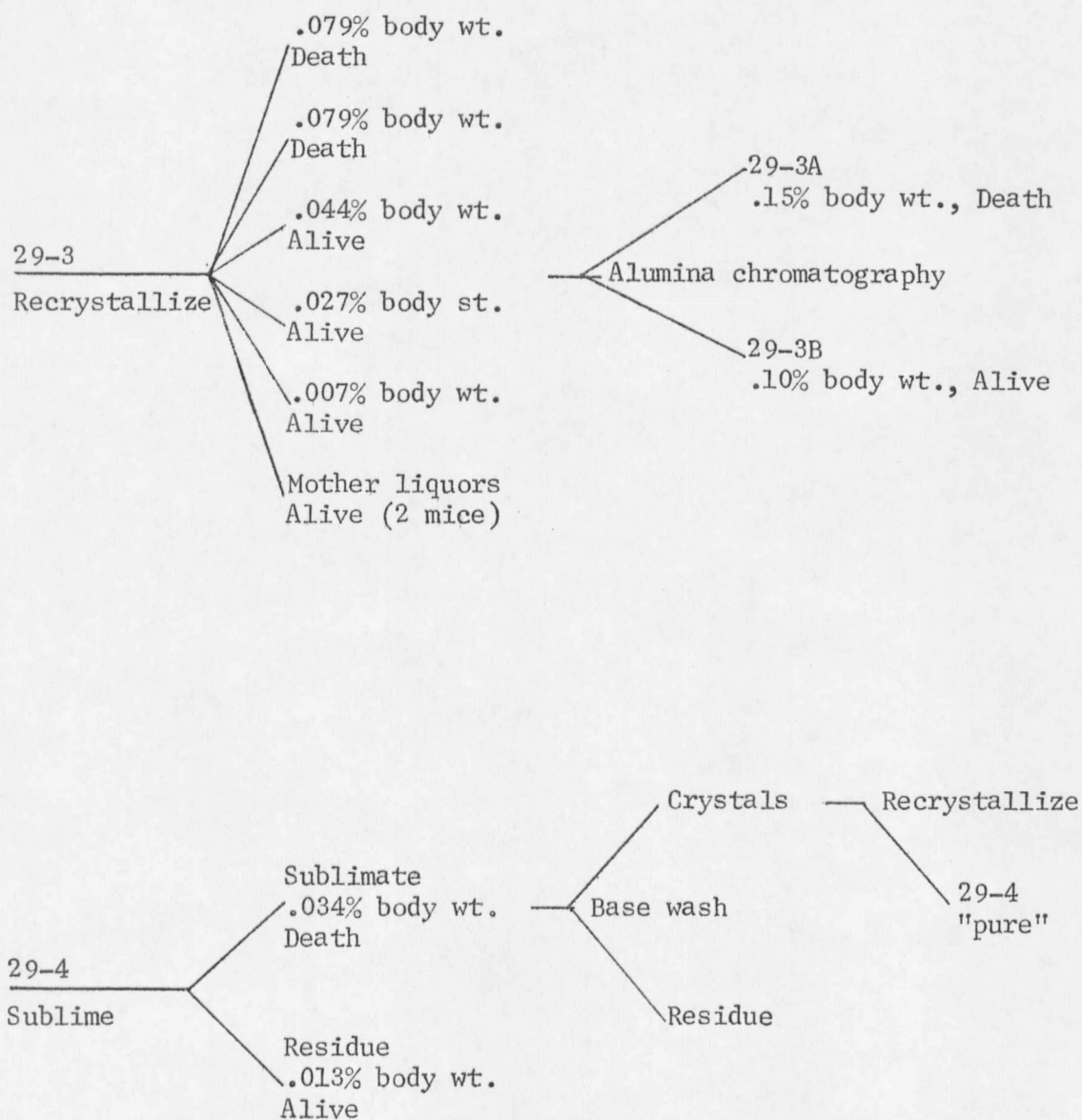
[†] Anasil S tlc, developed in 40:60 n-hexane:diethyl ether.

Next the non-polar fraction of the extract was divided into four fractions (C-F) shown in Scheme 1 by silica gel chromatography. These fractions were then administered to mice in a concentration about four times as great as they occurred in the toxic dosage of the original crude extract. Only the mouse receiving fraction (D), the materials labeled 29-3A through 29-4, demonstrated toxic effects. Since at these high dosages only fraction (D) displayed toxicity the other fractions were placed aside and fraction (D) was further separated by silica gel chromatography into two portions corresponding to 29-3 and 29-4 as shown in the scheme. These fractions were then administered to mice in the high dosage rate and both demonstrated toxicity.

The material labeled 29-3 was now further purified by recrystallization and fed as shown in Scheme 2. There were not enough feedings here to establish with any certainty the exact toxic dose but it must lie between 790 mg/kg and 270 mg/kg. At this point it was found that 29-3 was really two compounds by changing from Anasil to alumina tlc. Thus, 29-3 was fractionated by alumina chromatography into 29-3A and 29-3B. These materials were then fed in the concentrations shown in Scheme 2 with the result that the mouse receiving 29-3A died while the mouse receiving 29-3B remained alive. As the concentration in the plant of 29-3B is less than one half that of 29-3A, this amount of 29-3B is a much larger dosage based on yield from the plant than the amount of 29-3A fed to the mouse.

Scheme 2

Isolation of the Toxin Followed by Toxicity* (II)



* Mice were used as test animals for the above feeding experiments.

At this point 29-3A was set aside and further work was done on 29-4 which was given the trivial name tetradymol.

The tetradymol containing yellowish solid from the chromatographic separation of 29-3 from 29-4 was sublimed and fed to mice resulting in their deaths as shown in Scheme 2. The residue from the sublimation did not demonstrate toxicity. The white crystalline sublimate was further purified by base extraction and recrystallization to yield a pure compound whose LD₅₀ for mice lies between 170 and 330 mg/kg (see Table 4, page 31).

As was mentioned on page 27, the hexane extract from plant equivalent to 5 per cent of the body weight of a mouse is lethal to that mouse. It is quite possible that this dosage is greater than the minimum required for death, as the minimum toxic dosage of the crude extract was not pursued. However, when the quantities of the various fractions of the crude hexane extract are considered; as shown in Scheme 1, page 35; this dosage could not be too far out of line. It was also shown; as recorded in Table 4, page 31; that purified tetradymol (II) was lethal to all mice tested in a concentration of approximately 300 mg/kg. By the purification procedure outlined in Scheme 3, page 42 it was observed that sublimed tetradymol could be isolated (later shown to be at least 90 per cent pure) in a quantity equal to about 0.3 per cent of the wet buds weight. A consideration of these numbers showed that if the isolation procedure were quantitative, which it obviously was not, the

toxin from plant equivalent to 10 per cent of the mouse's weight was lethal. When the losses in isolation and extraction were considered along with the toxicity of 29-3A, another toxin isolated from T. glabrata (see page 36), the levels of toxicity pointed to the conclusion that tetradymol was mainly responsible for the toxicity of the hexane extract in mice.

Though this study did not directly show that tetradymol was the molecule responsible for the extensive range losses of sheep in the Great Basin, it did suggest that there was a good possibility of that conclusion. Support for the conclusion was found in similar necropsy findings and similar levels of toxicity in mice and sheep.

B. After it was determined that tetradymol, or 29-4, was the toxic principle of T. glabrata it was essential to isolate larger quantities for structural studies. In order to determine how best to extract the toxin from the plant, two samples (1165 g) of buds and new growth were randomly taken. One of these samples was then ground with a meat grinder in n-hexane while the plant was still frozen. It was then extracted three times, for 20-24 hours each time, with a total of 6 liters of n-hexane each time. The other sample was left unground and extracted in the same manner. After this was completed the unground, extracted material was ground and extracted two times with n-hexane. The results of these extractions are summarized in Table 5.

Table 5

Effect of Grinding on Plant Extraction

	Ground	Unground	Unground-ground
Weight of plant material	1165 g	1165 g	ND [*]
Weight of crude extract	20.7 g	9.3 g	14.1 g
Per cent of plant weight	1.78%	0.8%	1.2%
Crude extract concentration of tetradymol (II) [°]	0.6	1.0	0.2
Amount of tetradymol (II) ^{**}	1.4	1.0	0.3
Amount of 29-3 ^{°°}			

* Not determined

[°] Relative to a concentration of 1.0 in the unground crude extract

^{**} Relative to an amount of 1.0 in the unground crude extract

^{°°} Relative to an amount of 1.0 in the ground crude extract

All concentrations were determined by spot matching on alumina tlc visualized by charring with sulfuric-dichromate solution.

Since most of the tetradymol was extracted from the plant without grinding, and the unground extract had less contaminating substances, bulk extractions of the plant material were performed on unground material.

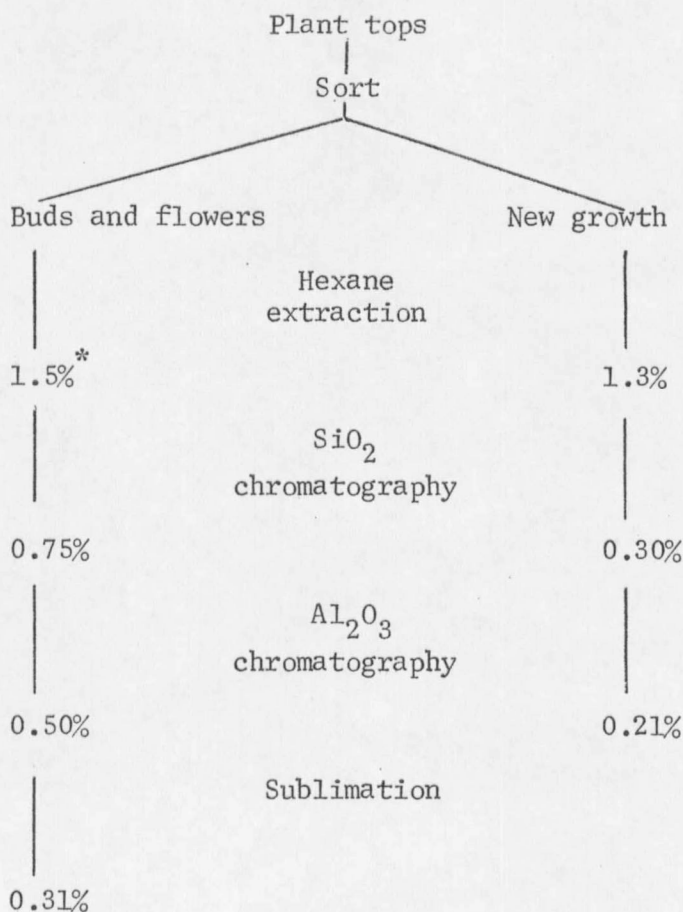
In order to determine the approximate yield of toxin from the plant a 100 g sample of buds and flowers and a 100 g sample of new growth were extracted separately without grinding and purified as outlined in Scheme 3, page 42. The yield after alumina chromatography of the two extracts

revealed that there was less toxin in the new growth than there was in the flowers and buds.

Keeping in mind the moderate instability of the toxin, which is discussed on pages 45-47, tetradymol was purified for chemical and physiological studies using a number of methods as shown in Scheme 3. Chromatography removed most of the contaminating materials leaving a yellowish solid that gave one slightly elongated spot on tlc. These yellowish impurities could be removed either by multiple recrystallizations or by sublimation. When the recrystallized material was sublimed, a residue remained behind that displayed an ir carbonyl band that had been present, but very small, in the material before sublimation. Thus, since sublimation removed both the yellow and the carbonyl containing impurity, the column chromatographed material was sublimed directly to yield white crystals. These crystals were still not a pure compound by elemental analysis, however, so they were dissolved in concentrated ethanolic potassium hydroxide and allowed to stand overnight in a closed, light protected, vial at about 25°C. When this mixture was worked up and the compound recrystallized it gave a satisfactory analysis. Whether the impurity reacted or was extracted with base was not certain. There was, however, quite a noticeable yellowing of the basic ethanolic solution when it was observed after standing overnight. This coloration did indicate some decomposition but not necessarily of the impurity. It might be suggested that tetradymol was modified during this rather

Scheme 3

Isolation Procedure for Tetradymol



* Percentages based on isolated material at each step relative to the whole green plant material.

vigorous purification step. However, when the purified material was checked by ir, nmr, uv, and ms there seemed to be no significant differences resulting from the base extraction. Also the position and shape of the spot on alumina tlc and the color visualization reactions

of the compound were identical before and after the extraction. Further the optical rotation of the compound increased from $+44^{\circ}$ to $+56^{\circ}$ as a result of the extraction. These physical findings coupled with the findings of physiological activities of about the same magnitude with material before and after extraction led to the conclusion that tetradymol was unchanged by this base wash.

C. A number of characteristics of tetradymol, the toxin from *tetradymia glabrata*, are given below.

Tetradymol crystallized as white needles from n-hexane and had a melting point of $92-92.5^{\circ}\text{C}$.

When tetradymol (29-4) was mixed with Ehrlich's reagent⁵⁵ there developed an immediate lavender color that changed to blue in one half hour and finally green in two hours. If instead tetradymol was added to 91 per cent formic acid and the p-dimethyl-amino benzaldehyde added an immediate intense dark blue color resulted that was stable for at least the hour until the solution was discarded.

Tetradymol showed an $R_f = .34$ in 50 per cent hexane in diethyl ether on alumina tlc. It gave a bright rose-lavender color when the tlc spot was sprayed with either vanillin-sulfuric or sulfuric-dichromate reagents. The compound 29-3A showed an $R_f = .60$ in the same system but displayed a bright blue-black color with the sulfuric-dichromate spray reagent and a bright violet color with the vanillin-sulfuric spray reagent.

Tetradymol displayed one well defined peak by glc using a 6 foot, 1 per cent OV-1 liquid phase on GC-Q support in a 1/8-inch copper column; a 6 foot, 2 per cent SE-30 liquid phase on GC-Q support in a 1/8-inch glass column; and a 6 foot, 1 per cent OV-17 liquid phase on GC-Q in a 1/8-inch aluminum column. When the SE-30 column or the OV-17 column were utilized, 29-3A would not demonstrate any peak in the gas chromatograph. It would, however, come through the OV-1 column. Microgram quantities of these compounds were captured from the gas chromatographic detector port in capillary tubes while the flame was extinguished. When these samples were spotted on tlc, they revealed the same Rf and color reactions as authentic tetradymol and 29-3A.

A consideration of the results recorded in Table 5, page 40, suggested that tetradymol was mainly a surface lipid as it was easily extracted in hexane from the whole green plant. The other possible toxic constituent, 29-3A, was only slightly extracted by hexane under these conditions but was easily extracted after the plant is ground up. Since both of these compounds were soluble in hexane and seemed to extract in a manner equal to each other from the ground plant the difference in extractability from the unground plant was possibly due to the location of the molecules in the plant. There was also the possibility that tetradymol was concentrated in the intercellular spaces as one of the sap components rather than appearing on the plant's surface. The above extraction experiment would not differentiate between the two

possibilities.

The instability of tetradymol was noted on numerous occasions during isolation and characterization studies. Fleming⁵⁶ showed that the plant material lost toxicity on drying and storage. Though the molecule does sublime it does so to an appreciable extent only under vacuum, or with heat near its melting point. If the molecule was not lost in this manner it probably was decomposed or altered on storage. This problem was avoided in this laboratory by storage of all plant material and extracts at -20°C in sealed containers.

Another demonstration of instability was in the behavior of the compound on alumina chromatography. Elution chromatography on highly activated alumina could not be effected with tetradymol as the compound decomposed or became bound so tightly to the support that it could not be eluted with 5 per cent methanol in diethyl ether. The total yield from the column amounted to only 55 per cent of the material placed on the column. Further the upper section of the column took on a yellow color suggestive of decomposition on the column but no attempt was made to find the decomposition products. Alumina is known to mediate a considerable number of reactions⁵⁷ as condensations, dehydrations, oxidations, and rearrangements so this proposed decomposition was not too surprising. To avoid the above problem, the column support material was first deactivated with methanol.

Lederer and Lederer⁵⁸ reference methods of deactivating alumina by

the addition of water, but this requires a fairly long mixing time in the attempt to avoid the problems of inhomogeneity due to the small volume of water being added to a very hygroscopic adsorbent. This problem was avoided by the use of methanol mixed with benzene. When this solvent mixture was stirred with alumina for a short period and then allowed to cool, (to remove the heat of adsorption) it yielded a homogeneous adsorbent of a suitable activity for these compounds as was demonstrated by fairly sharp bands and little tailing. This slurry could be poured directly into the column, eluted with a small amount of hexane, and used without further preparation. When this column was used there was still a small amount of material that could not be eluted from it (approximately 15 per cent) but this was not deemed to be significant.

Florisil was tried as an adsorbent but was not effective in separating tetradymol from 29-3A. It also led to some decomposition as was demonstrated by a pink color that developed on the column.

Silica gel chromatography was quite useful for removing the colored polar materials and the very non-polar compounds from the crude green-brown hexane extract but did not give as good a separation of 29-3A and B from tetradymol as did alumina. There seemed to be no loss of compound on the silica gel column as the yields were greater than 98 per cent from the chromatography.

Besides instability to chromatography, tetradymol exhibited light sensitivity. When it was dissolved in hexane and left in a capped,

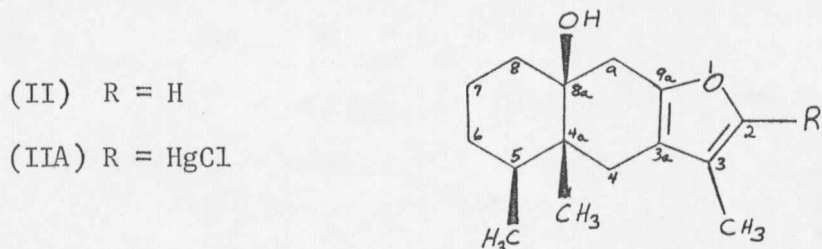
clear glass vial on the desk top near a north facing window a white solid collected on the glass nearest the light. This solid was insoluble in diethyl ether and was much more polar than tetradymol by tlc. In one instance tetradymol also decomposed while stored in solution in a sealed vial under refrigeration. This decomposition has not been observed, however, when the compound was stored in a crystalline form at -20°C under a nitrogen atmosphere. The product(s) of the proposed decomposition have not been investigated.

III. Spectral and Chemical Elucidation of Structure

The structural elucidation of tetradymol (II), 8a(S)-hydroxy-3, 4a(R), 5(S)-trimethyl-4, 4a, 5, 6, 7, 8, 8a, 9-octahydro-naphtho [2, 3-b] furan, has proved to be an interesting problem. The compound presented a number of unusual features including a furan ring; the comparatively rare, non-isoprenoid eremophilane skeleton; and an angular hydroxyl function, which has been found in only one other eremophilane.⁵⁹ This elucidation was complicated by the moderate instability of the compound which was noted in previous sections of this thesis. Beyond these demonstrations of instability the compound was very labile to concentrated formic or hydrochloric acid giving as products black tars which were also the eventual products when tetradymol was dissolved in deuteriochloroform or carbon tetrachloride for nmr studies. The instability of tetradymol to acids could be rationalized as due to the reactivity of the furan nucleus⁶⁰ but the decomposition in the chlorinated solvents is as yet unexplained.

By combining the elemental analysis (Calcd. for $C_{15}H_{22}O_2$: C, 76.88%; H, 9.04%. Found: C, 76.84%; H, 9.25%.) and the ms parent peak ($M^+ 234$ m/e) the molecular formula of $C_{15}H_{22}O_2$ was ascertained. A cursory inspection of this formula revealed that any structure proposed for tetradymol (II) must incorporate five unsaturations, two oxygens, and fifteen carbons. Three of these unsaturations, one of the oxygens, and five carbons are included in the methyl furan ring as will presently be

shown. The other oxygen must be part of a hydroxyl moiety as in the ir spectrum of this compound shown in Figure 7 demonstrated the typical strong O-H stretching frequency at 3400 cm^{-1} .⁶¹ From the nmr spectrum,



shown in Figure 8, two more of the carbon atoms could be assigned to a secondary methyl group, resonance at 0.66 ppm (3H d, $J=6$ cps), and a tertiary methyl group, resonance at 0.90 ppm (3H s). These assignments left eight carbons and two unsaturations unaccounted for in the molecular formula. They will be shown below to be incorporated in a [4.4.0] bicyclic ring system.

The presence of a furan ring in the molecule was indicated by several means. The compound gave the usual color reactions for a trisubstituted furan ring as lavender with the Ehrlich reaction, red with the Lieberman-Burchard reaction, and pink with vanillin in dilute, alcoholic sulfuric acid.⁶²

Beyond these general reactions, tetradymol displayed spectral evidence characteristic of a trisubstituted furan. The uv max $222\text{ m}\mu$ ($\log \epsilon 3.83$) (Figure 8) is in excellent agreement with that of menthofuran (III) which demonstrates uv max $220\text{ m}\mu$ ($\log \epsilon 3.78$).⁶³ Menthofuran has the same proposed methyl furan chromophore. The nmr spectrum

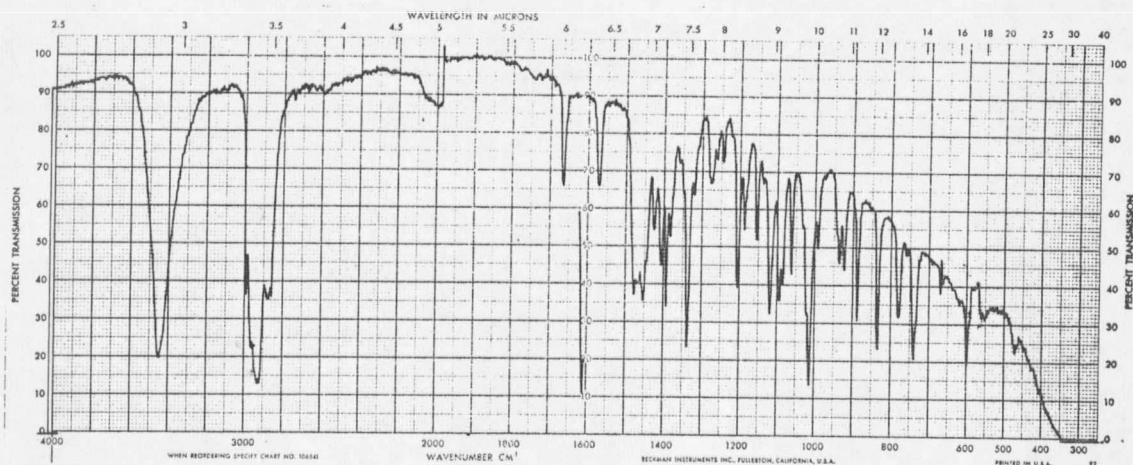


Figure 7. IR Spectrum of Tetradytol (II) (Micro KBr Pellet)

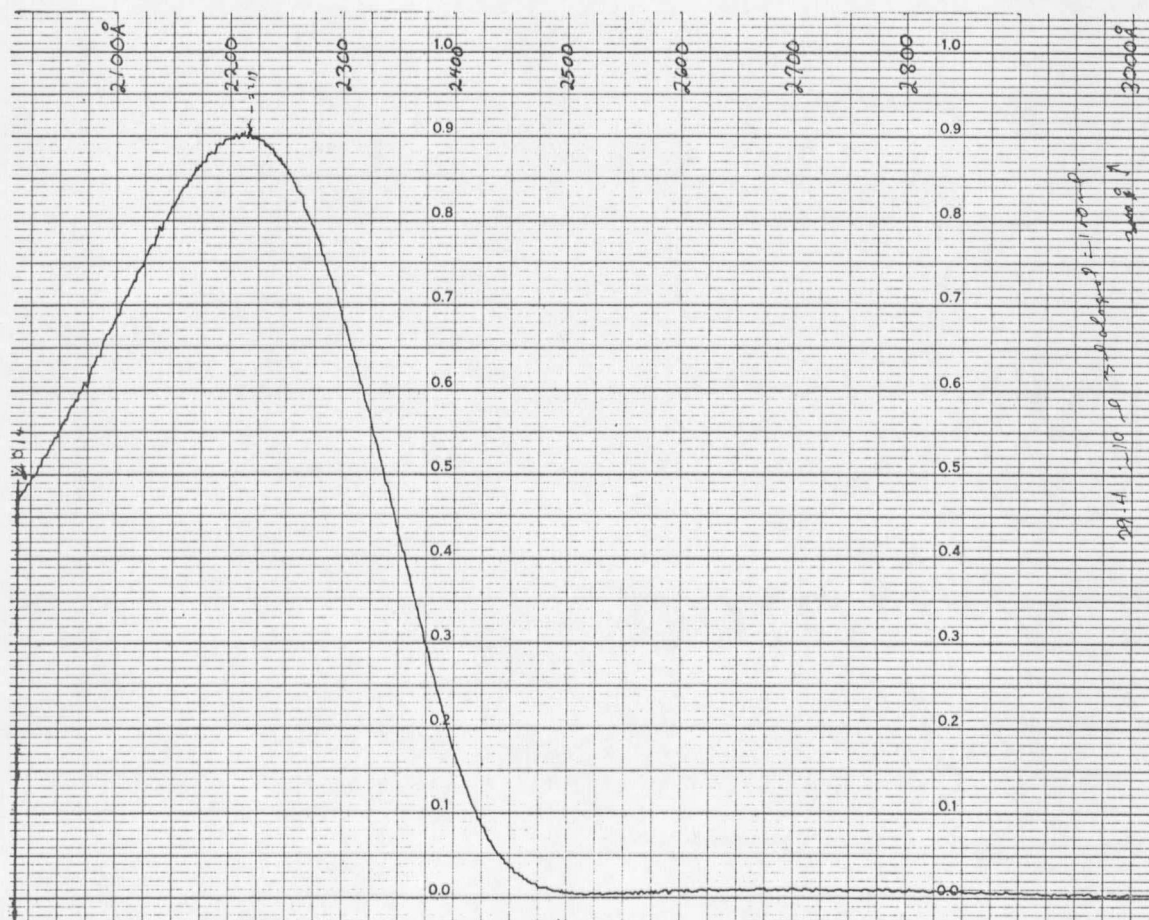
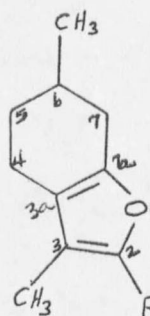


Figure 8. UV Spectrum of Tetradytol (II) (Methanol, 1.35×10^{-4} Molar)

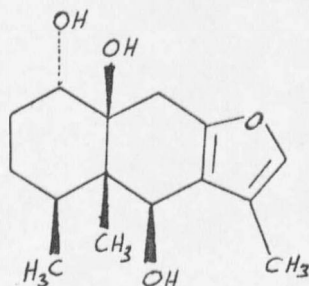
of (II), Figure 9, showed a single proton at 7.04 ppm weakly coupled (shown by spin decoupling) to a three proton resonance doublet at 1.77

(III) R = H

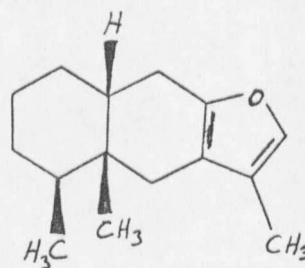
(IIIA) R = HgCl



ppm ($J=1.2$ cps) which agreed very well with the shift position and coupling of the α (or 2) proton and the β (or 3) methyl group respectively of the trisubstituted furan nucleus of such compounds as euryposal⁶⁴ (IV) (7.12 and 2.04 ppm $J=1$ cps), petasalbine⁶⁵ (V) (7.05 and 2.05 ppm $J=1.1$ cps), and menthofuran⁶⁶ (III) (6.84 and 1.85 ppm $J=1.0$ cps). The ir spectrum of (II) Figure 7, displayed two bands at 1655 and 1560 cm^{-1} that are characteristic of the trisubstituted furan nucleus^{67,68} along with the strong ring breathing band at 1005 cm^{-1} .⁶⁹



(IV)



(V)

In addition to these spectral characteristics tetradymol gave the typical furan derivatives when treated with mercury (II) chloride, chromium trioxide, hydrogen, and maleic anhydride. Each of these derivatives is discussed in detail below.

A furan nucleus with an α (or 2) position unsubstituted is very easily mercurated at that position.⁷⁰ As an example, simply adding 3,4,5-trimethyl furan to a buffered solution of mercury (II) chloride at room temperature immediately gave high yields of the 2-chloromercury derivative.⁷¹ When tetradymol was added to the mercury (II) chloride solution there was an immediate precipitation of the mercury chloride derivative (IIA) which was recrystallized from ethanol to yield beautiful white needles. A good elemental analysis was not obtained on the compound from the one sample submitted but mass spectral data (M^+469 m/e plus all the other peaks clustered about the parent ion peak for the various isotopes of chlorine and mercury) was obtained that agreed with the assigned structural formula. The nmr spectrum (Figure 10) of this adduct displayed the disappearance of the resonance of the furan proton from the region of 7 ppm when run in $DCCl_3$ (not shown), while the ir spectrum (Figure 11) still displayed the three furan bands near 1650, 1550, and 1000 cm^{-1} along with the hydroxyl stretching frequency at 3400 cm^{-1} . When this adduct was dissolved in aqueous ethanol, and hydrogen sulfide bubbled through the solution, tetradymol (II) was regenerated⁷² as demonstrated by tlc. Thus, the compound was not

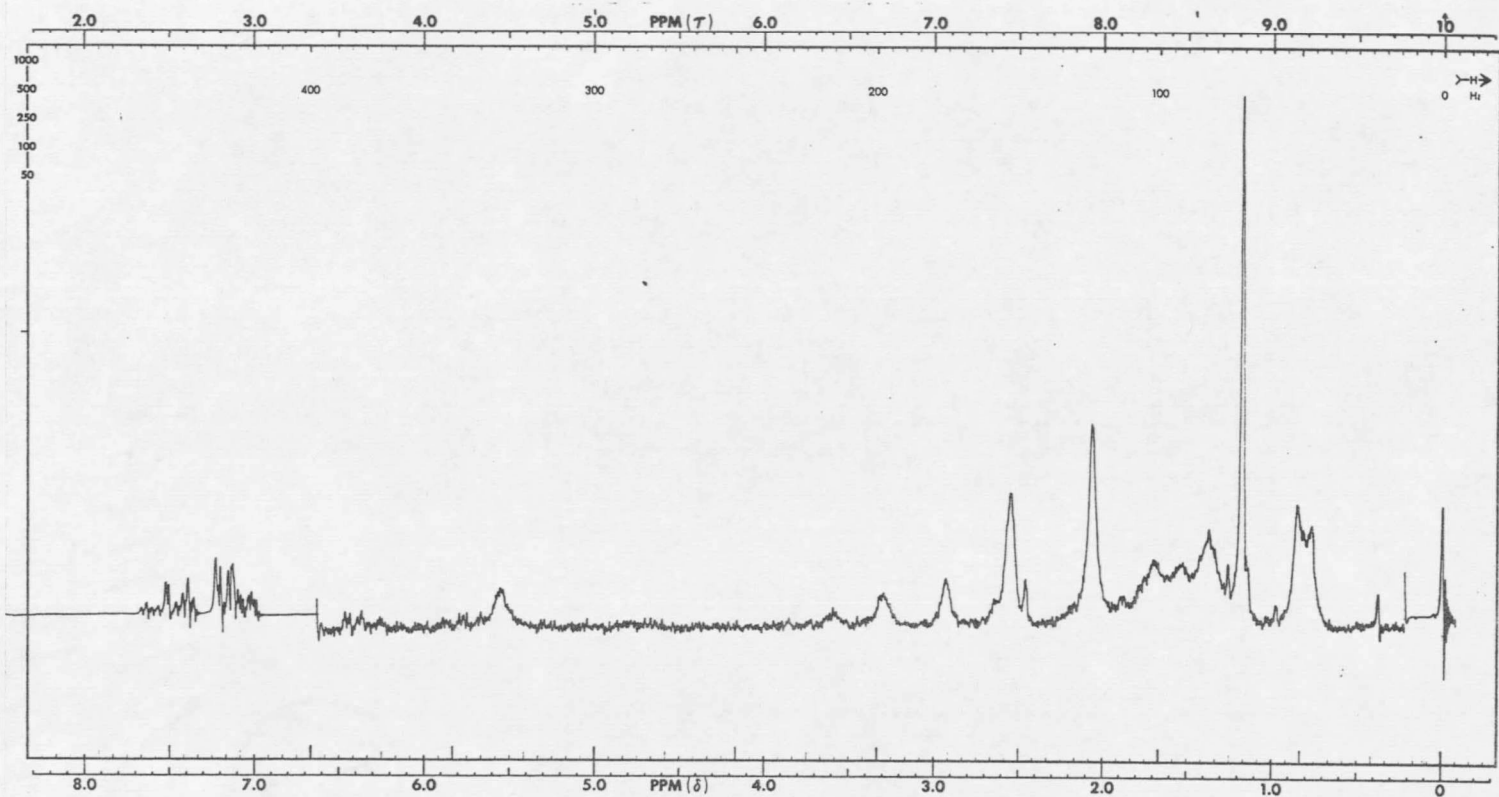
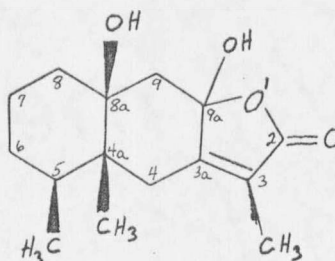


Figure 10. A 60 MHz Spectrum of (IIA) run in Pyridine with TMS as an Internal Standard.

changed by the substitution reaction. This was analogous to the work of Eastman⁷³ who made the 2-mercury chloride derivative (IIIA) of menthofuran. Finally, this chloromercury substituted compound from tetradymol was submitted to X-ray crystallographic analysis to yield the structure shown as (XXVII) on page 90.

When (II) was reacted with the Sarett reagent,⁷⁴ a chromium trioxide-pyridine complex, it yielded a compound ($C_{15}H_{22}O_4$, mp 161-162.5°C, $M^{+}266$ m/e) which had added two oxygen atoms to the molecular formula of (II). To this compound was assigned the structure (VI), a hemiketal lactone or pseudo-acid. This structure was analogous to the

(VI)

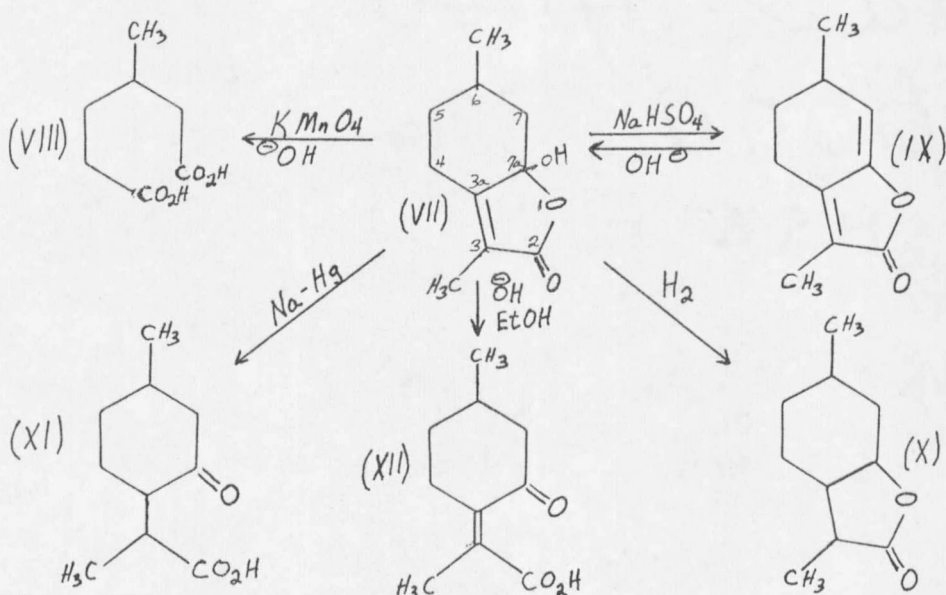


chromium trioxide oxidation product, peperic acid, derived from menthofuran (III) whose structure was proved by Woodward and Eastman.⁷⁵

These workers assigned the structure (VII) on the following bases (shown in Scheme 4): (a) Oxidative cleavage with potassium permanganate yielded β -methyl adipic acid (VIII). (b) Dehydration with sodium hydrogen sulfate yielded the anhydro compound (IX) which reverted to (VII) on treatment with alkali. (c) Catalytic hydrogenation was

difficult, but under drastic conditions yielded (X). Reduction with sodium amalgum yielded the γ -keto acid (XI). (d) Titration to phenolphthalien did not open the lactone ring but treatment with sodium hydroxide in ethanol revealed uv max 265 m μ , which is assigned to an α,β -unsaturated γ -keto acid (XII). Other workers have found the same

Scheme 4
Reactions of Peperic Acid (VII)



type of compounds in the oxidation of other highly substituted furan derivatives as (XIII) from atrolactylon⁷⁶ and (XIV) from isoserice-nine.⁷⁷ The oxidation product (VI) from tetradymol was a white crystalline high melting solid that demonstrated uv max 221 m μ

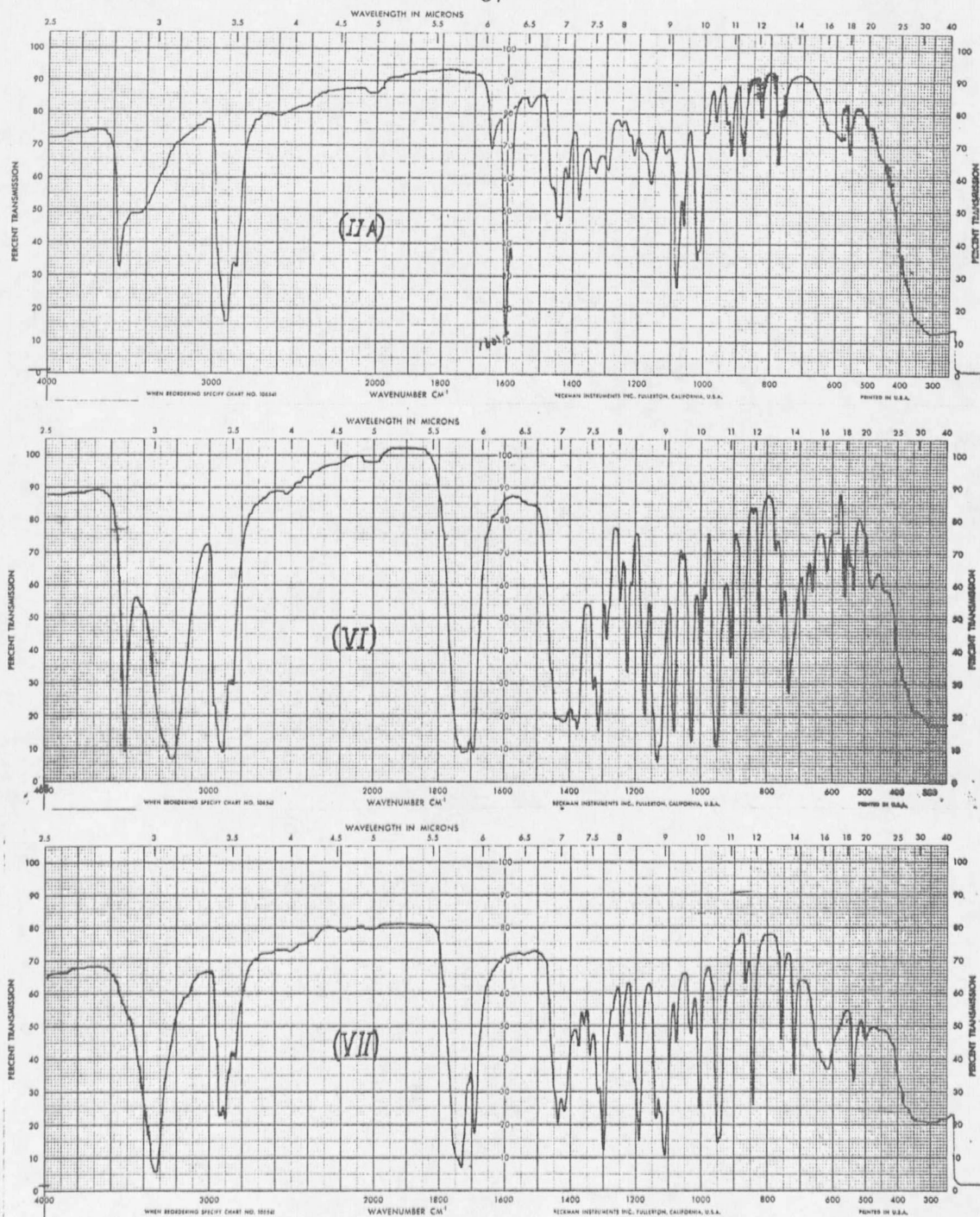
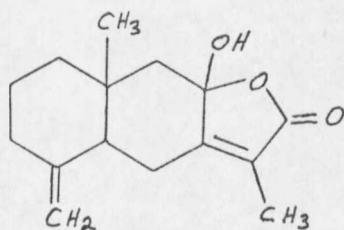
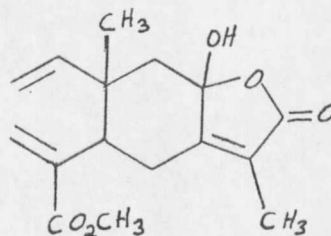


Figure 11. IR Spectra of (IIA), (VI), and (VII).
(all spectra run in micro KBr pellets)



(XIII)



(XIV)

(log ϵ 4.05) which compared favorably with the uv absorption of the oxidation product of menthofuran (VII), uv max 216 m μ (log ϵ 4.07), made in this laboratory. Also the ir spectra of these two compounds showed the same type of unsaturated carbonyl band (Figure 11) but differed in the oxygen hydrogen stretching frequency area possibly due to the two hydroxyls in (VI) and only the one in (VII). The nmr spectra (Figures 12 and 13) of these two compounds also displayed distinct similarities in the location of the resonance of the methyl group on carbon 3 at 2.75 and 2.85 ppm for (VI) and (VII) respectively and the resonances around 2.5 ppm that were assigned to the protons on carbons 4 and 9 in (VI) to carbons 4 and 7 in (VII).

When tetradymol (II) was treated with maleic anhydride a mixture of three compounds (by tlc) resulted. A mass spectrum of this mixture was taken which showed a parent peak (M^+ 332 m/e) which had the correct molecular weight for a Diels-Alder type adduct similar to those with furan (XV) and (XVI).⁷⁸

This mixture was not further characterized as it turned pink then

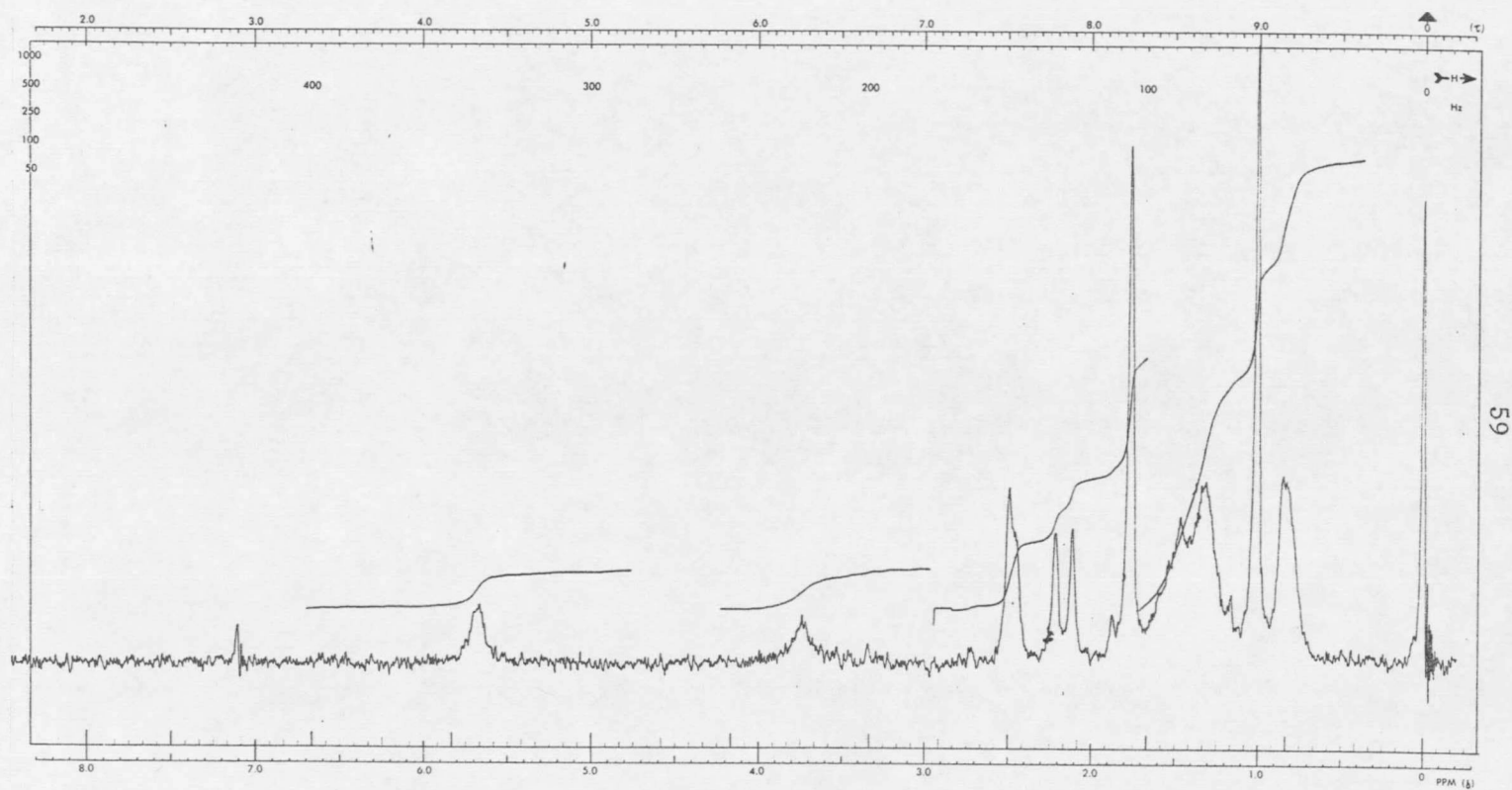


Figure 12. A 60 MHz Spectrum of (VI) run in DCCl_3 with TMS as an Internal Standard

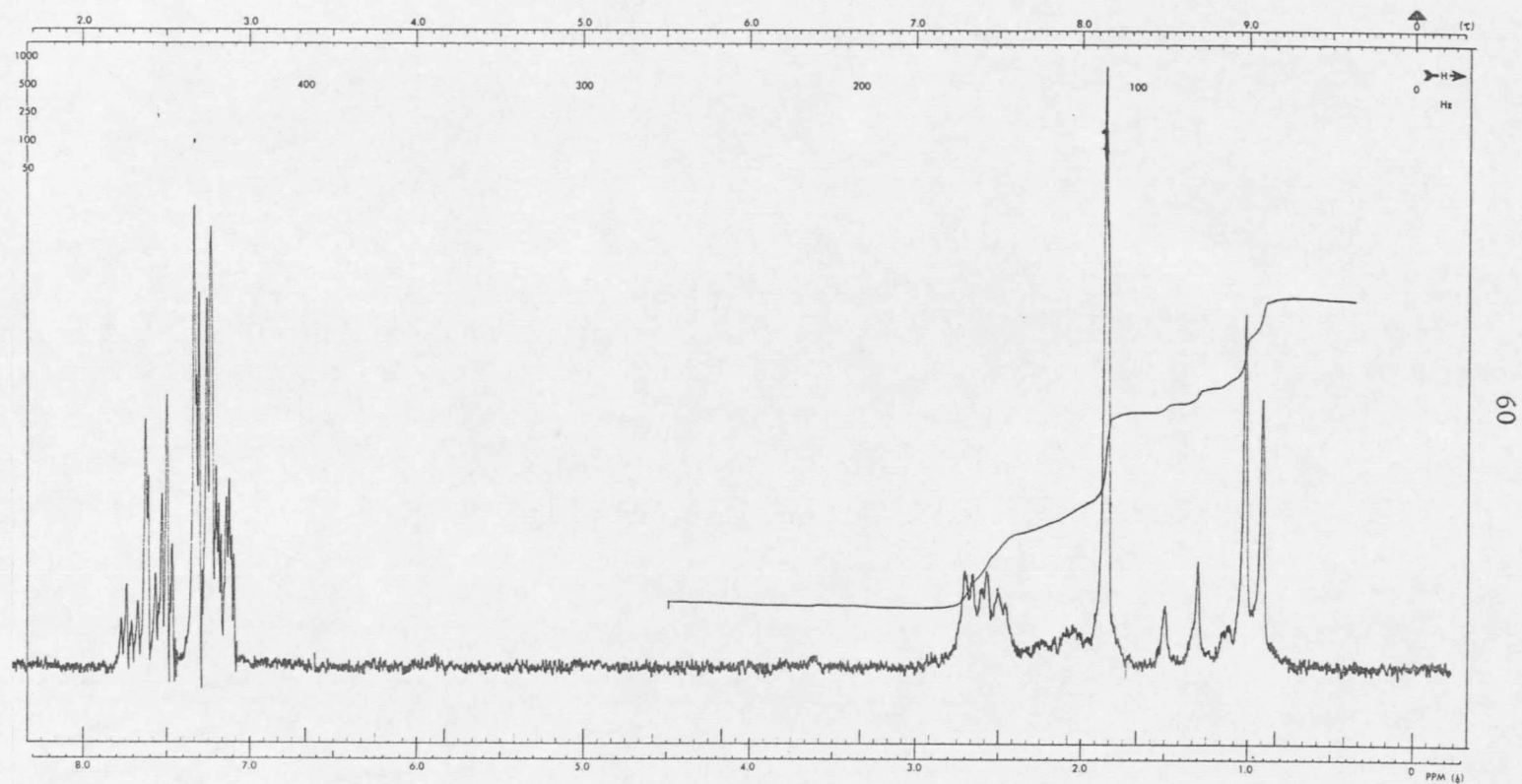
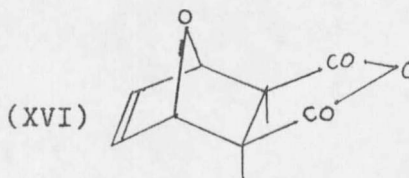
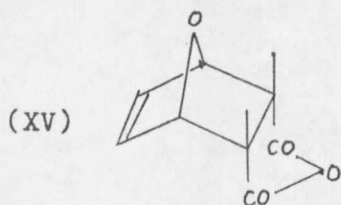


Figure 13. A 60 MHz Spectrum of (VII) run in Pyridine with the Pyridine Protons as an Internal Standard.



brown on exposure to air indicating instability and decomposition. This was not surprising as maleic anhydride adducts of furan compounds are many times unstable⁷⁹ and besides tetradymol itself was not very stable under aerobic conditions.

Perhaps the most useful of the furan derivatives, for characterization purposes, were the hydrogenation products. Due to the essentially planar character of the furan ring and substituents surrounding it, catalytic hydrogenation yielded a number of isomeric products. These included two major compounds (XVIIA) and (XVIIB) which had added two moles of hydrogen to (II), as demonstrated by the ms, and one major compound (XVIII) which had added one mole of hydrogen, by ms. In addition to these products a mixture of isomeric dehydrated-hydrogenated products as (XIX) were also obtained, as revealed by ms. These latter compounds are shown below to have lost the hydroxyl and be fully hydrogenated. All of these hydrogenation products could be separated into four fractions; (XVIIA), (XVIIB), (XVIII), and (XIX); by elution chromatography on alumina. The reaction and products are given in

Scheme 5.

The ratios of the products could be changed by the use of different catalysts and solvents as is illustrated in Table 6. Note especially that the yield of the dehydrated-hydrogenated products was decreased from 18 per cent to 6 per cent by changing from Pd on charcoal in ethanol to Rh on alumina in methanol. Since these products (XIX) were shown to be a difficultly separable mixture, Rh on alumina was used as the catalyst in methanol. This increased ease of dehydration with Pd had been observed by other workers.⁸⁰

The dehydrated-hydrogenated compounds (XIX) were shown to be a mixture of at least six compounds by glc. All of the compounds yielded a parent peak (M^{+222} m/e) by glc/ms which corresponded to the loss of water and the addition of three moles of hydrogen to (II). One of these isomers was partially purified for spectral use by repeated alumina chromatography. The ir spectrum (Figure 14) of this partially purified compound no longer demonstrated the 3400 cm^{-1} band of the hydroxyl, nor the 1550 and 1650 cm^{-1} bands of the furan ring (the mixture of XIX likewise did not display these bands). The nmr spectrum (Figure 15) displayed resonances for three protons in the region between 3.5 and 4.5 ppm which could be assigned to those protons on the carbons bearing the ether linkage in the tetrahydrofuran nucleus.⁸¹ This spectrum also showed resonances for three methyl groups attached to carbons 3, 4a, and 5. These methyl groups were now all on saturated

Scheme 5

Hydrogenation of Tetradymol (II)

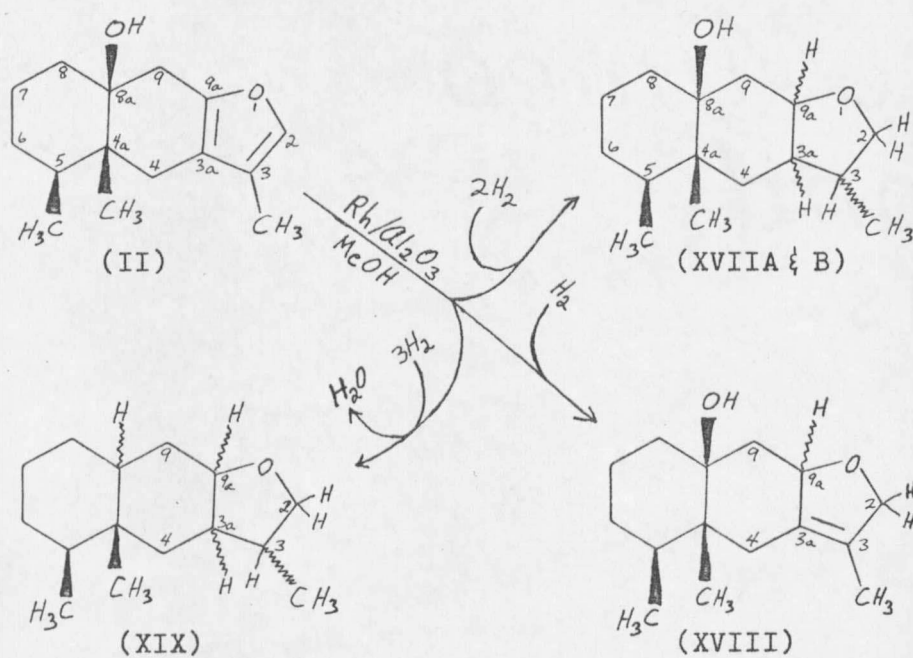


Table 6

Yields* of Hydrogenated Products Using Different Catalysts

Catalyst		
Product	Rh on alumina in methanol	Pd on charcoal in ethanol
(XIX)	6%	18%
(XVIIA)	22%	52%
(XVIIIB)	52%	18%
(XVIII)	19%	14%

*Yields based on isolated products

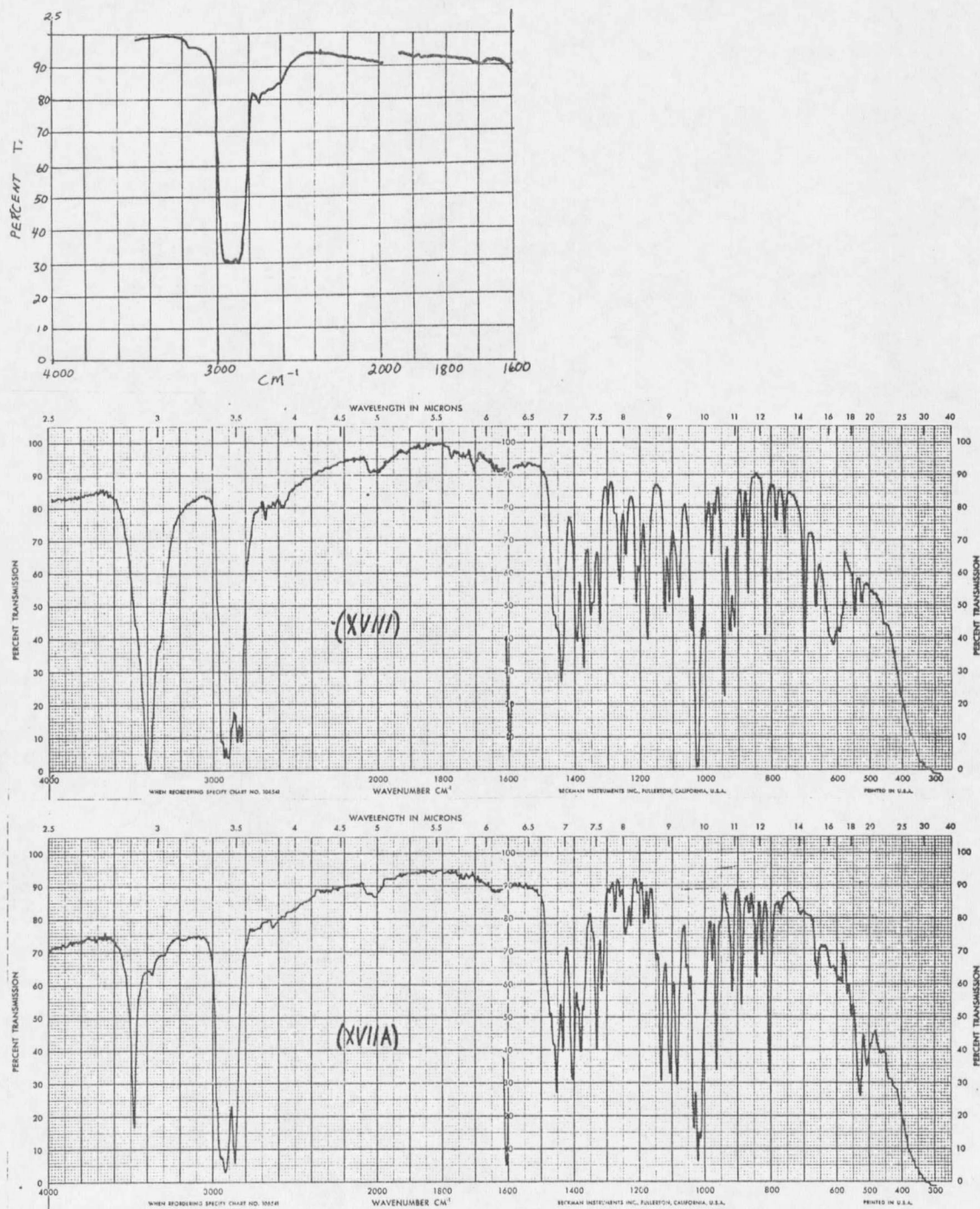


Figure 14. IR Spectra of (XIX) (spectrum run in CCl_4), (XVIII) and (XVIIA) (spectra run in micro KBr pellets).

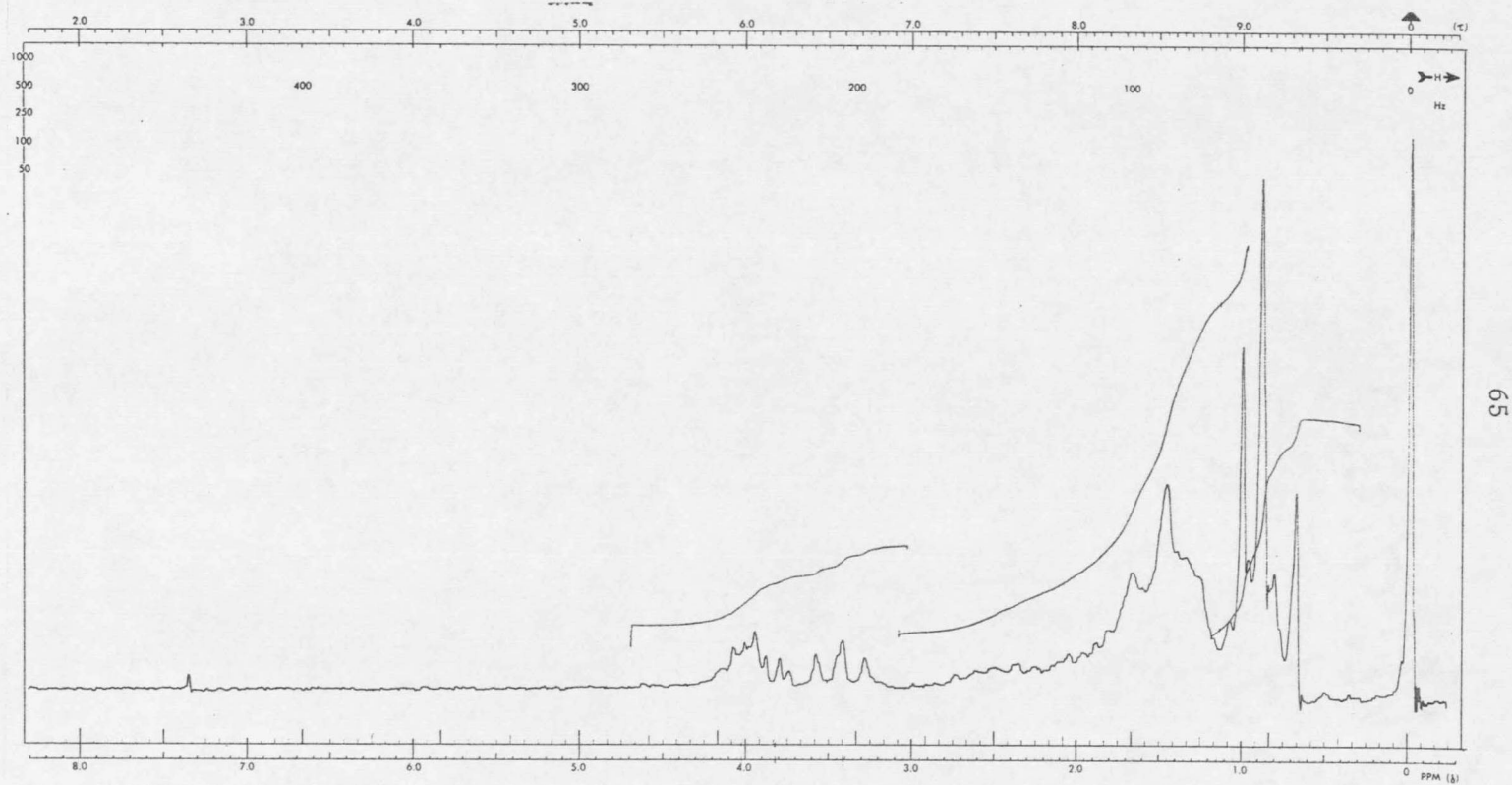


Figure 15. A 60 MHz Spectrum of (XIX) run in CDCl_3 with TMS as an Internal Standard.

carbon atoms and were expected to resonate at high field.

Compound (XVIII) ($C_{15}H_{24}O_2$, mp 145-146°C, $M^{+}236$ m/e) revealed by its physical constants that it represented the addition of one mole of hydrogen to (II). These two hydrogen atoms could most logically be added in one of three positions in the furan ring; 9a-3a, 9a-2, or 3-2 (Figure 16). They were most likely added to the 2-9a position (next to the ether linkage) since three protons appeared down field between 4.5 and 5.5 ppm in the nmr spectrum of (XVIII) shown in Figure 17. This shift position was only slightly below the expected shift position of the

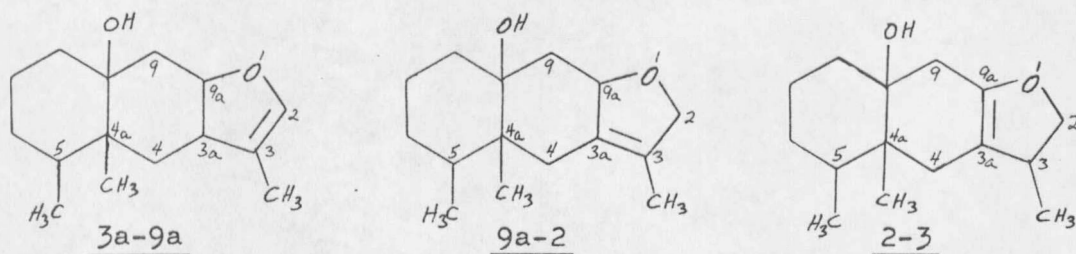


Figure 16. Probable Positions for the Addition of one Mole of Hydrogen to Tetradymol (II).

resonance of a proton allylic to a double bond and located on a carbon bearing an ether linkage.⁸² The resonance for the methyl attached to carbon 3 was not assigned in the nmr spectrum of (XVIII), but if that carbon were saturated the methyl attached to it would be expected to resonate above 1.3 ppm as a methyl group located on a saturated carbon next to a double bond is not usually shifted much below 1.0 ppm.⁸³

Since there were only two methyl resonances above 1.3 ppm in the nmr

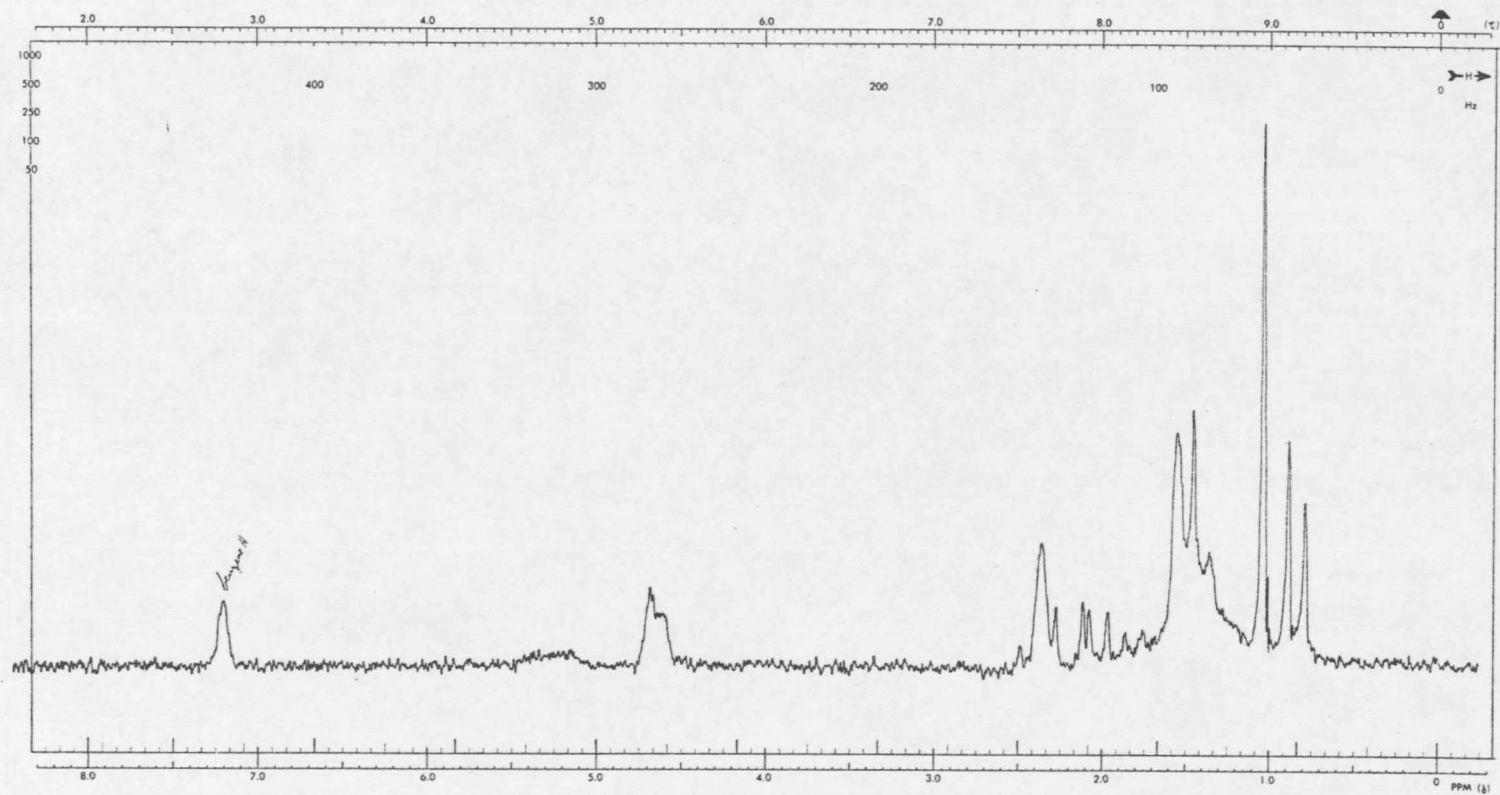


Figure 17. A 60 MHz Spectrum of (XVIII) run in D_6 Benzene.

spectrum of (XVIII) (which can easily be assigned to the methyls on carbons 4a and 5), the methyl on carbon 3 seemed most likely to be on an unsaturated position. In addition to this nmr spectral data there was additional evidence against the addition of the two hydrogen atoms in the 3a-9a or 2-3 positions from the ir spectrum of (XVIII) (Figure 14). If either of these structures was correct the compound would contain an enol ether. The observed ir spectrum did not contain the strong bands around 1250 cm^{-1} that are characteristic of an enol ether⁸⁴ and the alkene stretching vibration at 1700 cm^{-1} was so low in intensity that it also seemed unlikely to have arisen from an enol ether.⁸⁵ This compound was converted very slowly into (XVIIIB) on further catalytic hydrogenation with Rh on alumina in methanol which suggested that this compound did not arise from an impurity in the original compound (II) and thus, must have had the same general structure as tetradymol and the other hydrogenation products of that compound.

The other two major components of this hydrogenation mixture (XVIIA, $\text{C}_{15}\text{H}_{26}\text{O}_2$, mp $75-77^\circ\text{C}$, M^+238 m/e; XVIIIB, $\text{C}_{15}\text{H}_{26}\text{O}_2$, mp $104-105^\circ\text{C}$, M^+238 m/e) were evidently isomers of the compound which has the completely saturated furan ring. These two compounds were different by tlc and glc, and showed some difference in their spectra, but displayed the same molecular weight by ms and had the same elemental analysis. Their ir spectra (Figures 14 and 20) both demonstrated a rather sharp hydroxyl band in the 3400 cm^{-1} region and the loss of the furan bands at 1650 and 1550

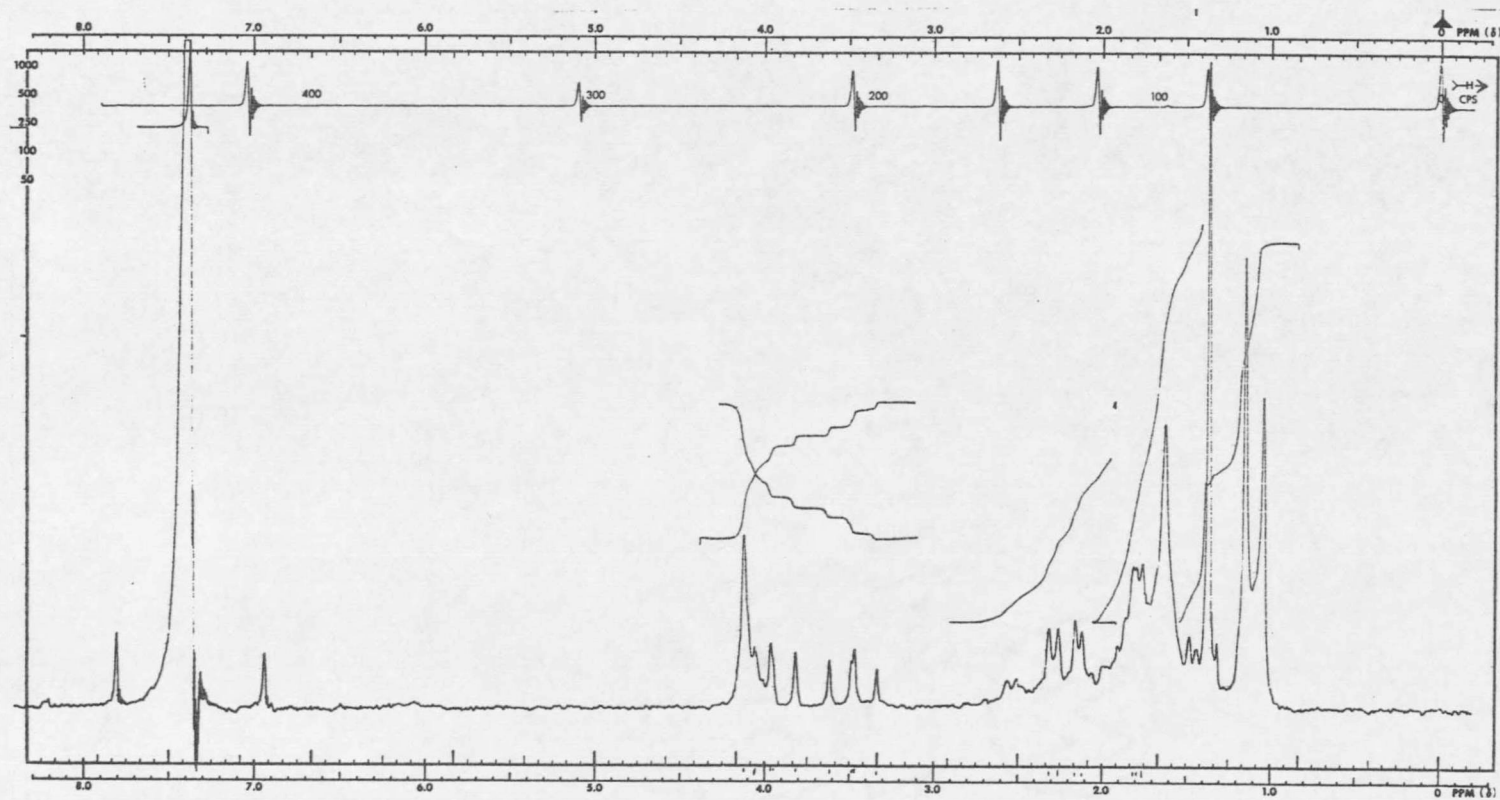
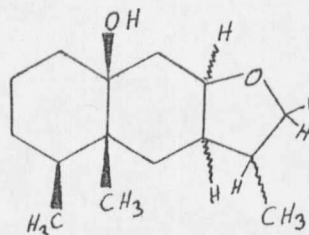


Figure 18. A 60 MHz Spectrum of (XVIIA) run in Benzene.

cm^{-1} which supported the assigned structure (XVIIA and B).

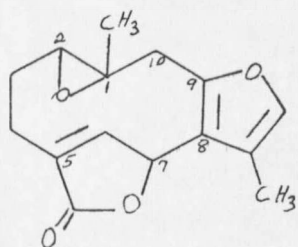
(XVIIA and B)



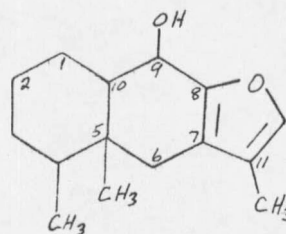
The nmr spectra of these two tetrahydro compounds (Figures 18 and 22) revealed the same three protons' resonances between 3.0 and 4.1 ppm which had been assigned to those protons on carbons 2 and 9a in all the hydrogenated compounds, and resonances for three methyl groups above 1.3 ppm for those methyls on carbons 3, 4a, and 5, which were all now saturated positions. One of these compounds (XVIIIB) will be discussed in more detail in a later portion of this section dealing with its dehydration. Further studies were not carried out to determine which of the eight possible isomers these two compounds represented and detailed analysis to confirm that both (XVIIA) and (XVIIIB) were a single isomer was not taken beyond the glc and tlc characterization on two supports, in each case, which showed both compounds to be homogeneous.

With the inclusion of a methyl furan in the molecular structure to account for three unsaturations, one oxygen atom, and five carbon atoms of the molecular formula there were remaining two unsaturations, one oxygen atom and ten carbon atoms to be accounted for. As was previously noted two more of the carbon atoms could be assigned to a secondary

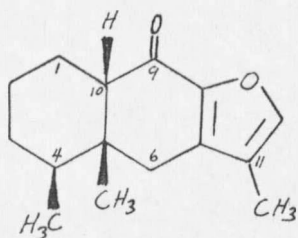
methyl group and a tertiary methyl group, while the other oxygen was accounted for in a hydroxyl moiety. This hydroxyl moiety was probably tertiary as the nmr spectrum of (II), Figure 8, showed no proton resonances in the region between 3.3 and 4.5 ppm where those protons on the



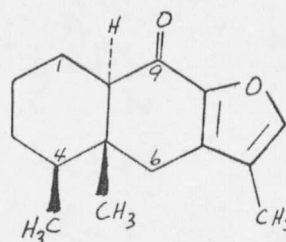
(XX)



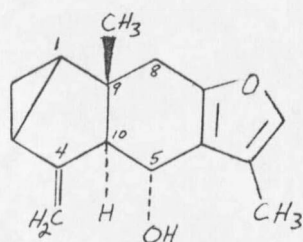
(XXIV)



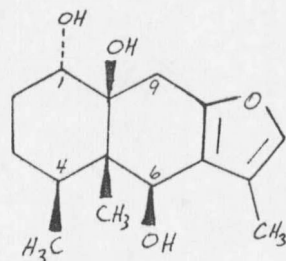
(XXI)



(XXII)



(XXIII)



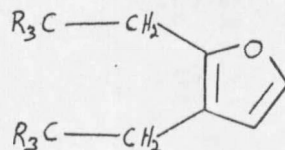
(IV)

(Numbering of these compounds follows the original literature.)

carbon bearing the hydroxyl should have resonated⁸⁶ if there were any present. Further the hydroxyl could not be acylated with pyridine and acetic anhydride, which is typically used as a test to confirm that a hydroxyl is tertiary.^{87,88,89}

The position of two more carbon atoms could be postulated from the nmr spectrum of (II). This spectrum (Figure 8) displayed two AB quartets due to geminal coupling at 2.1 and 3.0 ppm ($J=17$ cps) and at 2.2 and 2.4 ppm ($J=9$ cps) which could be assigned to protons on the carbon atoms 4 and 9a between the furan nucleus and tertiary carbon atoms. The assignment of these positions and splittings had analogy in neolinderane⁹⁰ (XX) (C_{10} AB spectrum 2.3 and 3.25 ppm, $J=15$ cps), 9-hydroxyfuroeremophilane⁹¹ (XXIV) (C_6 AB spectrum 1.90 and 2.57 ppm, $J=16.5$ cps), cis-furoeremophilone⁹² (XXI) (C_6 AB spectrum 2.20 ppm, $J=17.5$ cps), trans-furoeremophilane⁹³ (XXII) (C_6 AB spectrum 2.40 ppm, $J=16.0$ cps), linderene⁹⁴ (XXIII) (C_8 AB spectrum 2.63 and 2.80 ppm, $J=15$ cps), and euryopsol⁹⁵ (IV) (C_9 AB spectrum 3.00 and 2.78 ppm, J not given but statement made that carbon 9 protons give a quartet). This assignment for the protons in tetradymol (II) gave the partial structure (A).

(A) $R \neq H$



Since tetradymol (II) would take up only two moles of hydrogen on catalytic hydrogenation the other two unsaturations of the molecular formula were accounted for as rings with the hydroxyl group at one ring juncture and a methyl group at the other. The hydroxyl group and tertiary methyl could not be on the same carbon since the tertiary methyl was too far upfield. If they were both on the same carbon atom the methyl would normally be below 1.0 ppm.⁹⁶

The remaining carbons and one side of the furan ring could be imagined as combined in at least six basic ring systems; 4,4,0; 5,3,0; 6,2,0; 7,1,0; 4,3,1; and 4,2,2 as illustrated in Figure 19. The other isomers with the furan ring on the four membered ring in the 6,2,0 system or on the five membered ring in the 5,3,0 system could be rejected since they did not have the two allylic secondary carbons that have been shown to be present next to the furan ring in the above paragraphs.

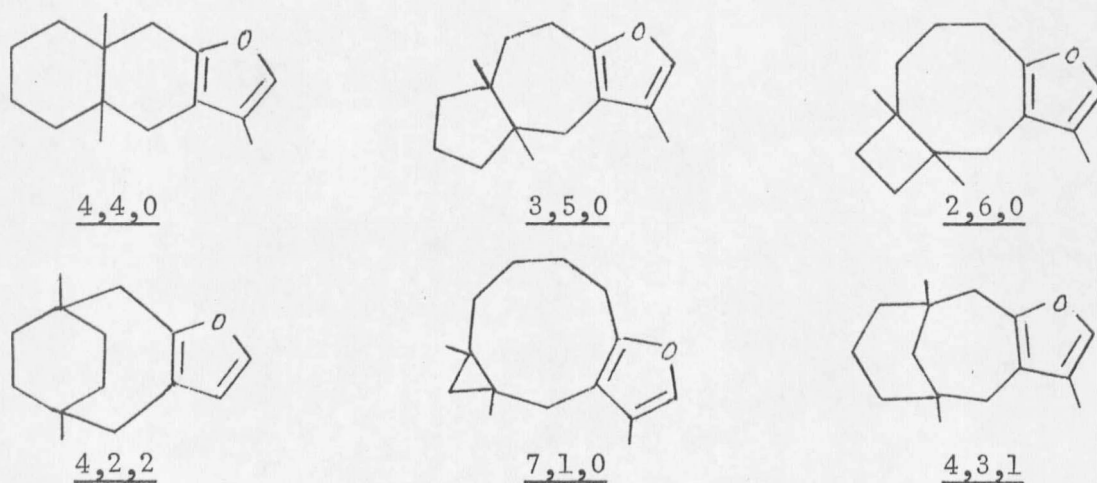


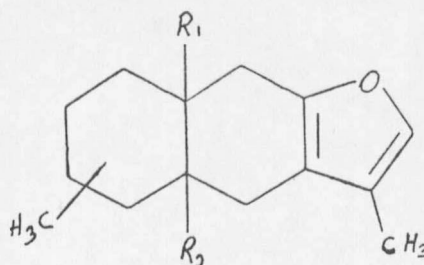
Figure 19. Possible Tricyclic Ring Systems for Tetradymol.

By the following process of elimination it was possible to postulate the 4,4,0 ring system as the most likely candidate for the structure of tetradymol. The 7,1,0 system was unlikely since there were no proton resonances for the cyclo-propane ring in the region from 0.0 to 0.5 ppm in the nmr spectrum of (II) which is the normal shift position for those proton resonances.⁹⁷ Second, the 6,2,0 system was also unlikely by the same type reasoning since the nmr spectrum did not reveal proton resonances around 3.5 ppm for those protons on the cyclobutane ring.⁹⁸ Third, a 5,3,0 system appeared unlikely since a dehydrogenation (aromatization) attempt with either S in boiling triglyme or Pd on charcoal at 320°C yielded either starting material or a mixture of light yellow non-polar compounds (by tlc) with no hint of the bright blue of an azulene. Since azulenenes have such an intense color⁹⁹ and would be expected to be formed in at least small yields from these reactions on a 5,3,0 system¹⁰⁰ that system could be quite safely ruled out. Fourth, if the 4,3,1 system was correct, a rather sharp signal in the nmr spectrum for the bridge protons would be expected since tertiary carbons adjoin the bridge carbon at both bridgeheads. This sharp signal was not observed. Now if partial structure (A) was correct it eliminated all except the 4,4,0; 4,3,1; and 4,2,2 systems since these alone of the suggested ring systems had two and only two carbons between the furan and the bridgehead carbons. Fifth, none of these systems except the 4,4,0 and 3,5,0 had very good precedent in known sesquiterpene systems.¹⁰¹ By the above reasoning it

was possible to postulate the partial structure (B) incorporating the 4,4,0 ring system.

There are two basic types of known sesquiterpene systems having the partial structure (B)¹⁰², the eremophilanes (B1) and the eudesmanes (B2). Both of these systems have the secondary methyl group at carbon 5 with varying stereochemistries of the ring juncture and methyl groups. In order to determine which of these two was represented in the structure of tetradymol (II), an attempt was made to dehydrate (II) using the mild

- (B) $R_1 \neq R_2$;
 $R_1, R_2 = \text{CH}_3 \text{ or OH}$
 (B1) $R_2 = \text{CH}_3$
 (B2) $R_1 = \text{CH}_3$



conditions of Florisil in refluxing benzene.¹⁰³ This did produce a colorless liquid product (XXV) which was purified by chromatography on deactivated alumina. The ir spectrum of this compound (Figure 20) indicated dehydration had occurred by the absence of the hydroxyl band. The shift in the uv absorption from $\lambda_{\text{max}} = 222 \text{ m}\mu$ in tetradymol (II) to $288 \text{ m}\mu$ in this dehydrated product (XXV) suggested that the new double bond was conjugated to the furan ring. The nmr spectrum (Figure 21) showed a one proton resonance at 6.8 ppm which could be assigned to the α or 2 proton on the furan ring and a one proton resonance at 5.8 ppm which could be assigned to the proton on the new double bond in (XXV)

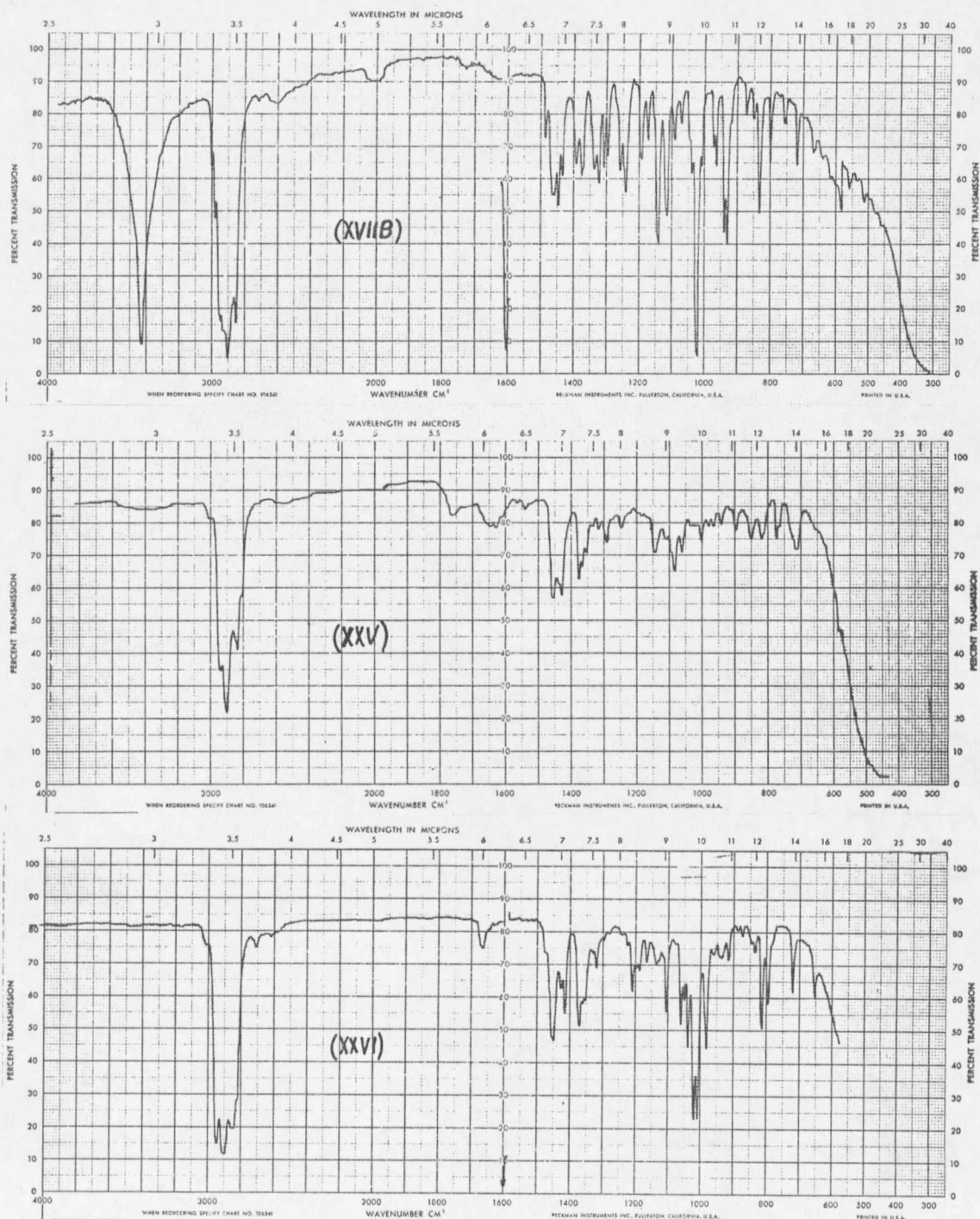


Figure 20. IR Spectra of (XVIIIB) (spectrum run in a micro KBr pellet), (XXV) and (XXVI) (spectra run neat on salt plates).

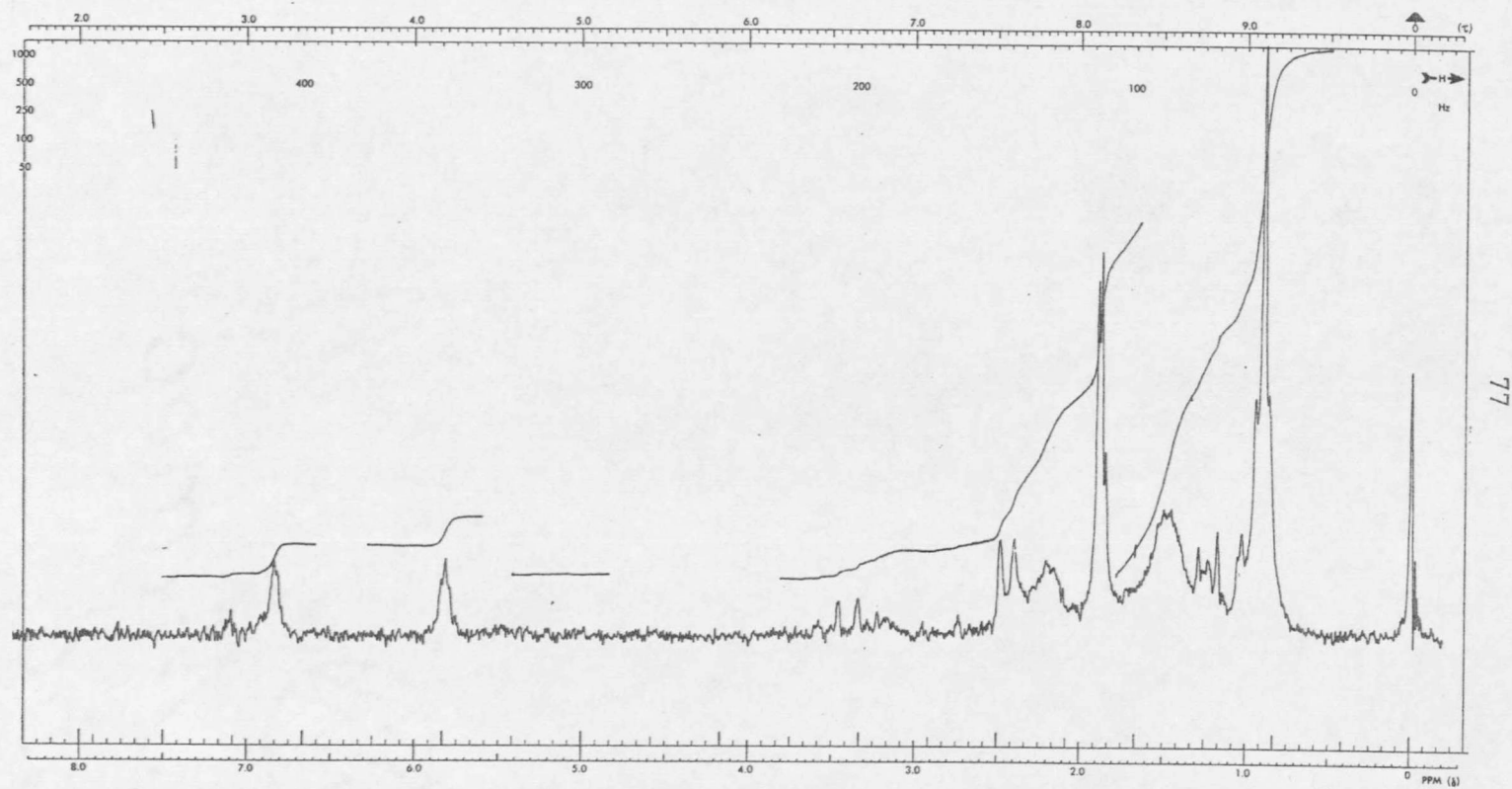
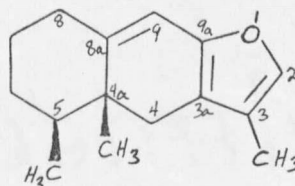


Figure 21. A 60 MHz Spectrum of (XXV) run in Silanor C (DCCl_3 with 1% TMS).

indicating it was a trisubstituted double bond. Compound (XXV) probably had the structure shown but it was not further characterized as it was decomposing quite rapidly while the spectra were being run. This decomposition was demonstrated by a change in the appearance of solution from colorless to orange and the appearance of a new more polar compound when the solution was checked by tlc. Because of this problem of instability, the tetrahydro product (XVIIB) was dehydrated to find if it was more stable and possibly easier to characterize.

(XXV)



This tetrahydro product (XVIIB) was successfully dehydrated to form a hydrogenated-dehydrated product (XXVI). With this molecule the location of the double bond could be ascertained, as is shown below, and a choice be made between the two sesquiterpene types.

When tetradymol (II) was catalytically hydrogenated it yielded, as has been already noted, a mixture of products which had added two or four hydrogen atoms or had lost water and added six hydrogen atoms. The product in highest concentration, utilizing Rh on alumina in methanol, had incorporated four hydrogen atoms to saturate the furan ring (XVIIB). Further assignments could be made, however, to this structure from the nmr spectrum.

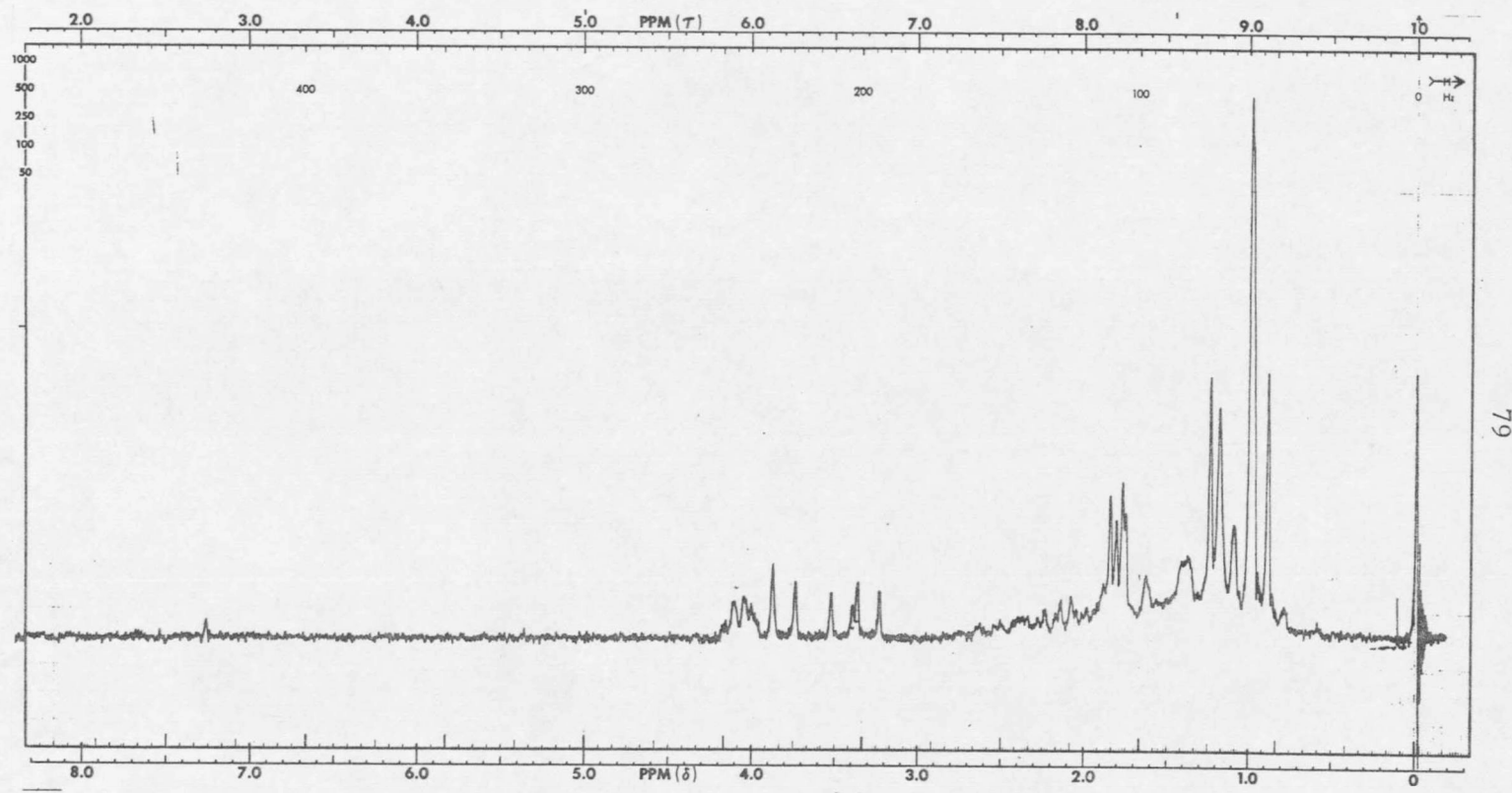
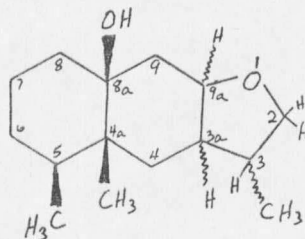


Figure 22. A 60 MHz Spectrum of (XVIIIB) run in Silanor C (DCCl_3 with 1% TMS).

(XVIIIB)

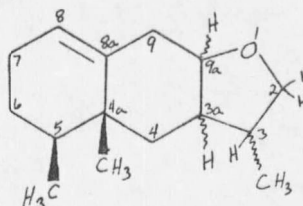


In this nmr spectrum (Figure 22) were displayed resonances for three protons at 4.07 ppm (1H m), 3.80 ppm (1H d $J=8$ cps), and 3.38 ppm (1H dd $J=8,9$ cps) which could be assigned to the protons on the carbons 2 and 9a bearing the ether linkage of the tetrahydrofuran (3.8 ppm typical for protons on the α position of the unsubstituted tetrahydrofuran).¹⁰⁴ The one proton resonance at 4.07 ppm was assigned to the proton on carbon 9a which was shown to be coupled to a two proton resonance at 1.80 ppm by a spin decoupling experiment. This latter resonance was assigned to the protons on carbon 9. The appearance of the resonances of the two protons at 3.38 and 3.80 ppm was probably due to geminal coupling between the protons on carbon 2 ($J=8$ cps), while the one proton on carbon 3 was coupled with only one of the carbon 2 protons ($J=9$ cps). This was evidently due to a rigid conformation that held these protons at such an angle as to allow efficient coupling between only one of them and the carbon 3 proton. This coupling constant angle dependence is mathematically expressed in the Karplus equation.¹⁰⁵ From the graph determined by this equation the dihedral angle between the protons on carbon 2 and that on carbon 3 must be approximately 90° and

$0^{\circ}.106$

When this hydrogenated product (XVIIIB) was dehydrated with I_2 in boiling heptane it yielded a colorless liquid having the proper molecular weight ($M^{+220} m/3$) for (XVIIIB) minus H_2O . This liquid displayed an ir spectrum (Figure 20) that was devoid of the O-H stretching frequency in the region around 3400 cm^{-1} and after purification by chromatography on alumina and careful vacuum distillation gave (XXVI) in 50 per cent yield based on starting material. This compound was homogeneous by glc

(XXVI)



on the one column used. The nmr spectrum of (XXVI), recorded in Figure 23 displayed a one proton resonance at 5.47 ppm (m) which could be assigned to the proton on a trisubstituted double bond attached to carbon 8. When this nmr spectrum was compared to that of (XVIIIB) (Figure 22), a two proton resonance was noted at 2.52 ppm which was downfield from the resonance position of these protons previous to the dehydration. This resonance could be assigned to protons allylic to the new double bond. Also it was noted in this comparison that the two proton quartet at 1.80 ppm (the two protons on carbon 9 in XVIIIB, Figure 22) was now either absent from or moved in the spectrum. Lastly it was observed that the resonances assigned to the protons on carbons 2 and 9a were only slightly

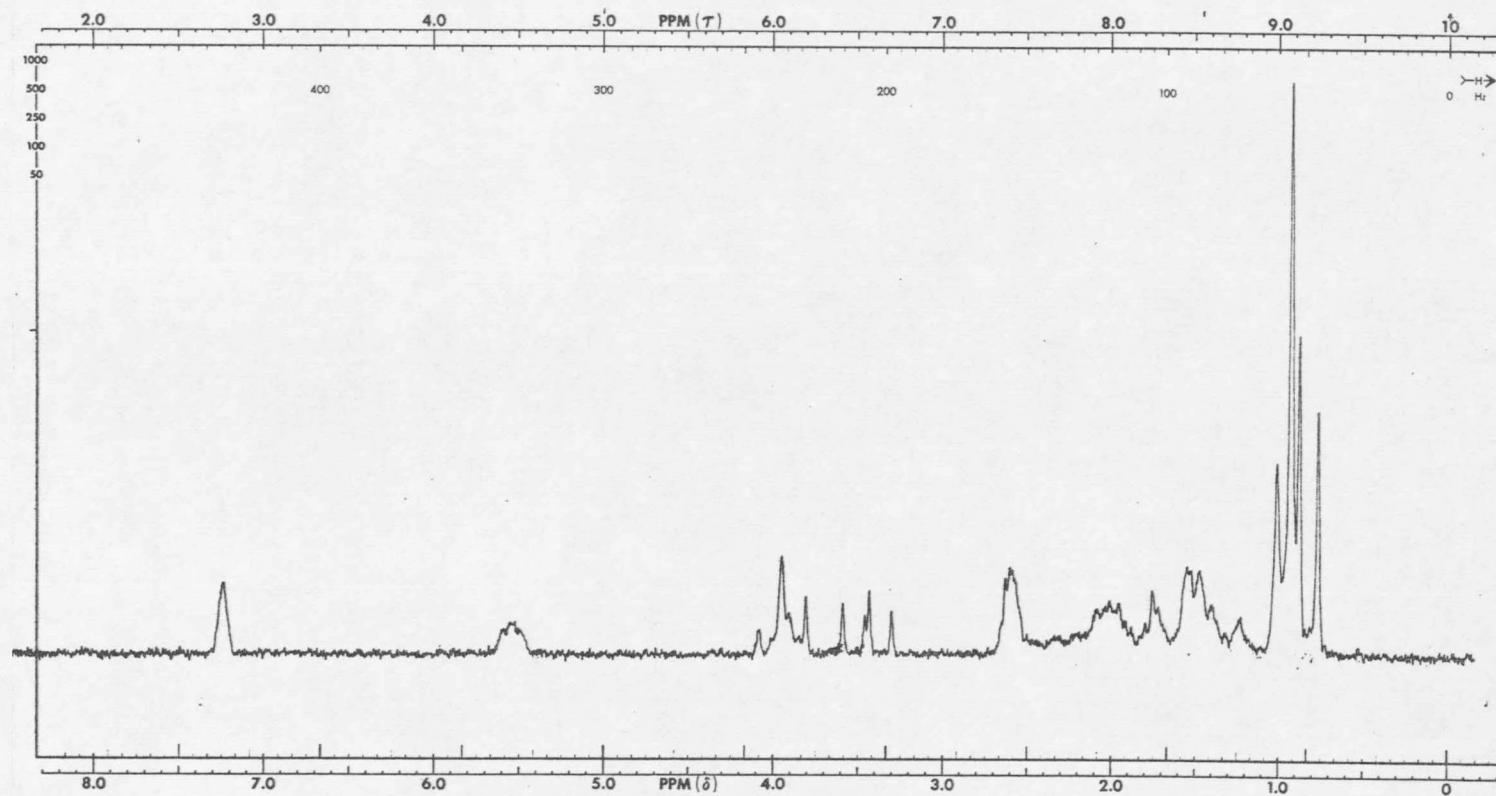


Figure 23. A 60 MHz Spectrum of (XXVI) run in D_6 Benzene.

affected by the dehydration.

Keeping the above facts in mind, it was possible to assign the double bond to the 8-8a position by the following process of elimination. First, the double bond could not be 4a-5 because it would be tetrasubstituted and there would be no proton resonance at 5.47 ppm. Second, if the double bond was located in the 8a-9 position the proton on carbon 9a should be greatly affected since it would be both allylic to a double bond and geminal to an ether linkage. This shift was not observed. Third, if the double bond was located 4-4a the two allylic protons (to the double bond) would be quite different and would not be expected to resonate at the same shift position. There was only one peak, though somewhat broad, for the two protons which could be seen to be affected by the new double bond. Also the resonance for the tertiary proton on carbon 5 in this instance would not be expected to be shifted below 2 ppm since the effect of a double bond is not sufficient to move an allylic proton below that shift position.¹⁰⁷ Further a 4-4a double bond would not be expected to so markedly affect the carbon 9 protons as was evidenced.

This left only the 8-8a position as indicated in the proposed structure (XXVI). The nmr spectrum matched this structure quite well. The resonance of the two protons on carbon 9 would be expected to be shifted from 1.80 ppm downfield to a position below 2.0 ppm due to the additive effect of both the double bond and ether oxygen; and thus this was a tri-

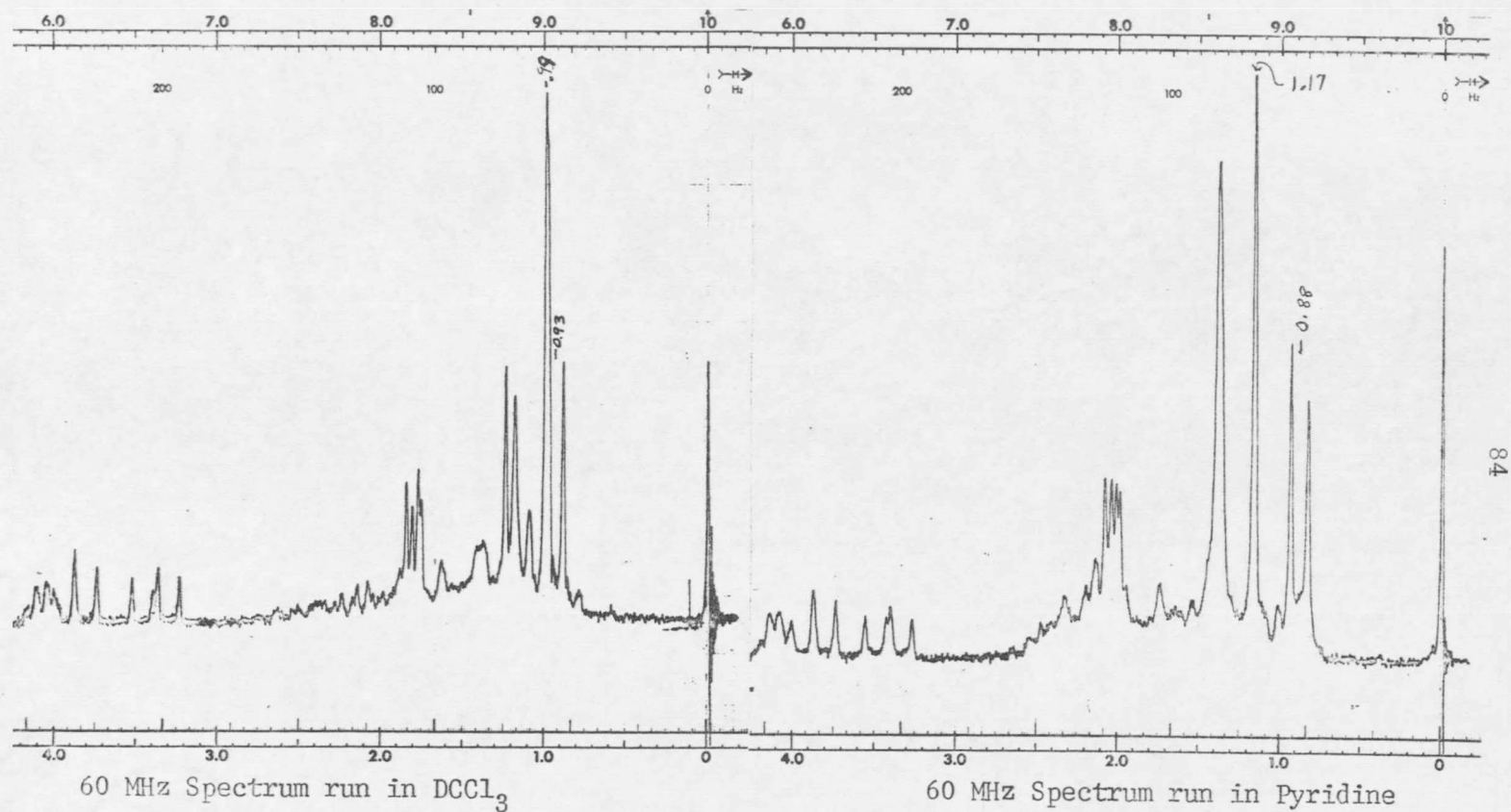


Figure 24. Pyridine Induced Shift in the NMR Spectrum of (XVIIIB)

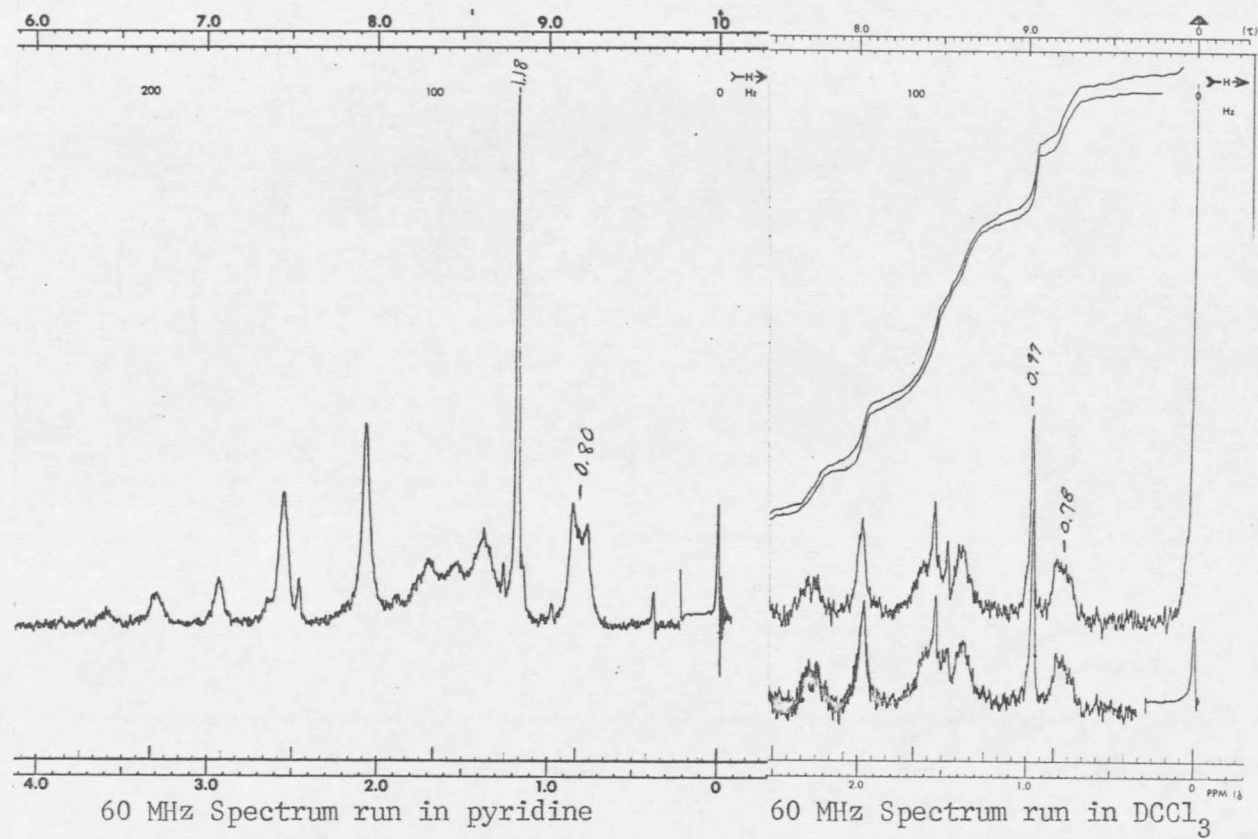


Figure 25. Pyridine Induced Shift in the NMR Spectrum of (IIA)

substituted double bond. The protons on the 7 position were not assigned as they were still not separated sufficiently to be observed, as was expected since they were affected by only a simple double bond.¹⁰⁸

The location of the double bond in this 8-8a position obviously requires the eremophilane rather than the eudesmane skeleton.

The cis type ring juncture at 8a and 4a was established on the basis of the solvent induced shift of the tertiary methyl group resonance in the nmr spectrum of (XVIIIB) as shown in Figure 24. A methyl group which is cis to a hydroxyl moiety is considerably more deshielded when the spectrum of the compound is run in pyridine than when it is run in deuteriochloroform.¹⁰⁹ From the available tables, the pyridine-induced shift ($\Delta_{\text{C}_5\text{H}_5\text{N}}^{\text{CDCl}_3} = -.18$ ppm) of the carbon 4a methyl resonance agreed with a dihedral bond angle between the hydroxyl group and methyl group of just over sixty degrees.¹¹⁰ This then was a cis ring juncture. The pyridine-induced shift ($\Delta_{\text{C}_5\text{H}_5\text{N}}^{\text{CDCl}_3} = -.22$ ppm) of the methyl on carbon 4a in the mercury chloride derivative (IIA) (Figure 25) also supported the cis ring juncture assignment as this shift was that given for a dihedral angle of 60°. This type ring juncture is typical of the known eremophilanes.¹¹¹

The secondary methyl of an eremophilane is typically cis with respect to the tertiary methyl group.¹¹² If this was the case in tetradymol two conformers (IIB) and (IIC) are possible (Figure 26). When the stabilities of the two conformers were compared it was observed that

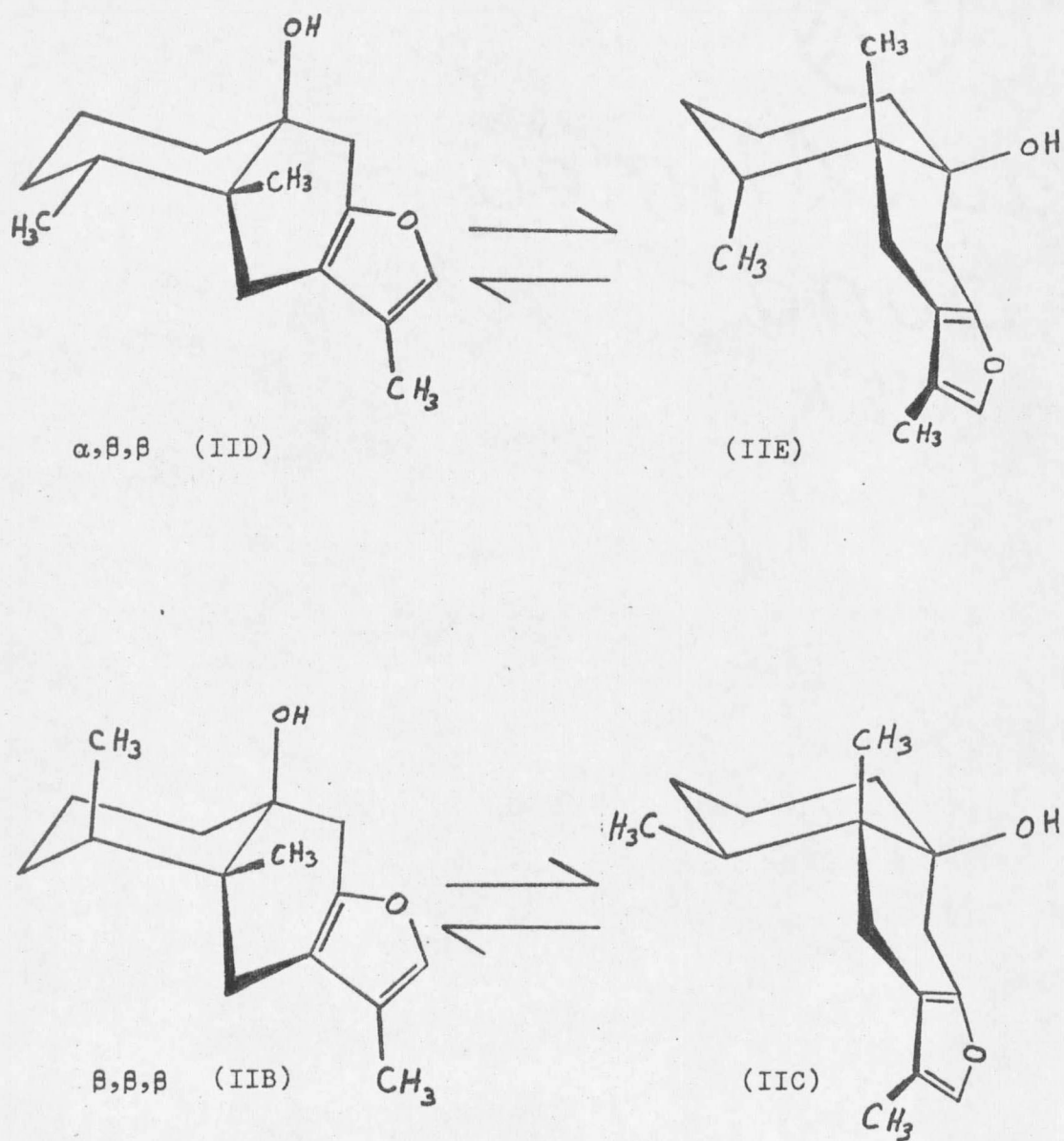


Figure 26. Tetradymol Isomers.

(IIC) has one less methyl, ring-carbon gauche interaction and did not have the 1,3 diaxial hydroxyl, methyl interaction. Thus, it would be expected that conformer (IIC) would predominate. The observed solvent induced shift ($\Delta_{\text{C}_5\text{H}_5\text{N}}^{\text{DCCl}_3} = -.03 \text{ ppm}$) of the methyl group on carbon 5 of the mercury chloride derivative was so small that structure (IIB), with the hydroxyl and methyl groups diaxial, seemed unlikely.¹¹³ This conformational assignment was further supported by the intensity (23 per cent of base) of the parent peak ($M^+ 234$) in the mass spectrum of (II) which indicated that the formation of the six membered ring for the most facile dehydration¹¹⁴ was not favorable. As could be readily seen conformer (IIB) was ideally arranged for this dehydration as shown in Figure 27. Also it was noted that the hydroxyl moiety in (IIB) was more highly hindered by the 1,3 hydroxyl, methyl interaction than it was in (IIC). This hinderance should have speeded the elimination of the hydroxyl group in the ms in line with Biemann's¹¹⁵ work. It might be expected, however, that the rapid inversion of this cis ring juncture would eliminate the above argument, i.e., the juncture could invert in time for the hydroxyl group to eliminate in the ms, but it was noted that the conformational change requires the eclipsing of two methyl groups and a hydroxyl group in the transition plus the interconversion of two chair forms of cyclohexane. This process obviously requires a considerable amount of energy.

The above arguments supporting the structure (IIC) gave just as

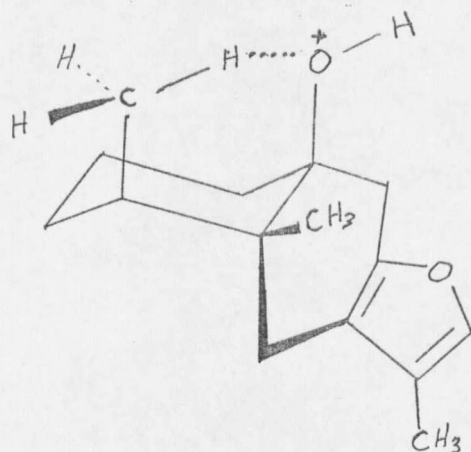


Figure 27. Conformation for Easy Dehydration in MS.

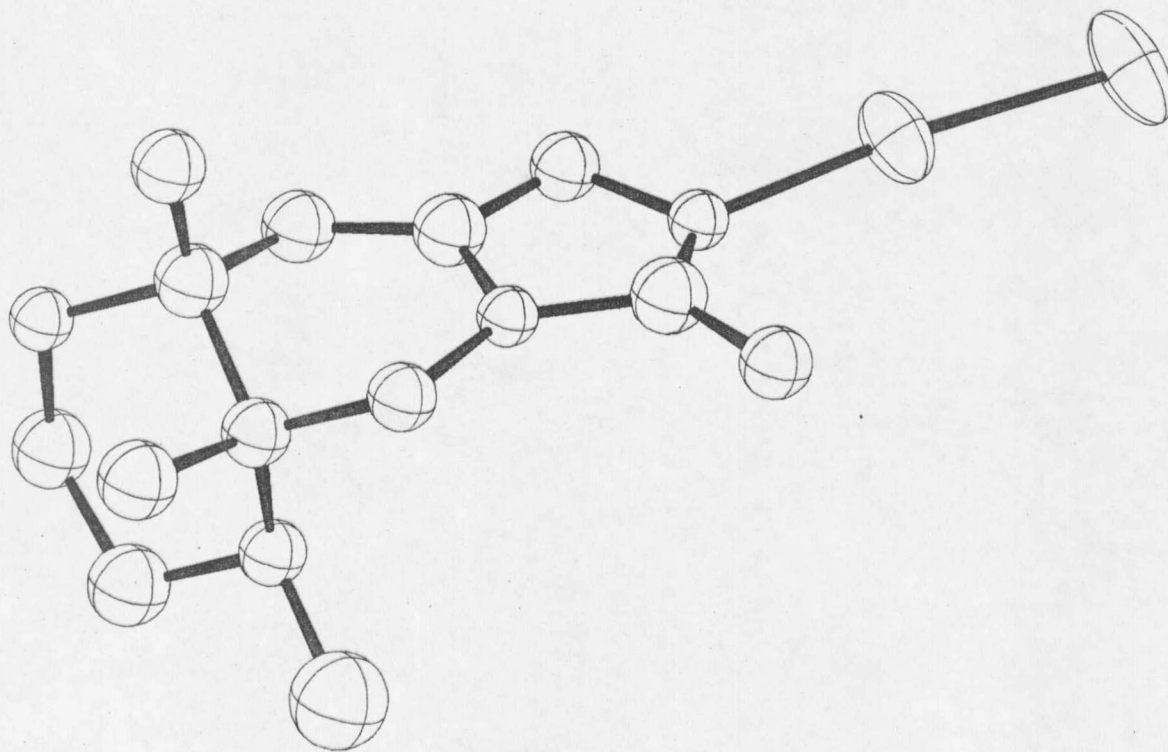
good support to both conformers (IID) and (IIE) which are isomeric with (IIB and C) about carbon 5. On the basis of these studies it was not possible to choose between the two isomers, nor was it possible to ascertain the absolute configuration of the molecule though the configuration shown is that of the typical eremophilane.

In order to determine the configuration about carbon 5 in tetradymol (II), confirm the rest of the proposed structure, and determine the absolute configuration, the mercury chloride derivative (IIA) of tetradymol was submitted to X-ray crystallographic analysis -- the results of which are outlined in the next section of this discussion.

IV. X-Ray Structural Analysis

The X-ray crystal structure of the mercury chloride derivative of tetradymol (XXVII) authenticated the proposed structure and further revealed that the carbon 5 methyl group was cis with respect to the hydroxyl group and carbon 4a methyl group. It also revealed that the absolute configuration was that shown below.

(XXVII)



Dr. D. Smith, a post-doctoral student of Dr. C. Caughlan who performed the X-ray crystal study, reported that the crystal displayed orthorhombic symmetry with unit cell dimensions of $a = 7.371(5) \text{ \AA}$, $b = 10.304(9) \text{ \AA}$, and $c = 19.759(17) \text{ \AA}$ as obtained from a GE XRD-5 diffractometer using $\text{Mo K}\alpha$ radiation. While data was being collected on the crystal, it partially decomposed in the X-ray beam. This was made evident by a decrease in the standard spots of approximately 30 per cent and a gradual darkening of the crystal.

Of the 1159 piece data set, 787 pieces were found to be more than twice the standard deviation of the intensities and were considered observed. The other 372 reflections were considered unobserved and were not included in the refinement.

After various refinement procedures the final R was found to be 5.4 per cent and the weighted R 5.9 per cent for the structure shown.

The positional and thermal parameters of the enantiomorph were also refined but the refinement stopped at an R of 6.5 per cent. This significant difference in R proves the absolute configuration of (XXVII).

The crystal structure revealed a number of other interesting features. When a least squares plane was drawn through the atoms of the furan ring and the methyl group on carbon 3, the maximum deviation from this plane was $0.07 \text{ \AA}$ by carbon 2 which was probably very nearly within the experimental error. Thus, the furan ring was essentially planar. The bond distances (Figure 28) indicated that there was a considerable

delocalization of electrons in the furan ring since the bonds were short (except the oxygen 1 to carbon 2 bond) and the bond between carbons 3 and 3a was very nearly the same length as that between carbons 3a and 9a. If this was simply a butadiene system held by an oxygen bridge, as has sometimes been suggested for furan,¹¹⁶ the above two bond lengths should differ appreciably. These findings of planarity and short bond lengths lent support to the assignment of aromatic character to this furan ring.

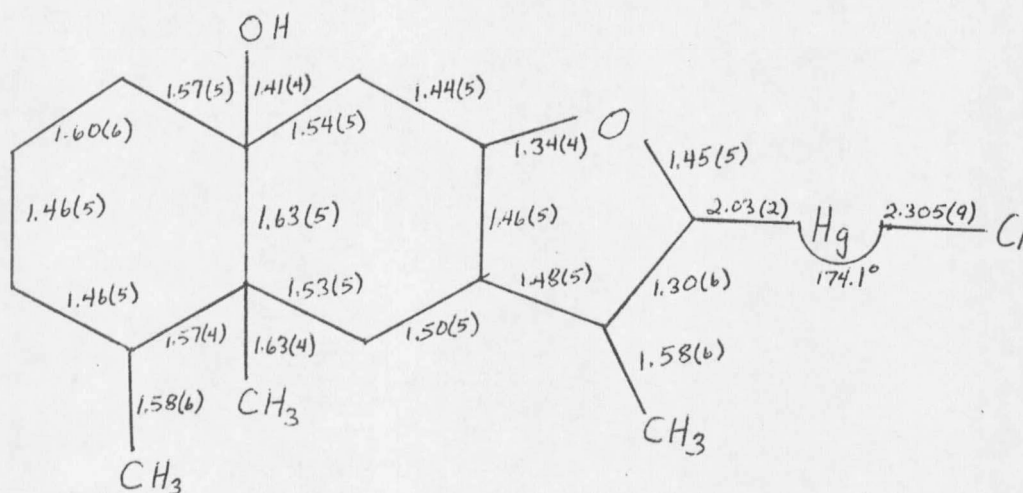


Figure 28. Tetradymol Mercury Chloride Derivative Bond Distances from X-Ray Structure.

From the crystal packing data, a unit cell is shown in Figure 29, it was determined that the compound exists in the lattice as a dimer held by hydrogen bonding between the hydroxyl group of one molecule and the mercury of the other. This could have accounted for the 6 degree

variance from linearity of the carbon, mercury, chlorine bonds.

The dihedral angle between the carbon 8a hydroxyl group and the tertiary methyl group on carbon 4a was 57.3° which was very close to the angle suggested from the nmr spectral data on page 86 of this thesis.

With the heavy atoms present, the X-ray structural analysis of this compound proved to be a rather straight-forward and simple problem. Since the compound was so easily prepared and, according to the literature¹¹⁷ furans with the α or 2 position unsubstituted characteristically form the chloro-mercury derivative so readily, the author suggests that for new furano-compounds of that type this procedure is the method of choice for the elucidation of structure.

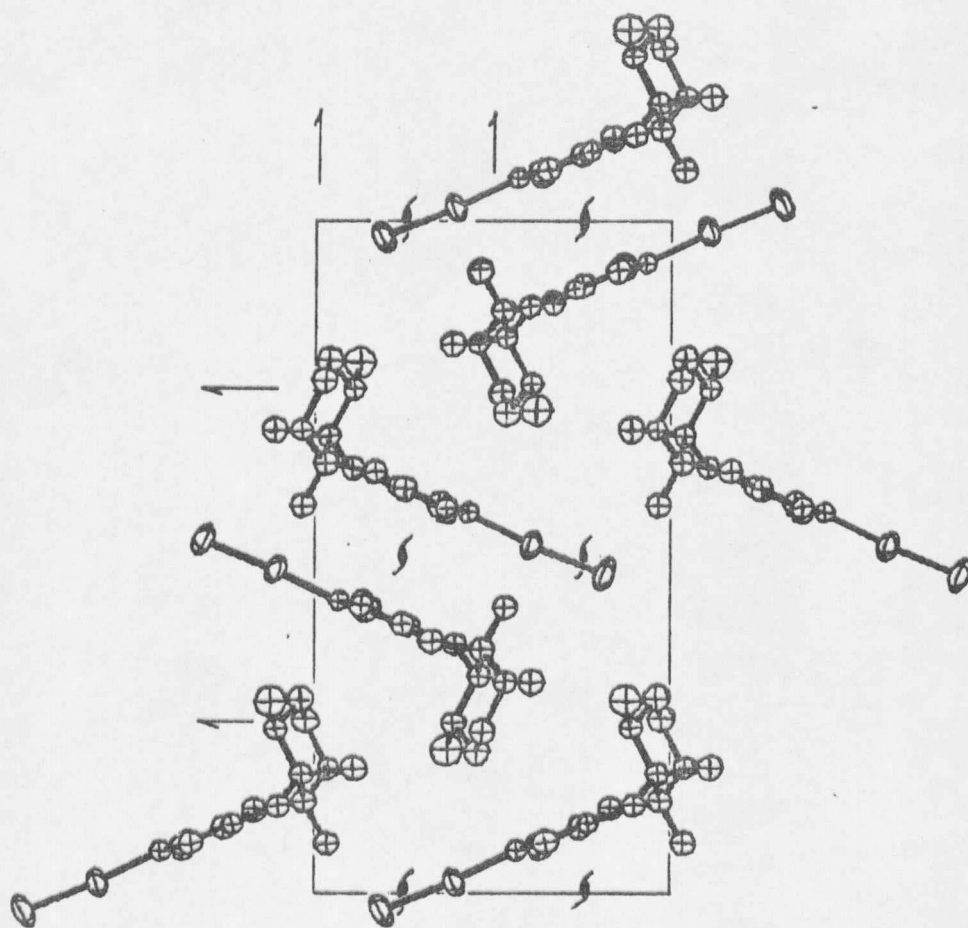


Figure 29. Crystal Packing Pattern for Tetradymol Mercury Chloride

EXPERIMENTAL SECTION

I. Reagents

Bio-Sil A, 100-200 mesh, obtained from Bio-Rad Laboratories and neutral alumina, 100 mesh, obtained from Ventron Corporation, were used directly, without activation, for elution column chromatography in all but one experiment. In this instance the alumina was activated overnight at 120°C. Florisil, 60-100 mesh, obtained from Floridin Company, was activated overnight at 120°C before use in chromatography.

For thin layer chromatography Anasil S, obtained from Analabs Inc., and aluminum oxide G (type E), obtained from E. M. Reagents, were used. The thin layer chromatographic plates were poured, as a water slurry with a Desaga spreader giving a 250 micron thickness, and then activated at 120°C for one hour.

The following solvents were found to be satisfactory for use in extraction, column elution, and thin layer development directly from the container without further purification: n-hexane, Phillips Petroleum; anhydrous diethyl ether, anhydrous methanol, ethyl acetate, pyridine, and benzene, Baker reagent.

For making the micro-pellets used in obtaining infrared spectra, anhydrous KBr, SPEX Industries Inc., was found to be quite satisfactory directly from the supplier's small containers. The solvents Silanor C, Merk; D₆ benzene, Diaprep Inc.; pyridine, carbon tetrachloride, and absolute methanol, Baker analyzed reagent were used as solvents in

spectral analyses.

The vanillin-sulfuric spray reagent was made up by adding 1.5 g vanillin, USP Merk, to 50 ml absolute ethanol and then adding 0.25 ml of concentrated sulfuric acid.

Ehrlich's¹¹⁸ reagent was made up by adding 50 mg of p-dimethyl-amino benzaldehyde, Eastman reagent, to 1 ml 95 per cent ethanol and then adding 2 drops of concentrated hydrochloric acid.

A fresh Chromerge, Manostat Corp., solution in sulfuric acid was used directly as the sulfuric-dichromate spray reagent for tlc.

The other reagents and solvents were used as received with the exception of n-heptane which was washed with sulfuric acid, dried and distilled.

II. Instruments

Infrared spectra were obtained using either a Beckman IR-4 or IR-20 spectrophotometer. Spectra were run in micro KBr pellets, as a neat, thin film between sodium chloride plates, or in solution in carbon tetrachloride using sodium chloride infrared cells.

Nuclear magnetic resonance spectra were obtained using a Varian model A-60 for the 60 MHz spectra and a Bruker instrument for the 90 MHz spectra. Spin decoupling experiments were performed on compound (II) with the 90 MHz instrument while these experiments were performed on (II) and (XVIIIB) with a 60 MHz instrument.

Ultraviolet spectra were obtained in anhydrous methanol solution using matched quartz cells on a Cary Model 14.

Analyses of dye concentrations obtained in animal feeding experiments were determined using a Beckman DU.

Mass spectral data were obtained using a Varian Mat CH 5 with both a solid probe and a glc interface.

Optical rotations were determined on a Rudolph Model 180 in anhydrous methanol.

For determination of purity a Beckman Model GC 4 gas chromatograph was utilized. It was equipped with various columns, a flame detector, and temperature programmed oven.

Elemental analyses were performed by Chemanalytics, Tempe, Arizona.

X-Ray crystallographic data were taken on a GE XRD-5 diffractometer.

III. Plant Material

Four pickings of plant material were made which are designated Tg I-IV. The Tg I picking was made approximately fifteen miles south of Lucin, Utah, which is in the northwestern part of the state, on May 4-5, 1968. The plant had flowers, buds, and new growth present when picked. The top four to eight inches of the plant was broken off and stuffed into "gunny" sacks which were cooled with dry ice and placed in an enclosed trailer for transportation to Bozeman. Upon arrival at Bozeman, the plant material was placed in a refrigerator at 40°C for one day. It was

then chopped and frozen for storage at -20°C .

The Tg II picking was made at the same location as Tg I on June 18-19, 1968. The plant was in the full bloom or fruit stage of growth. This time only new growth, flowers, and buds were picked. This plant material was placed in plastic bags, along with dry ice, and these bags were stored in an insulated chest for transportation to Bozeman. Upon arriving at Bozeman, the plant material was stored in the cold room at -20°C .

The Tg III picking was made July 1-4, 1968, approximately fifty miles north of the area where Tg I and II had been obtained. By this time the plant was beginning to dry out, but a few of the bushes were found that were still quite succulent with buds, flowers, and new growth present. Again the older growth was discarded and the newer growth packed in plastic bags with dry ice and taken to Bozeman.

The Tg IV picking was made on June 15, 1969, along the highway about 20 miles south of Park Valley, Utah. This was in the same general region where the other pickings were made. The plant was very lush with many buds and flowers along with considerable new growth. The buds and new growth were picked and placed in plastic bags. These were taken to a cold room (0°C) at the University of Utah and stored for four days, then transported to Bozeman and stored at -20°C .

Tg I was identified by Dr. W. E. Booth, curator of the herbarium at Montana State University, as authentic Tetradymia glabrata. Tg II was

confirmed as T. glabrata by Dr. Holmgren, curator of the herbarium at Utah State University. Samples of both of these pickings were filed for reference at the Montana State University Herbarium as numbers 63708 and 63737.

The region where this plant material was picked was very sandy and arid with a salt flat just a few miles away. The elevation was about 4000 feet above sea level.

When the hexane extracts of each of the four pickings were analyzed by tlc they were all found to contain the compound that was later shown to be the active principle of T. glabrata. Also it was possible to extract this principle from plant material that had been stored frozen for two years.

IV. Animal Testing

A. Feeding Techniques: Sheep feedings of the whole plant were first attempted with a balling gun utilizing large gelatin capsules containing ground plant material. This method was found to be very slow for both feeding and preparation of the capsules. Forced feeding through a stomach tube was then attempted and found to be quite satisfactory.

The plant material was prepared for direct stomach feeding by first grinding it, while frozen, with a meat grinder. The resultant fibrous mass was further pulverized in a Waring blender to yield a thick slurry. This slurry was then forced by moderate positive air pressure directly

into the stomach of the sheep through a 10 mm Tygon tube. The apparatus is illustrated in Figure 30.

Plant extracts were prepared for sheep feeding in the following manner. The still frozen plant material was first ground in the cold solvent in which it was to be extracted. Then more cold solvent was added and the resultant mixture allowed to stand in a cool room (10°C) for 24 hours. At the end of this time the liquid was filtered off, the solvent evaporated and resubmitted to the plant material, and the extracted materials stored at 10°C in preparation for feeding. This cycle was performed three times.

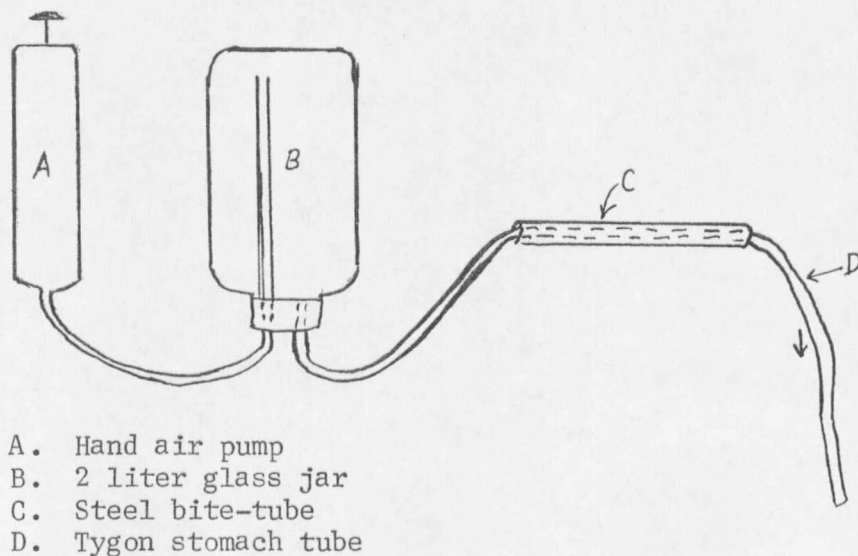


Figure 30. Apparatus for Sheep Feedings.

The ethanol extract was further treated by extracting the pentane to give a "polar" and a non-polar" fraction which were fed separately.

These plant extracts were administered to sheep with a balling gun and capsules. The plant that had been extracted was fed as a slurry with the stomach tube.

Feedings of the whole plant were not attempted with mice or other laboratory animals. They were fed only extracts and purified compounds which were all administered orally. The early feedings were accomplished using a syringe with a blunt needle covered with plastic. This method of feeding had the problems of losing part of the dosage in the animal's mouth and suffocation of the animal by injecting material into the throat, not the esophagus. It was later found that mouse feedings could be better accomplished by injecting the dosage directly into the stomach. This was done by means of a 7 cm PE 20, "Intramedic", polyethylene tube (Clay Adams) attached to a 26 gauge needle on a $\frac{1}{4}$ cc tuberculin syringe. Almost the entire length of the tube could be passed down the esophagus of the mouse and into the stomach while the mouse was held securely in a supine position. This method of feeding was used for the mouse feedings numbered 8-20 in Table 4.

All mice used were Swiss Manor males from the colony operated by Dr. J. Jutila at Montana State University. The usual age was approximately 6 weeks at the time of feeding.

B. Tests of Function: The following tests, BSP clearance and ammonia level, were run only on sheep. To perform the BSP clearance test¹¹⁹, 5 ml of a standard solution, 50 mg/ml, of sulfobromophthalein (XXVII) (Hynson, Westcott and Dunning) was injected into the jugular vein in the sheep's neck. Aliquot portions of blood were withdrawn from the jugular vein on the other side of the sheep's neck at appropriate intervals. A typical time sequence for blood samples would: 0 minutes (before injection for a blank reading), 5, 15, 30, and 60 minutes. Each blood sample was mixed immediately after drawing with an EDTA solution prepared from EDTAP tablets (Cambridge Chemical Products) to prevent clotting. The blood was then centrifuged. One ml of the serum was pipetted into 10 ml of water to which was added 3 ml of a 0.1N sodium hydroxide solution to develop the dye color. The absorbance of the solution was read at 580 m μ . Log A was then plotted against time to obtain the usual first order rate constant. From this was calculated the half-time for the dye retention.

To perform the ammonia level test¹²⁰ an aliquot portion of blood was drawn and immediately mixed with a 10% TCA solution and stored in ice. This blood sample was then centrifuged and 0.5 ml of the resultant serum placed in a test tube. To this was added 2.5 ml of a solution of 11.2 g liquid phenol and 0.050 g sodium nitroferricyanide made up to one liter with water. To this solution was added 2.5 ml of a solution of 5.0 g sodium hydroxide and 7.0 g sodium hypochlorite (Purex) made up to one

liter with water. This serum mixture was agitated in a thermostated (37°C) water bath for thirty minutes to develop the color reaction and then read at $625\text{ m}\mu$ against water. This reading was then converted to $\mu\text{g/ml}$ using a standard curve formulated using a range of ammonium chloride solutions.¹²¹

During the time of the sheep feeding experiments their heart action was monitored by the electrocardiograph (EKG) utilizing four leads. These leads were the standard leads I, II, and III¹²², and a back lead IV which extended from the back at the shoulder to the left front leg.

V. Isolation Techniques for Tetradymol (II)

A. Extraction Procedure: The plant material was transferred while still frozen into a large stainless cone in a room kept at about 10°C . This cone was fitted with an all metal (brass) gate valve at the bottom supporting a wad of glass wool. Cold n-hexane was added in sufficient quantity to completely cover the plant material. The cone was covered with aluminum foil and allowed to stand for from 8 to 20 hours. The solvent and extracted materials were then drained off through the bottom valve and the solvent removed from the extract under water aspirator vacuum in a steam heated still. The n-hexane was then resubmitted to the extraction cone and the dark green-brown residue further concentrated under water aspirator vacuum with the rotary evaporator at a temperature of 50°C . This crude extract was then stored in the cold (-20°C) until

chromatographed. The plant material was extracted three times in this manner.

B. Silica Gel Chromatography: The silica gel, Bio-Sil A, was made up as a slurry in 10% diethyl ether in n-hexane. This slurry was poured into a typical glass chromatography column. After draining the solvent to the top of the adsorbent the column was charged with crude extract at the ratio of 1 g crude extract to 50 g dry silica gel. The column was then eluted in the manner shown in Table 7.

The fractions from the column were monitored by alumina tlc and those containing tetradymol were concentrated and stored at -20°C in preparation for alumina elution chromatography.

The column and adsorbent could be re-used effectively at least two more times by reversing the eluotropic series to n-hexane after each run, and then recharging the column and eluting as usual.

C. Alumina Chromatography: The alumina was made up as a slurry in 50% benzene in n-hexane to which had been added anhydrous methanol in a ratio of 3 ml methanol to each 100 g of alumina to be slurried. This slurry was poured into a typical glass column and the solvent drained to the adsorbent surface. The column was next eluted with a 50% benzene in n-hexane solution at a ratio of 1 ml for each 1 g of dry alumina. When this solution had drained to the top of the adsorbent the column was charged at the rate of 1 g tetradymol mixture, from the silica gel

column, to 200 g alumina and eluted as shown in Table 7.

Table 7

Elution of Chromatographic Columns

	Solvent mixture used			Solvent quantity l/kg
	n-hexane	diethyl ether	methanol	

Silica gel elution

1	90%	10%		6
2	85%	15%		2
3	70%	30%		2
4	40%	60%		2
5		100%		2
6		97%	3%	4

Alumina elution

1	50%	50%		1
2		100%		1
3		97%	3%	1

The fractions from the column were monitored by alumina tlc and those fractions containing only tetradymol were concentrated and stored at -20°C in preparation for sublimation.

The column and adsorbent could be re-used effectively two more times by reversing the elutotropic series to n-hexane after each run, and then recharging the column and eluting as usual.

In one instance the alumina was activated before use and packed using 30 per cent diethyl ether in n-hexane. In this instance tetradymol and 29-3 did not come off the column until it was washed with 2 per cent methanol in diethyl ether. When they did come off they did so together. Total yield from the column was about 55 per cent.

D. Florisil Chromatography: Freshly activated Florisil (27 g) was made up as a slurry using 1 per cent diethyl ether in n-hexane. This slurry was poured into a typical glass chromatography column and the solvent drained to the surface of the adsorbent. The column was then charged with 350 mg of the mixture of tetradymol (II) and 29-3 from the silica gel column. This column was then eluted using a gradient from n-hexane diethyl ether mixtures to diethyl ether methanol mixtures. As the elution proceeded the column turned a bright pink, and remained that color. The total yield from the column was about 90 per cent.

F. Sublimation: The yellow solid from the alumina column, that was shown to contain tetradymol (II) by tlc, was recrystallized four times from n-hexane to give white crystals. These white crystals (173 mg) were then sublimed twice at 0.15 mm Hg with an oil bath temperature of 55 to 60°C while the cold finger was maintained at tap water temperature of from 5 to 9°C. The sublimation gave 135 mg (80%) of white crystals; mp 91.5-92.5°C, $[\alpha]_D^{25} + 44^\circ$ (C 0.90, MeOH): which were still impure.

Anal. Calcd. for $C_{15}H_{22}O_2$: C, 76.88%; H, 9.4%. Found: C, 75.29%;

H, 8.99%; N, less than 0.1%.

Later sublimations were carried out as above on the yellow solid directly from the alumina column which gave the white crystals. This sublimed material was used for the various degradative reactions described herein and for the base extraction that finally gave the pure compound.

F. Base Extraction: The sublimed white crystals (0.360 g) were dissolved in a 0.6M ethanolic potassium hydroxide solution and kept in a capped, light-protected vial overnight at approximately 25°C. This material was then extracted three times with n-hexane. The n-hexane extract was washed with salt solution, dried with Na₂SO₄, and evaporated. The resultant crystalline material (0.282 g, 78%) was recrystallized twice from n-hexane to yield tiny transparent white needles; mp 92.5°C, $[\alpha]_D^{25} + 56^\circ$ (C 4.3, MeOH). The mass spectrum showed m/e 234 (M⁺, 24%), 216 (5%), 126 (28%), 111 (38%), 109 (100%), 108 (45%).

Anal. Calcd. for C₁₅H₂₂O₂: C, 76.88%; H, 9.40%. Found: C, 76.84%; H, 9.25%.

VI. Reactions

A. Hydrogenation of Tetradymol: In a typical reaction 1.9 g of 5% Rh on alumina was placed in 300 ml of absolute methanol and shaken under 35 pounds square inch pressure of hydrogen for one half hour in a Parr shaker. The reaction bottle was then opened and 2.57 g (0.011 mole) of

sublimed tetradymol (II) added. The bottle was then reclosed, hydrogen pressure of 35 pounds/square inch re-established, and shaking continued for 18 hours (shorter times of from 1 to 5 hours were insufficient for complete reaction). At the end of this time the solution was filtered through a sintered glass funnel to remove the catalyst. (Caution! If the funnel is allowed to go completely dry during filtration it spontaneously ignites.) The solvent was then evaporated from this mixture on a rotary evaporator under water aspirator vacuum. The yield of the products was 2.60 g.

The resultant mixture of compounds was separated by repeated column chromatography on alumina using an eluotropic series of n-hexane, benzene, diethyl ether, and ethyl acetate. The individual compounds, with the exception of (XIX), were further purified by sublimation (at 60°C and 0.05 mm Hg) and repeated recrystallization from n-hexane. The purification was monitored by glc using a 6 foot, 1 per cent OV-1 liquid phase on GC-Q support in a 1/8-inch copper column. Purity was confirmed by tlc and elemental analysis.

The products were as follows with their isolated form, melting point, R_f (on alumina tlc using 40% n-hexane in diethyl ether), elemental formula, mass spectral parent peak, and analysis given in that order. The yields of individual products are given in Table 6, page 63.

- (XIX) Colorless liquid (at least 7 components by glc), .90, $C_{15}H_{26}O$, 222 m/e, no analysis obtained.
- (XVIIA) Colorless plates from n-hexane, 75.5-77°C, .47, $C_{15}H_{26}O_2$, 238 m/e. Calcd.: C, 75.63; H, 10.92. Found: C, 75.88; H, 10.92.
- (XVIIB) Colorless blocks from n-hexane, 104-105°C, .36, $C_{15}H_{26}O_2$, 238 m/e. Calcd.: C, 75.63; H, 10.92. Found: C, 75.45; H, 11.00.
- (XVIII) Colorless needles from n-hexane, 145-146°C, .10, $C_{15}H_{24}O_2$, 236 m/e. Calcd.: C, 76.27; H, 10.17. Found: C, 76.18; H, 10.18.

B. Mercury Chloride Derivative of Tetradymol:¹²³ To 2.72 g (0.01 mole) $HgCl_2$ in 25 ml hot H_2O was added 5.44 g (0.04 mole) NaOAc in 10 ml H_2O , then 24 ml ethanol. The solution was allowed to stand at room temperature overnight, when it was filtered and used.

When 600 mg of sublimed tetradymol (II) (0.0026 mole) was dissolved in 8 ml of 95% ethanol and slowly added to 17 ml (0.0028 moles Hg) of the above solution there was an immediate formation of the flocculent precipitate of the mercury chloride derivative. The solution was filtered to give 1.49 g (crude) of a white solid. This product was recrystallized from hot ethanol to yield transparent, white needles, mp 205-206.5°C (with some decomposition).

When a few milligrams of the mercury chloride derivative of tetradymol was dissolved in an aqueous ethanol solution and H_2S bubbled through the solution for a few minutes, tetradymol was regenerated as

demonstrated by its mobility and color reaction on tlc.

C. Maleic Anhydride Adduct of Tetradymol: To a solution of 140 g maleic anhydride in 40 ml of diethyl ether was added 50 mg of tetradymol (II). This solution was capped and allowed to stand in the dark at room temperature overnight. The resultant mixture was spotted on Anasil tlc and developed with 50% acetone in methanol to reveal three spots made visible by spraying with sulfuric-dichromate solution. These spots had an Rf .82, .35, and .18. (Maleic anhydride had an Rf .27 in this system.) The solvent was evaporated under vacuum and the white solid submitted to mass spectral analysis to reveal a parent peak $M^+ 332$ m/e. Before the material could be purified and further characterized it turned pink and then brown overnight. No attempt was made to protect from air or light after the solvent was evaporated.

D. Sarett Oxidation of Tetradymol:¹²⁴ To an ice cooled flask containing 200 ml of pyridine was slowly added, with magnetic stirring, 20 g of CrO_3 . After this mixture had stirred for 10 minutes, 2.02 g of tetradymol (II) in 200 ml of pyridine was added. The cooling was now removed, a condenser fitted, and the suspension allowed to stir in the dark for 8 hours at which time no tetradymol was detectable by tlc. Diethyl ether was added¹²⁵ to the suspension to precipitate the rest of the chromium salts and the suspension filtered. The solution was acidified with 6N HCl and repeatedly extracted with ethyl acetate and diethyl

ether. The extract was washed with an acidified salt solution, dried and evaporated. The gummy product crystallized with difficulty after the addition of n-hexane and recrystallized from diethyl ether to yield 0.92 g (40%) of the product (VI) as white crystals, mp 161-162.5°C, uv max 221 m μ (log ϵ 4.05). The mass spectrum showed m/e 266 (M^+ , < 0.1%), 123 (100%).

Anal. Calcd. for $C_{15}H_{22}O_4$: C, 67.65; H, 8.33. Found: C, 67.50; H, 8.41.

E. Sarett Oxidation of Menthofuran: In a manner analogous to the above procedure 10 g of menthofuran was oxidized. In this instance the reaction was worked up by adding water¹²⁶ and extracting with n-hexane and diethyl ether, to remove the unreacted menthofuran, then acidifying with HCl and extracting with diethyl ether. A monstrous emulsion resulted and it was only with difficulty that 1.9 g (16%) of the oxidation product (VII) was obtained as white crystals from methyl acetate and n-hexane, mp 190-191°C (reported 188°C)¹²⁷, uv max 216 m μ (log ϵ 4.07).

F. Attempted Acetylation of Tetradymol: Tetradymol (II) (4.8 mg) was dissolved in 1 ml pyridine to which was added 1 ml acetic anhydride.¹²⁸ The system was flushed with nitrogen, sealed and allowed to stand in the dark at room temperature overnight. When this solution was worked up in the usual manner starting material was isolated in about 50 per cent yield and no acetate could be detected by tlc.

When acetylation was attempted with acetyl chloride in n-hexane, the solution turned blue and gave an unstable non-polar compound that behaved on tlc in much the same manner as the dehydration product of tetradymol using Florisil.

Acetylation was also attempted using acetic-formic anhydride.¹²⁹ To 51 g of acetic anhydride (0.5 mole) was added 23 g of 91 per cent formic acid at 0°C. This solution was then heated to 45°C for 1 hour and used directly when cool. When 37 mg of tetradymol was added to 0.5 ml of this solution it immediately turned yellow. At the end of 2 minutes the solution was red turning lavender in a few more minutes. The reaction mixture turned green when it was quenched with aqueous NaHCO₃ after 2 hours. It was extracted with diethyl ether and spotted on Anasil S tlc and developed in diethyl ether. The spotted material turned blue as soon as it touched the adsorbent and this blue spot did not move on development. There was, however, one colorless substance in the reaction mixture that did move to R_f .8 and was made visible by spraying with sulfuric-dichromate solution. This compound was not further characterized due to low yield.

G. Dehydration of Tetradymol: Sublimed tetradymol (130 mg) was dissolved in 10 ml of benzene and refluxed over 2 g of Florisil.¹³⁰ The Florisil almost immediately turned lavender and in 20 minutes was blue-black. In 90 minutes almost all the tetradymol was gone, as evidenced by tlc, so the benzene solution was decanted and the Florisil extracted with

methanol. The methanol extract was orange leaving the Florisil a sky blue color. (This color was very stable on the Florisil as it remained with little visible change for weeks while open in the laboratory.) The benzene solution yielded a non-polar component which could be isolated by elution chromatography. However, this compound rapidly decomposed to a more polar compound while spectra were being run.

H. Attempted Aromatization: Sublimed tetradymol (85 mg) was dissolved in 15 ml of triglyme to which was then added 27 mg of sulfur. This solution was refluxed for 1 hour, then steam distilled.¹³¹ The resultant light yellow solid showed itself by tlc and ms to be identical with tetradymol (II) plus a little elemental sulfur. When another sample was refluxed under the same conditions for 12 hours much of the tetradymol disappeared leaving a reddish solution. When this solution was analyzed by tlc there was still tetradymol remaining along with some more polar compounds in very low concentration.

An attempt was also made to aromatize the tetradymol by the procedure of Minato.¹³² An intimate mixture of 50 mg of tetradymol and 200 mg of 5 per cent Pd on carbon was heated under a nitrogen atmosphere to 320°C for 5 minutes. The resultant yellow oil was chromatographed on silica gel to yield two fractions that were much less polar than tetradymol. The materials were analyzed by glc on a 15 foot, 1 per cent OV-1 liquid phase on GC-Q support in a 1/8-inch copper column to reveal at least 7 components that were not further characterized.

I. Dehydration of (XVIIIB): To 50 ml of acid washed, redistilled heptane was added 20 mg of iodine then 680 mg of (XVIIIB).¹³³ The flask was fitted with a condenser and magnetic stirrer and the solution refluxed for 2.5 hours. The resultant mixture of products was chromatographed on alumina using hexane and diethyl ether to yield four fractions with at least eight components, by glc. The overall yield was 430 mg. The major component (XXVI) was present in a yield of 50 per cent from starting material, and of isolated material 75 per cent. The compound (XXVI) was carefully vacuum distilled from a small sealed tube to a dry ice acetone trap at 0.05 mm Hg as a further purification for spectral analysis.

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