

THE INVESTIGATION OF AN ORGANIC METABOLITE
OF PENICILLIUM PINOPHILUM

by

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INTRODUCTION

One of Pasteur's statements according to Ramsbottom (15), " We are convinced that a day will come when molds will be utilized in certain industrial operations, on account of their power of destroying organic matter," is now being realized. No more mention can be made of the production by fungi of glycerol, citric acid, fats, ethyl alcohol, lactic acid, vitamins, and enzymes, or of the immense fermentation industries, especially of Penicillin, Streptomycin and other antibiotics, prepared in recent years. The scheme followed by the chief worker in this field, Raistrick (14), has been to use glucose as the sole source of carbon in a synthetic culture medium, usually a Czapek-Dox medium. In this long-continued and productive research, about a hundred substances previously unknown to science have been prepared and many of them synthesized. It may well be that some of these will be found useful in some way or other, but their present interest lies chiefly in giving a picture of what happens inside the cell.

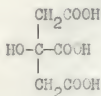
The molds are a large group of lower or true fungi. Fungi are usually classified as part of the plant kingdom and are divided into the Myxomycetes or slime molds, the Schizomycetes or bacteria, and the Eumycetes or the true fungi (5). The Penicillia molds are a subdivision of the true fungi.

The formation of acids as a result of the decomposition of organic substances has become a commonly recognized characteristic of many species of Penicillium. The usual acids are often polybasic

and some examples are oxalic (I), citric (II), gluconic (III) and gallic acids (IV). The real interest in the subject may be said



I



II



III



IV

to date from Carl Wehmer's classical observations, according to Raistrick (14), that Aspergillus niger in sugar solutions produced oxalic acid in considerable quantities, and that citric acid was a metabolite of species of Citromyces discovered in 1891. Since then a very large number of mold metabolic products of many types have been reported from different laboratories. The range of compounds (5) includes oxalic, fumaric, malic and citric acids, polyhydric alcohols, derivatives of furane, pyrone, tetrionic acid and cyclopentanone. In addition, there have been found derivatives of benzene, benzoquinone, anthraquinone, xanthone, pigments, sterols, fats, phospholipids and complex polysaccharides. Vitamins of the B group and provitamins A and D were also found amongst mold products. In the presence of arsenic, certain species such as Scopulariopsis brevicaulis have produced volatile organic arsenic compounds. Substances containing chlorine as part of the organic molecule were also synthesized from inorganic chlorides, and organic nitrogenous compounds including proteins were synthesized from inorganic nitrogen. The series of reports entitled "Studies in

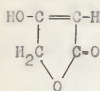
the Biochemistry of Microorganisms " (1) deals with the work which has been carried out by a group of workers under the leadership of H. Raistrick at the London School of Hygiene and Tropical Medicine since 1922.

A series of mold metabolic products, which may be regarded as derivatives of tetronic acid (V), has been described in recent years and affords an excellent example of a close structural relationship (Table 1).

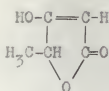
The metabolic products of Penicillium charlesii G. Smith, isolated from moldy Italian maize, were examined by Clutterbuck, Haworth, Raistrick, Smith and Stacey (6) and led to the isolation (in a total yield of over ten per cent of the glucose metabolised) of the following substances: γ -methyltetronic acid (VI), carolic acid (VII), carolinic acid (VIII), carlic acid (IX) and carlosic acid (X).

These acids on boiling with 2 N sulfuric acid were broken down quantitatively into a series of simple compounds. Under these conditions (7) γ -methyltetronic acid (VI) gave one molecule of carbon dioxide and one molecule of acetoin; carolic acid (VII) gave one molecule each of carbon dioxide, acetoin and butyrolactone; carolinic acid (VIII) gave one molecule each of carbon dioxide, acetoin and succinic acid; carlic acid (IX) (8, 9) gave two molecules of carbon dioxide and one molecule each of acetoin and butyrolactone; and carlosic acid (X) gave two molecules of carbon dioxide and one molecule each of acetoin and n-butyric acid.

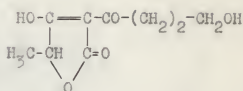
Table 1. Structurally Similar Mold Metabolites.



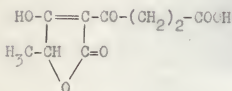
V



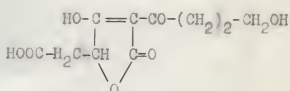
VI



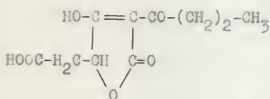
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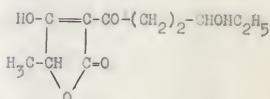
VIII



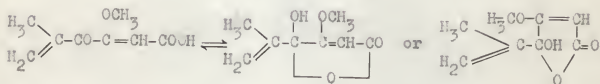
IX



X



XI

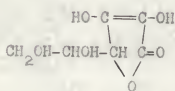


(A)

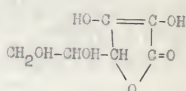
(B)

(C)

XII



XIII

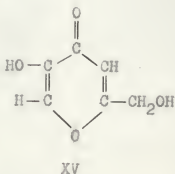


XIV

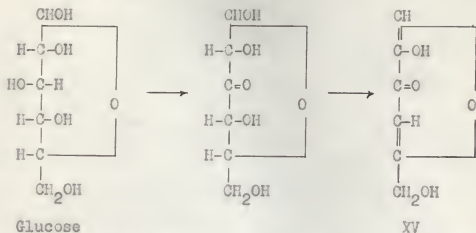
In 1936, Birkinshaw and Raistrick (1), in an investigation of the metabolic products of strains in the Penicillium terrestre Jensen series, isolated terrestric acid (XI), $C_{11}H_{14}O_4$, which was very similar in its properties to carolic acid. On acid hydrolysis, it gave one molecule each of carbon dioxide, acetoin, and the l-isomeride of n-hexanolactone.

Penicillic acid (XII), $C_8H_{10}O_4$, from Penicillium cyclopium was investigated by Birkinshaw, Oxford and Raistrick (2). Its properties are best illustrated by assigning to it the interchangeable structural formulas (A) and (B). The latter may be written as structure (C) from which penicillic acid is seen to be the β -methyl ether of γ -hydroxy- γ -isopropylidene tetronic acid. These series of metabolic products have structures related to ascorbic acid (XIV).

Kojic acid (XV) was first isolated in 1907 from cultures of Aspergillus oryzae which were used in Japan to ferment rice and produce saké, an alcoholic beverage (22). It was named by Yabuta (22, 23) in 1924 and he established the structure as 5-hydroxy-2-hydroxymethyl- γ -pyrone (XV).



Iwanoff (11) proposed that the transformation of glucose into kojic acid might be represented as follows. It was also

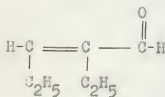


produced by members of Aspergillus and Penicillium species (11). These organisms not only produced kojic acid from mono- and polysaccharides but also from solutions containing glycerol or xylose. This has been studied in detail by Birkinshaw, Charles, Lilly and Raistrick (3), who suggested that the source of carbon supplied, whatever its nature, was anabolised into a reserve carbohydrate which was later hydrolyzed by the micro-organism into a monosaccharide which in turn gave rise to kojic acid.

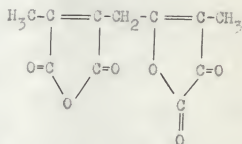
Wijkman (21) obtained a great quantity of kojic acid from different species of Aspergillus, but Penicillium glaucum in a new glass vessel under similar conditions produced a good yield of colorless crystals with a molecular weight over twice that of kojic acid. This observation confirmed the fact that small amounts of accessory mineral substances greatly influenced the growth of molds in synthetic culture media. Since the material obtained by Wijkman appeared to be similar to that under investigation here a review of that worker's results follows.

The mixture of colorless crystals was separated into gluconic acid I and II by crystallization from ethyl alcohol. Gluconic acid I analyzed as $C_{13}H_{20}O_7$, had a melting point of $202^{\circ}C$. was soluble in alcohol and was first thought to be optically inactive. It contained no methoxyl group, gave no color with ferric chloride solution and from its behavior on titration appeared to contain one or more lactone groups. It was not esterified with methyl alcohol and hydrochloric acid. With diazomethane, nitrogen was evolved and a non-crystalline product was obtained. The acetyl and benzoyl derivatives were prepared by Wijkman. Reduction of gluconic acid I with zinc and glacial acetic acid gave the compound $C_{13}H_{22}O_7$. Pyrolysis (18) in a nitrogen atmosphere at $215^{\circ}C$. gave the unsaturated aldehyde $C_7H_{12}O$ having a boiling point of $146^{\circ}C$. at 720 mm, which absorbed two molecules of bromine and two molecules of hydrogen. By oxidation, this aldehyde gave propionic acid. The nonvolatile substance after pyrolysis analyzed as $C_{11}H_{18}O_6$ and had a melting point of $174^{\circ}C$. Gluconic acid II $C_{13}H_{20}O_6$, m. p. $186^{\circ}C$. could be distilled successfully in a high vacuum and titrated as a tetra-basic acid. Later, Sutter and Wijkman (18, 19) reported that gluconic acid I was optically active with a specific rotation of $[\alpha]_D^{20} = -42.6^{\circ}$. They also investigated again the easily oxidized distillate of pyrolysis by using the newly distilled pure substance. The distillate was obtained when gluconic acid I was heated to $240^{\circ}C$. The formula was deduced from the results of ozonisation to be

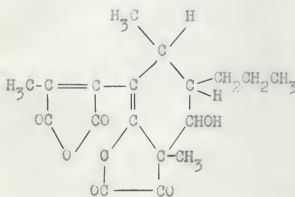
α,β -diethylacrolein (XVI). The constitution was confirmed by the ultra violet absorption spectrum, which indicated a double-bond conjugated with a carbonyl group. It was synthesized and proved to be identical with the liquid distillate. In regard to the non-volatile fraction of pyrolysis, Kraft and Porsch (13) have postulated the structure (XVII) and also mentioned that the structure of glauconic acid I might be as follows (XVIII).



XVI



XVII



XVIII

Yuill (24) obtained an acid metabolite by using a Penicillium parasitic upon Aspergillus niger in a sucrose-containing acidified nutrient solution. This substance appeared to be identical with glauconic acid I of Wijkman. An acetyl derivative was also prepared.

Glauconic acid II was not recovered by Yuill, but it might have been present in the mother liquor as he mentioned.

The organic compound under investigation in this laboratory was a crystalline metabolite of Penicillium pinophilum in a nutrient solution containing glucose. Penicillium pinophilum has been reported by Thom (20) as a cause of discoloration in pine wood. Organisms indistinguishable from this species have been isolated from various sources. It is available from the Thom and Church collection according to the Catalogue of Cultures (4). The investigation reported here deals with preliminary efforts to elucidate the structure of the metabolite, which in many respects resembles glauconic acid I of Wijkman. It may be related to glauconic acid I as will be demonstrated by the work we have done. We suggest that this compound be named pinophilic acid.

EXPERIMENTAL

Purification of the Pinophilic Acid

Crude crystals of pinophilic acid were very easily soluble in methyl ethyl ketone, acetone, ethyl alcohol and glacial acetic acid, were slightly soluble in ether and benzene and were insoluble in water.

A 20 gm sample of the crude product was dissolved in 50 ml of hot acetone. The brown solution was boiled on a steam bath and was filtered while hot. Water was added to the clear filtrate

until it was cloudy and the solution was allowed to cool slowly. The large brown crystals which separated out were removed by filtration and were dried. This material had a melting point of 185-189°C. These brown crystals were dissolved in 40 ml of ethyl alcohol, the solution was heated and charcoal was added. The charcoal was removed by filtration of the hot solution and the filtrate was allowed to cool. In some cases water was added to hasten crystallization.

An alternative method of purification was as follows. A sample of the product was dissolved in an excess of five per cent sodium hydroxide solution. The dark brown solution was decolorized by two charcoal treatments while hot, and the colorless alkaline solution was acidified with hydrochloric acid until it was acidic toward Congo red paper. The solution became turbid and the white precipitate was removed by filtration after twelve hours. This product had a melting point of 190-192°C. After two further recrystallizations from alcohol the colorless crystals melted at 197.5-198°C. (corrected). Samples of pinophilic acid so purified were used in the tests described in this thesis.

Physical Properties

Melting Point. The pure pinophilic acid had a melting point of 197.5-198°C. The melting points were determined in capillary tubes immersed in a rapidly stirred and heated liquid. Corrections for emergent stem were made in all cases.

Optical Activity. The specific rotation was measured by means of a standard polarimeter. The data were obtained for solutions of methyl ethyl ketone, acetone and ethyl alcohol and the compound was found to be dextro-rotatory. A 2.54 gm sample of the pure crystals dissolved in 25 ml of methyl ethyl ketone gave an observed reading of +4.08 and a specific rotation $[\alpha]_D^{20} = +40.2^\circ$. A 1.00 gm sample in acetone gave an observed reading of +1.57 and a specific rotation $[\alpha]_D^{20} = +41.8^\circ$. A 1.12 gm sample in alcohol gave an observed reading of +1.80 and a specific rotation $[\alpha]_D^{20} = +40.2^\circ$.

Molecular Weight. The molecular weight was determined by the method of melting point depression. Camphor and β -naphthol were employed as solvents. A molecular weight range of 240 to 400 was obtained, and individual runs are tabulated in Table 2. The camphor was purified by sublimation and gave a product with a m. p. of 177°C . The molal freezing point depression of camphor was found to be 38.7 when checked with benzhydrol and biphenyl. The molal freezing point depression of β -naphthol, m. p. 124.4°C ., was found to be 11.9 when checked with benzophenone. A 0.05 gm sample was weighed carefully and was transferred into a test tube, to which twenty times the weight of solvent was added. The test tube was stoppered and heated in the hot liquid paraffin or with a free flame. When the mixture melted, it was shaken frequently in order to mix thoroughly the two components. After cooling, the solid substance was removed from the test tube and put on a piece of clay plate. It was powdered and mixed thoroughly, then several

samples were taken from each mixture and the melting points were determined. The determination of melting point was carried out in a capillary tube by the ordinary method and the temperature was recorded when the mixture just became entirely liquid. The values given by β -naphthol were thought to be more reliable since camphor solutions were gummy. This may have been caused by the decomposition of pinophilic acid at the high temperatures.

Table 2. Molecular Weight Measurements of Pinophilic Acid.

Weight of solvent : (gram)	Weight of sample : (gram)	M. P. depression : (average value)	Mol. wt. ΔT^*
Camphor			
0.5328	0.0499	10.4	364
0.4990	0.0508	10.2	407
β -naphthol			
0.5095	0.0505	3.8	293
0.5009	0.0503	4.6	237
0.5018	0.0533	3.2	341
0.4994	0.0508	4.0	297
0.5170	0.0491	4.0	282
0.5008	0.0511	4.5	266
0.5047	0.0519	5.1	236
0.4972	0.0569	5.1	262
Average			303.5

* M. P. depression, ΔT , was in degree centigrade, obtained from the difference of the m.p. of the solvent and the m.p. of the mixture.

Freezing point depression in acetic acid failed to give satisfactory results.

Carbon Hydrogen Analysis. The pure pinophilic acid used for quantitative analysis was dried over phosphorous pentoxide in an Abderhalden vacuum drying apparatus. Macro- and micro-combustion methods were employed and the results are summarized in Table 3. Table 3.* Carbon and Hydrogen Analysis of Pinophilic Acid.

Weight of sample (gram)	Carbon	Hydrogen	Oxygen **
	Percent		
0.1065	62.84	6.07	31.09
0.2009	62.59	6.00	31.41
0.1183	62.63	5.99	31.33
0.1066	62.34	5.90	31.76
0.008548	61.50	6.12	32.38
0.005003	61.70	6.04	32.36
0.004839	59.96	5.69	34.35
0.004609	59.98	5.72	34.30
Average	61.69	5.69	32.62

* The first six samples were purified by recrystallization from alcohol and acetone and had a m.p. of 188-189°C. The last two samples were purified by reprecipitation with acid from alkaline solution and had a m.p. of 197.5-198°C.

** The percentage of oxygen was obtained by difference.

Chemical Properties

Preliminary Examination. When heated on the porcelain lid of crucible, the crystals first melted to a yellowish brown liquid and then burned with a non-luminous flame. An odor similar to that of burning sugar was noted. The final carbon stain was oxidized and there was no residue.

Elementary Analysis. Duplicate sodium fusions were carried out according to the method of Shriner and Fuson (17). Nitrogen, sulfur and halogens were found to be absent. Magnesium and anhydrous potassium carbonate fusion gave the same results. This compound therefore contains carbon, hydrogen and oxygen only. The absence of nitrogen was checked twice by sodium fusions in which sucrose was mixed with powdered pinophilic acid in a relatively large amount. Similar results were obtained.

Solubility Tests. This compound was slightly soluble in water and gave a solution acidic to litmus. It was soluble in five percent sodium hydroxide solution and insoluble in five percent hydrochloric acid and in 85 percent phosphoric acid. In five percent sodium bicarbonate solution no carbon dioxide gas evolved visibly, however, the acid dissolved slowly after long standing. In concentrated sulfuric acid the color changed from yellow to brown as it dissolved. It was only slightly soluble in ether. Based upon these solubility tests the crystals were con-

cluded to be an organic acid, either of phenolic or enolic nature or one containing easily hydrolyzed lactone groups. The insolubility of pinophilic acid in water may account for the time required for solution in sodium bicarbonate.

Classification Reagents. Classification tests were applied to the powdered pinophilic acid. Cold neutral potassium permanganate solution was reduced slowly by a water solution of this acid. In alcoholic solution the reaction was faster than a blank test with alcohol and complete decolorization eventually resulted. It did not decolorize bromine in carbon tetrachloride nor bromine water. There was no color developed in alcohol with dilute ferric chloride solution, thus a phenolic or enolic system may not be present. With both phenylhydrazine and 2,4-dinitrophenylhydrazine reagents, the alcoholic solution yielded precipitates, which indicated the presence of ketonic or aldehydic carbonyl groups. The negative results shown by the reaction with ammoniacal silver nitrate and Fehling's solution excluded the presence of an aldehyde group or other easily oxidizable group. The acid was recovered unchanged after refluxing in 20 percent sodium hydroxide which indicated stability toward alkali. It also was unaffected by boiling with 20 percent sulfuric acid.

Neutral Equivalent Weight. The titration of this acid was carried out in two different ways. Direct titration with dilute alkali gave an neutral equivalent weight of 144 (average). In alcohol solution alkali was consumed gradually and since there was

no sharp end point shown by the phenolphthalein indicator, readings were recorded when the pink color given by the indicator persisted one minute. The results are summarized in Table 4.

Table 4. Neutral Equivalent weight Measurements of Pinophilic Acid (Direct Titration).

Weight of sample (gram) :	Volume of 0.1038 N NaOH :	N. E.
0.1110	7.80	137
0.1196	8.00	144
0.0553	3.64	146
0.0343	2.20	150
Average		144

This experiment was repeated by using about 0.2 gm of sample dissolved in 50 ml alcohol and titrating with dilute sodium hydroxide. A blank titration of 50 ml alcohol required 0.18 ml of sodium hydroxide solution and this was accounted for in the calculation. The results are summarized in Table 5.

Table 5. Neutral Equivalent Weight Measurements of Pinophilic Acid (Direct Titration).

Weight of sample (gram) :	Volume of 0.0956 N NaOH :	N. E.
0.2099	15.95	133
0.2007	13.71	148
0.1696	11.21	153
Average		145

The second method employed appeared to give what may be termed saponification equivalents. In general, the samples were added to excess standard alkali and back-titrated to the end point of phenolphthalein. Some samples were kept at room temperature for 12 hours and some were heated before titration. The results are summarized in Table 6.

Table 6. Neutral Equivalent Weight Measurements of Pinophilic Acid (Back Titration).

weight of sample: (gram)	Treatment before titration with acid:	Volume of 0.1038N NaOH:	Volume of 0.1034N HCl:	N.E.
1. 0.1203	12 hrs. at 20°C.	20	6.12	83.3
0.1284	12 hrs, at 20°C.	20	5.45	84.9
2. 0.1062	1 hour at 100°C.	20	7.66	82.7
0.1113	1 hour at 100°C.	20	6.35	81.4
3. 0.1183	Immediate titration	25	11.26	82.7
0.1488	Immediate titration	25	8.02	84.2
Average				83.2

Since there appeared to be no difference in neutral equivalent weight when the acid was titrated immediately after addition of excess base or by heating and standing with alkali, this experiment was repeated with freshly prepared alkaline and acid solutions. The samples were dissolved in an excess amount of alkali and back titrated immediately with acid to the phenolphthalein end point.

The results are summarized in Table 7.

Table 7. Neutral Equivalent Weight Measurements of Pinophilic Acid (Back Titration).

Weight of sample (gram)	Volume of : 0.0996N NaOH :	Volume of : 0.1021N HCl :	N. E.
0.155	20	2.18	89.4
0.114	20	6.41	84.4
0.154	25	5.50	79.9
0.225	32	5.63	86.1
0.230	30	2.42	85.9
0.155	30	10.51	80.9
0.184	31.17	9.10	84.6
Average			84.4

Attempts to detect a multiplicity of acid functions by potentiometric titration failed to give curves with significant inflection points in the range of the neutralization points.

Pyrolysis Products. A 10 gm sample of purified acid was heated in a small flask by means of a Glascol heater under a pressure of 0.1-0.2 mm mercury at 175°C. for two and one half hours. Two ml of colorless liquid condensed in the dry-ice cooled trap. Yellow crystals formed on the walls of the distilling flask above the residue which, after cooling, was a reddish-brown brittle glassy substance. The following information was obtained about (1) the distillate and (2) the yellow crystals.

(1) Distillate. The b. p. of 138.3-140.3° C. was determined on a micro-scale by the Smich method (16). The liquid was not soluble in water, dilute alkali and dilute acid, and was soluble in 85 percent phosphoric acid. In concentrated sulfuric acid there was color formation and on dilution a brown oily layer separated. This liquid substance gave positive tests with phenylhydrazine and 2,4-dinitrophenylhydrazine. Baeyer's test showed quick decolorization of potassium permanganate solution but the result with bromine in carbon tetrachloride was uncertain. It dissolved in a hydrochloric solution of zinc chloride with brown color but without phase separation. With Tollen's reagent, it gave a silver precipitate after one hour, however, blanks were positive with both ethyl alcohol and acetone. The bisulfite test and sodium test were both negative. From this evidence the distillate seemed to be an unsaturated aldehyde.

Two derivatives were prepared by using one drop of the distillate for each. One drop of the aldehyde was treated with 1 ml of water solution containing 0.1 gm semicarbazide hydrochloride and 0.15 gm sodium acetate. The mixture was warmed in a hot water bath, then chilled in ice water and the walls of the test tube were scratched vigorously by means of a glass rod in order to induce crystallization. The colorless crystalline precipitate was collected, washed with water until free from reagent and then recrystallized from dilute alcohol solution. The fine crystals were air dried and the melting point was found to be 183-185° C. Carbon

hydrogen micro-analyses showed carbon 55.48 and 55.88 percent and hydrogen 8.47 and 9.00 percent.

One drop of the unsaturated aldehyde was used to react with 2,4-dinitrophenylhydrazine reagent. The latter was freshly prepared by adding 2 ml concentrated sulfuric acid and 3 ml of water to 0.4 gm of solid 2,4-dinitrophenylhydrazine with stirring to form a clear solution. Ten ml of 95 percent ethyl alcohol was added to this mixture. When the aldehyde was added a crystalline orange red precipitate was obtained. The solid was recrystallized first from ethyl acetate and then from ethyl alcohol. The 2,4-dinitrophenylhydrazone was a red crystalline solid with a melting point of 172-173°C. Carbon hydrogen micro-analyses gave carbon 53.08 and 52.93 and hydrogen 6.14 and 5.28 percent.

(2) Condensed crystals. The solid which had formed on the walls of the distilling flask was scratched out and found to be easily soluble in ethyl alcohol, acetone, ether, acetic acid and chloroform, and less soluble in benzene and pyridine, in which it developed a blue color. It was readily soluble in 5 percent sodium hydroxide solution, slightly soluble in 5 percent sodium bicarbonate solution, and insoluble in 5 percent hydrochloric acid, water, 85 percent phosphoric acid, carbon tetrachloride and petroleum ether. In concentrated sulfuric acid, the solid dissolved without color change. These crystals were freed from the yellow gummy residue by washing with 20 percent acetone in petroleum ether and recrystallized from chloroform as colorless needle-shaped crystals with a m. p. of 169.1-170.6°C. The melting point of the mixture of this compound

and the original acid before distillation was 145-155° C., evidence that this was a new compound.

The neutralization equivalent of this acid obtained from pyrolysis was determined. Duplicate samples of about 50 mg each were dissolved in 10 ml of alcohol and 4 drops of phenolphthalein were added as an indicator. The solutions were then titrated directly with standard sodium hydroxide solution and the results are summarized in Table 8. Four drops of indicator in 10 ml alcohol

Table 8. Neutral Equivalent Weight Measurements of Pyrolysis Acid.

Weight of sample (gram)	Volume of 0.0107 ^N NaOH	N. E.
0.0497	63.90	72.8
0.0499	64.71	72.5
Average		72.7

consumed 0.34 ml of alkali solution and this consumption was accounted for in the calculation of neutral equivalent weight. The acid was recovered by distillation of the solvent, acidification of the aqueous residue and extraction with chloroform. This acid did not decolorize bromine water and potassium permanganate solution and ferric chloride gave no color change. Carbon hydrogen analysis gave Carbon 57.30 percent and hydrogen 3.45 percent.

Derivatives. Several derivatives of pinophilic acid were prepared but some of them failed to give definite melting points.

(1) 2,4-dinitrophenylhydrazone. A 0.5 gm sample of pinophilic acid was dissolved in 10 ml alcohol, water was added dropwise

until it became cloudy and then two more drops of alcohol were added to give a clear solution. Freshly prepared 2,4-dinitrophenylhydrazine reagent was introduced and a yellow precipitate slowly formed. It was recrystallized from alcohol and water and gave a yellow solid with a m. p. of 163-165° C. Carbon hydrogen analysis gave carbon 43.61 percent and hydrogen 4.13 percent.

(2) Oxime. One gm of hydroxylamine hydrochloride was dissolved in 5 ml of water and 4 ml of 10 percent sodium hydroxide solution and 0.4 gm of the acid were added to it. Sufficient ethyl alcohol was added to the mixture to give a clear solution. The mixture was warmed on a steam cone for 10 minutes and then was cooled in an ice bath. Water was added dropwise and a white precipitate formed. After recrystallization from water the long colorless needle-like crystals had a m. p. of 190-193° C. Carbon hydrogen analysis gave carbon 41.65 percent and hydrogen 6.39 percent.

(3) Semicarbazone. One gram of the acid was dissolved in 100 ml of ethyl alcohol. Water was added until the solution was fairly turbid, and then the turbidity was removed with a few drops of ethyl alcohol. One gram of semicarbazide hydrochloride and 1.5 gm of sodium acetate were added. The mixture was vigorously shaken, and the flask was placed in a beaker of boiling water and the system was allowed to cool to room temperature. A small quantity of crystals separated out after a long period. They were recrystallized from 30 percent ethyl alcohol and the resulting substance had a m.p. of 225-230° C.

Attempts to prepare the phenylhydrazone, p-nitrophenylhydrazone

and acid amide derivatives were unsuccessful.

Other Tests. Acetyl derivative was prepared from acetic anhydride. A one gram sample was dissolved in 10 ml of acetic acid and a few drops of concentrated sulfuric acid were added. This mixture was heated on a steam cone for several minutes and the color became dark brown. After cooling it was poured into 500 ml of water and a white gummy precipitate formed which on standing became a brittle solid. Two recrystallizations were made from acetic acid and a white powder resulted which melted at 57-60°C.

The preparation of an acid anilide was attempted with a 0.4 gm sample of pinophilic acid, one ml of aniline and 0.3 ml of concentrated hydrochloric acid. The mixture was refluxed for one hour at 150-175°C. in an oil bath. After cooling, the product was washed with 15 ml of hydrochloric acid and 15 ml of cold water. The residue was boiled with 40 ml of 50 percent alcohol and filtered. The filtrate was chilled in ice and a pale yellow precipitate was obtained, m. p. 100-110°C. Neither alcohol nor acetone were suitable for recrystallization of this product. It was finally recrystallized twice from mixture of cold ether-petroleum ether. The resulting product melted at 114-115°C.

Saponification was carried out twice with different concentrations of alkali and the original acid was recovered in both cases. A 0.5 gm sample was dissolved in 20 ml of 10 percent sodium hydroxide solution and heated on a steam cone for 3 hours under a reflux condenser. When the solution was acidified a white solid precipitated

and had a m. p. of 186-187°C. and mixed m. p. 186-188°C. with the original acid. Another 1.7 gm sample was dissolved in 20 ml of 20 percent sodium hydroxide solution and refluxed over a free flame for seven hours. After cooling it was acidified with dilute hydrochloric acid to the Congo red end point. On standing a crystalline white precipitate was obtained with a m. p. of 193-195°C. and mixed m. p. of 192-195°C. with authentic pinophilic acid.

A reduction was attempted by dissolving a 3.4 gm sample of pinophilic acid in 30 ml of glacial acetic acid containing 3.2 gm of zinc dust. The mixture was heated on a steam cone under a reflux condenser. Another 2 gm portion of zinc dust was added and the flask was shaken vigorously when the former portion agglomerated. At the same time, a small amount of water and ethyl alcohol was added to start the reaction. After sixteen hours, the zinc was filtered off and the filtrate was distilled at ordinary pressure at 98-105°C. to reduce the volume of solution. Water was added to the solution and a white gummy substance precipitated. Overnight, it became solid and when isolated it was found to be slightly soluble in ether and benzene and easily soluble in acetone, from which it was recrystallized. Recrystallization from acidified alcohol yielded colorless crystals, m. p. 103-105°C. This acid gave a neutral equivalent weight of 157. Ether and benzene extracts of the filtrate yielded colored residue but recrystallization of this was not successful. No analysis was made because of insufficient sample.

Catalytic reduction of pinophilic acid with Raney nickel in

alkaline solution was attempted but no significant amount of hydrogen was absorbed and the original substance was recovered unchanged.

A sodium amalgam reduction was tried also. A 2 gm sample was dissolved in dilute sodium hydroxide solution (100 ml solution containing 1.5 gm of sodium hydroxide) until it was just alkaline toward phenolphthalein. This was added to freshly prepared sodium amalgam (10) (390 gm 2 percent sodium in mercury) and the mixture was mechanically stirred for one hour. Finally it was heated on a steam cone for thirty six hours. The solution was decanted and neutralized with dilute hydrochloric acid. The solid obtained had a m. p. of 168°C . and a mixed m. p. with the starting material of $156-158^{\circ}\text{C}$. However, this product could not be purified further.

Alkaline potassium permanganate solution was used to oxidize the acid. The resulting product which was obtained by extraction with benzene and ether, had a m. p. of range $163-175^{\circ}\text{C}$. but no pure crystalline substance was obtained.

When pinophilic acid was refluxed with 20 percent sulfuric acid for several hours, the crystals failed to dissolve and the original acid was recovered unchanged. Pinophilic acid dissolved in cold concentrated sulfuric acid but when this solution was poured into ice and water, only a very small amount of precipitate was obtained. Neutralization and extraction of the filtrate gave only a small amount of gummy material.

Esterification with methyl alcohol and acid also failed to

yield a pure derivative. Methyl alcohol and dimethyl sulfate apparently reacted with pinophilic acid but a gummy non-purifiable substance with indefinite melting point always resulted. After purifications a small amount of original material was recovered. With diazomethane, nitrogen gas was evolved, the ether solution changed to an orange color on standing and a small amount of precipitate formed. The solvent was removed under reduced pressure and the yellow colored residue was purified by recrystallization from methyl alcohol to give a yellow powder with m. p. around 200°C. Because of the small quantity and doubtful purity no analysis was made.

DISCUSSION AND CONCLUSION

The pinophilic acid was obtained in pure state by two methods. Recrystallization from alcohol gave a product with m. p. of 185-189°C. Perhaps a preferable method was that in which the crystals were dissolved in alkali and reprecipitated by addition of acid. The product obtained by this method had a m. p. of 197.5-198°C. Wijkman (21) and Yuill (24) both reported that glauconic acid I ($C_{18}H_{20}O_7$) had a m. p. of 202°C.

The optical activity was about the same magnitude but opposite in sign to that reported by Sutter and Wijkman (19). They reported $[\alpha]_D^{20} = -42.6$. This is perhaps the greatest difference found between glauconic acid I and the pinophilic acid under investigation here. There is no doubt as to the dextrorotatory nature of our product.

The molecular weight determinations gave a wide range of values, two extremes being 236 and 407. The Rast method of freezing point depression was used with two different solvents, camphor and β -naphthol. The former proved to be unsatisfactory due to the gummy nature of the mixtures. Later work on pyrolysis of pinophilic acid suggested that the mixing of sample with camphor at its melting point was too high a temperature and partial decomposition of the pinophilic acid may have occurred. Because β -naphthol melts about 50 degrees lower than does camphor, β -naphthol was used as a solvent in most of the determinations. The results of two camphor runs and eight β -naphthol runs are summarized in Table 2. The average of the ten determinations was 303, considerably below the theoretical molecular weight (343) of $C_{18}H_{20}O_7$, glauconic acid I. Attempts to obtain molecular weight by freezing point depression of low melting substances, such as benzene and acetic acid, were unsuccessful, but probably should be reexamined in the future.

Combustion analysis methods were used to determine percentage composition of pinophilic acid. Both macro- and micro-analytical procedures were followed and the results are summarized in Table 3. A surprisingly wide range of values was obtained, which may be accounted for partially by the fact that samples of different purity were used. The carbon and hydrogen percentages calculated from the macro-analysis were of the same magnitude as those obtained by Wijkman (21) for glauconic acid I. The theoretical carbon and hydrogen percentages for $C_{18}H_{20}O_7$ are 62.04 and 5.79, respectively.

The average values for pinophilic acid were carbon, 61.69 percent and hydrogen, 5.69 percent, both lower than theory. However, our values are not completely out of line with the possibility that pinophilic acid is closely related to glauconic acid I. Assuming 348 was the molecular weight of pinophilic acid and using average values for carbon and hydrogen content (Table 3) the carbon, hydrogen and oxygen ratios in pinophilic acid were calculated and gave $C_{17.9}H_{19.8}O_{7.1}$ as the result. The assumption of close similarities between pinophilic acid and glauconic acid I is supported further by the qualitative and quantitative data described below.

A preliminary examination of chemical properties was carried out. No inorganic material was left as residue upon ignition of pinophilic acid. Careful qualitative tests for nitrogen, sulfur and halogens were all negative. The solubility of the acid in various solvents was determined. Insolubility in water and dilute hydrochloric acid and solubility in dilute sodium hydroxide indicated that this compound was an acid. Slight solubility in ether indicated a polyfunctional compound and the slow rate of solution in sodium bicarbonate indicated that lactone or anhydride linkages may be present in pinophilic acid.

Certain of the classification tests listed by Shriner and Fuson (17) were carried out with pinophilic acid. It was slowly oxidized by neutral potassium permanganate but was unaffected by Fehling's solution and ammoniacal silver nitrate. This indicated olefinic unsaturation and was evidence against the presence of an easily oxidized phenol or aldehyde system within the molecule. Bromine

in carbon tetrachloride or bromine water was not decolorized by the compound which indicated that the unsaturated bond (or bonds) was sterically protected or was part of a stable conjugated system. No phenolic-enolic color reaction was given with ferric chloride solution which indicated the absence of such a system in the molecule. The presence of a carbonyl group (or groups) was indicated by the formation of a phenylhydrazone and a 2,4-dinitrophenylhydrazone. Boiling solutions of pinophilic acid in 20 percent alkali or 20 percent sulfuric acid failed to alter the compound and it was recovered unchanged in each case.

It was apparent at this time that the compound was polyfunctional and was an acid, therefore neutral equivalent determinations were made. The end point was not definite when samples of the compound were titrated directly with standardized sodium hydroxide solution. Arbitrarily, the end point was taken as that point at which the phenolphthalein color persisted for one minute. Inconsistent results were obtained by this method and were thought to support further the idea that lactone or anhydride linkages were present. However, consistent results were obtained if the samples were added to excess standard alkali and the solutions were back-titrated with standard acid. Immediate back-titration gave same results as in these cases when the alkaline solution stood overnight, or was heated for a period of time. The values obtained by this method are summarized in Tables 6 and 7 and gave an average neutral equivalent weight of 83-84. Pinophilic acid as a tetra-basic acid would then

have a molecular weight of 332 to 336 which is close to the 348 calculated for glauconic acid I. Furthermore, the neutral equivalent weight values are quite similar in magnitude to those obtained by Wijkman (21). Potentiometric titrations were resorted to in order to attempt the detection of several plateaus corresponding to the polybasic nature of the compound, but the results were inconclusive. Slope changes were too gradual to permit accurate determination of the neutral point.

As in the case of glauconic acid I as reported by Wijkman (21, 18), pinophilic acid underwent pyrolytic decomposition. When held at a temperature about its melting point, a liquid volatile fraction, a crystalline sublimate and an orange residue were obtained. The boiling point of the liquid was 138-140 C. and compared favorably with that reported by Sutter and Wijkman (18), which was 145° C. for the liquid they showed to be α,β -diethylacrolein. The liquid we obtained gave positive tests with phenylhydrazine and 2,4-dinitrophenylhydrazine which indicated the presence of a carbonyl group. A Baeyer test for unsaturation was positive as was the test with ammoniacal silver nitrate, which indicated olefinic unsaturation and the presence of an aldehyde or other easily oxidizable group. The Tollen's test (ammoniacal silver nitrate) was doubtful since acetone and alcohol both gave similar results, but the latter may have been due to aldehydic impurities in the two solvents mentioned.

The semicarbazone of the liquid distillate had a m. p. of 183-185° C., which was similar to the m. p. of 183° C. reported by Wijkman (21) for the semicarbazone of α,β -diethylacrolein. Carbon and

hydrogen percentages calculated for the semicarbazone were carbon, 56.76 and hydrogen, 8.94. The average values obtained for the semicarbazone prepared in this laboratory were 55.67 percent and 8.73 percent for carbon and hydrogen respectively. The carbon value is more than one percent lower than theory for the semicarbazone of the compound $C_7H_{12}O$. Due to the small quantities of liquid distillate obtained, which precluded further purification, it was thought that the semicarbazone was probably impure.

The 2,4-dinitrophenylhydrazone of the liquid pyrolysate was prepared and had a m. p. of $173-175^{\circ}C.$, and Wijkman (21) had obtained a derivative with a m. p. of $173^{\circ}C.$ Carbon and hydrogen percentages calculated for the 2,4-dinitrophenylhydrazone of α, β -diethylacrolein were carbon, 53.40 and hydrogen 5.52. The average values obtained for the 2,4-dinitrophenylhydrazone we obtained were 53.00 percent and 5.71 percent for carbon and hydrogen, respectively. These values were closer to theory than those obtained for the semicarbazone derivative. The similar melting point values of our derivatives and those reported by Wijkman strongly indicated that the liquid distillate was identical with that obtained by Wijkman, although the analytical values were not good.

The crystalline portion of the residue, when recrystallized, had a m. p. of $169.1-170.6^{\circ}C.$, which compared favorably with the m. p. of $170^{\circ}C.$, reported by Wijkman (21) for the similar pyrolysate obtained from glauconic acid I. The qualitative tests showed this material to be an acid and the neutral equivalent weight values averaged 72.7, somewhat higher than the value 59 reported by Wijkman

(21) for her product. However, the titration in this laboratory was conducted at room temperature and Wijkman conducted her titration at elevated temperature. The small amount of product precluded further investigation of this acid insofar as neutral equivalent weight was concerned. Carbon and hydrogen percentages calculated for $C_{11}H_8O_6$ were 55.92 and 3.42 respectively. The average values obtained for the crystalline pyrolysate in this laboratory was 57.30 and 3.45, respectively, a considerable deviation from what Wijkman obtained for carbon and hydrogen percentages of her product.

A number of derivatives of pinophilic acid were prepared. The 2,4-dinitrophenylhydrazone had a m. p. of 163-165°C. No corresponding derivative was prepared by the German workers. Analysis gave values for carbon and hydrogen of 43.61 and 4.13 percent, respectively. The derivative was difficult to purify and no interpretation of this analytical data could be made. The carbon percentage was far too low to correspond to a simple monophenylhydrazone of pinophilic acid (assuming it to be $C_{18}H_{20}O_7$).

An oxime was prepared and had a m. p. of 190-193°C., but again no corresponding derivative of glauconic acid was prepared by the German workers. Analysis gave values for carbon and hydrogen of 41.65 and 6.89 percent, respectively. The carbon content was surprisingly low and attempts to interpret these results were unsuccessful.

The semicarbazone of pinophilic acid was prepared in very

small yield and had no sharp melting point. No analysis of this derivative was attempted.

A product was obtained from an attempt to prepare an acetyl derivative. This product had a m. p. of about $57-60^{\circ}\text{C}$., whereas Wijkman (21) reported a m. p. of 175°C . for the acetyl derivative of glauconic acid I, and Yuill reported the same result. An anilide of pinophilic acid was prepared which had a m. p. of $114-115^{\circ}\text{C}$. This product after repeated purification was in powder form instead of fine crystals. There was no corresponding derivative of glauconic acid I reported. The purity of both of these derivatives was doubtful hence carbon and hydrogen analysis of these two derivatives was not attempted.

Saponification, a usual method of degradation, was applied to pinophilic acid, but it was not affected even in a twenty percent sodium hydroxide solution heated under a reflux condenser for a long period. The original material was recovered unchanged. This characteristic was similar to the stability of glauconic acid I toward alkali as Kraft reported (13).

The small yield of reduction product of pinophilic acid obtained by the reduction with glacial acetic acid and zinc dust had a m. p. $103-105^{\circ}\text{C}$., and a neutral equivalent weight of 157. For glauconic acid I, a m. p. of 235°C . and a neutral equivalent weight of 113 for the reduction product was reported by Wijkman (21). Our experiment was considered unsuccessful and probably should be repeated in the future.

Catalytic reduction of pinophilic acid with Raney nickel in alkaline solution failed to show absorption of hydrogen and the original material was recovered quantitatively. However, catalytic reduction with a platinum catalyst was carried out on glauconic acid I by Wijkman (21).

Sodium amalgam was used in the attempt to carry out a general reduction of lactone to alcohol and small amount of solid product was obtained from pinophilic acid. It had a m. p. of 164-169°C. and the sizable depression of melting point of the mixture with the original substance showed that a new compound was formed. However, this product could not be purified sufficiently and was of small quantity.

Oxidation of pinophilic acid with alkaline potassium permanganate resulted in a product which was obtained by extraction with benzene and ether, but no crystalline substance was obtained. There was also no corresponding reaction product reported by the German workers for glauconic acid I.

Pinophilic acid was found to be unaffected by hot 20 percent sulfuric acid, a further fact which demonstrated the stability of this compound.

Attempted preparations of methyl ester of pinophilic acid were made by means of methyl alcohol and hydrochloric acid, dimethyl sulfate and diazomethane. Pinophilic acid did not react with methyl alcohol and the original substance was recovered. Wijkman reported the same behavior of glauconic acid I (21). With dimethyl sulfate a gummy product resulted which had a large melting point range,

lower than pure pinophilic acid. Further purification failed to give a compound of definite melting point. It was assumed to be a mixture. The treatment of diazomethane with pinophilic acid yielded an orange colored product with the evolution of nitrogen. After purification the m. p. of this product was around 200°C. This product was not clearly definite, but was similar to the dimethyl glauconate reported by Kraft (13) in later work on glauconic acid I. The reported m. p. of dimethyl glauconate was around 200°C. also and not sharp.

In conclusion, this thesis reports a preliminary investigation of the structure of pinophilic acid. Much more work is necessary. Initially, this further work should be directed toward obtaining acetyl and benzoyl derivatives, reinvestigation of molecular weights, additional studies of the pyrolysis products and application of more degradation methods to the compound. Many similarities exist between pinophilic acid and the reported glauconic acid I but a few differences are also apparent. It would be of interest also to examine this compound from a physiological point of view.

SUMMARY

The organic compound under investigation was a colorless crystalline metabolite of the mold Penicillium pinophilum, thus named pinophilic acid. This thesis reported here deals with preliminary efforts to elucidate the structure of the metabolite. In many phases pinophilic acid resembled glauconic acid I of Wijkman, which was reported by the German workers as a tetra-basic acid containing lactone or anhydride groups. On the other hand, the significant difference between these two acids was that pinophilic acid was dextrorotatory while glauconic acid was levorotatory. Pyrolysis of pinophilic acid has been carried out and the decomposition products, an aldehyde and an acid, seemed to be identical with those pyrolysates of glauconic acid I. Some reactions were studied and derivatives were prepared in this laboratory, however, no definite structural formula of pinophilic acid could be postulated by the work carried out so far.

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