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MICROBIOLOGICAL OXIDATION OF SUBSTITUTED NAPHTHALENES

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This invention relates to the microbiological oxidation of alkyl napthalenes to form the corresponding monoacids. More particularly, this invention relates to novel processes for preparing alkyl naphthalene monocarboxy acid in high yield by the microbiological oxidation of one of the alkyl groups of a dialkyl naphthalene substrate.

While the use of microbiological means in the conversion of complex organic compounds is well-known, such methods have rarely been successful in converting hydrocarbons to useful compounds. Most often, microbiological oxidation of hydrocarbons has resulted in the conversion of these hydrocarbons to carbon dioxide and water, so that the effective microbiological oxidation of any hydrocarbon is at best a highly unpredictable art. Amongst those hydrocarbons whose oxidation products are of considerable commercial value are alkyl naphthalenes, the carboxylic acid derivatives of which are useful as antifungal agents. Heretofore, these compounds, as for example the monocarboxylic acid of an alkyl naphthalene such as methyl naphthalene, have been prepared by the elaborate and expensive chemical oxidation of the corresponding dimethyl naphthalenes such as, for example, 2,6-dimethylnaphthalene, and methods have long been sought for converting such compounds by less expensive means and in higher yield.

It has now been found, in accordance with the present process, that the oxidation of dialkyl naphthalenes to form the corresponding monoacids in high yield may be achieved microbiologically by subjecting said alkyl naphthalenes to the oxygenating action of a microorganism of the genus Streptomyces, and particularly the species *S. achromogenes* ATCC 15,077.

The substrates employed in this invention are naphthalenes having two alkyl groups substituted for each of two different hydrogen atoms on the nucleus. The substituent alkyl groups can be the same or different and may be branched or straight-chain groups having from 1–6 carbon atoms. Surprisingly, it has been found that when the alkyl side-chain contains an odd number of carbon atoms, the resulting acid moiety will contain only one carbon atom, whereas when the alkyl side-chain contains an even number of carbon atoms the corresponding naphthalene acetic acid derivative is formed. Illustrative examples of the dialkyl naphthalene substrates which may be used to prepare the products of this invention are: 1,2-dimethyl-naphthalene; 1,3-diethyl-naphthalene; 1,4-dipropyl-naphthalene; 1,5-dibutyl-naphthalene; 2,6-dimethyl-naphthalene; 2,7-dimethyl-naphthalene; 1-methyl-8-ethyl-naphthalene; 2-ethyl-3-propyl-naphthalene; 2-propyl-6-butyl-naphthalene; 2-butyl-7-pentyl-naphthalene and the like, and mixtures thereof.

When the aforementioned starting materials are thus treated in accordance with this invention, there are obtained the corresponding alkyl-substituted monoacids, as for example, 6-methyl-2-naphthoic acid, 7-methyl-2-naphthoic acid, 6-propyl-2-naphthoic acid, 7-ethyl-2-naphthyl-acetic acid, 1-methyl-2-naphthoic acid and the like.

In the practice of this invention, the novel microbiological oxidation process is desirably carried out under conditions used in conventional aerobic fermentations known to the art. Thus, for example, a suitable mineral salts-hydrocarbon medium is inoculated with a viable strain of the microorganism *Streptomyces archromogenes,* and

aerated and agitated under submerged conditions during the incubation period of from about two to eight days and preferably for about five to six days. The pH of the fermentation medium may vary from about 4.5–8.5, with a preferred range of from about 6.8–7.2. The temperature at which the fermentation is carried out may range from 25–40° C. and preferably is from 26–37° C.

As in conventional submerged aerobic carbohydrate fermentations, aeration, and dispersion of the broth during fermentation is essential. The aeration can be controlled by passing air or oxygen-nitrogen mixtures through the broth in either fine, coarse, or as both fine and coarse bubbles. The aeration should be coupled with a means of agitating or dispersing the broth. For example, the broth can be aerated by the above method while rotating or tumbling the fermentor or while stirring vigorously with an impeller or propeller type stirrer. In any event, a consistent means of dispersing and aerating the broth during fermentation is necessary.

Where the hydrocarbon substrates are volatile, it is preferred to use a closed system to insure maximum utilization of the substrate by the microorganism and to prevent loss of the alkylated naphthalene substrate to the atmosphere. An adequate sterile air supply should be maintained during the oxidation by the submerged cultures. If desired, the substrate vapors and gases produced during fermentation can be vented off, recovered, and recycled back to the fermentation medium.

The quantity of hydrocarbon substrate employed in this process may range from 1 to 9% of the total nutrient medium and preferably should be from about 3 to 7%. It is desirable in carrying out this process that the dialkyl naphthalene substrate be introduced into the fermentation broth in amounts below growth-limiting concentrations. Thus, for example, at the beginning of the fermentation, as little as 0.2% of the alkyl naphthalene may be added, followed by an additional 1% after the first 24 hours, another 1% after 48 hours, and the remainder up to 9% after an additional 24 hours. Depending upon the total amount of dialkyl naphthalene to be utilized, however, proportionably larger amounts may be added at 12 to 24 hour intervals until the fermentation is complete.

The resulting monoacids are conveniently recovered from the fermentation broth by first adjusting the pH of the broth to about 8.5–10, preferably with a strong base such as NaOH, at which pH the monoacid is soluble while the unconverted alkyl naphthalene is not. Following filtration, the filtrate may then be treated with a strong acid such as HCl until, at a pH of 2.5 or below, the monoacid is precipitated out, recovered by filtration, and further purified, if necessary, by known methods.

A suitable nutrient medium for the oxidative fermentation should contain a source of carbon, nitrogen, and mineral elements. Also, since the microorganisms used in this transformation are incapable of utilizing the alkylated naphthalene substrates as their sole source of carbon, carbohydrate sources such as sugars, starches, preferably pearl starch, paraffinic hydrocarbons (straight chain C_4–C_{20}), and the like must be added to the medium. Suitable sources of nitrogen include natural products such as yeasts, corn, steep, liquor, cotton, seed, meal, beef extract, enzymatically digested proteins, various proteinaceous products such as peptones and amino acids, as well as ammonium salts, nitrates, nitrites, quaternary bases and salts, urea, and the like. The minerals and elements required for the fermentation such as phosphorus, magnesium, and trace metals can be obtained from the appropriate natural products used as nitrogen sources or can be added in the necessary amount as the salts and ions of the metals.

While the organisms described in this application are capable of good growth in a simple mineral salts-hydro-

carbon medium, it has been determined that their growth and their oxidation to the naphthoic acid is potentiated or at least accelerated by a class of compounds known as "growth stimulators" or "stimulators." These compounds, whose mode of action is unknown, produce a greater growth of microorganism than a simple nutrient medium can achieve. Among the various substances which have been shown to possess this activity in this fermentation are cotton seed oil, debittered brewers yeast, wheat germ, pearl starch, soybean oil, corn flour, peptone, and the like. One particularly suitable fermentation medium has the following composition:

	Percent
P.T. pearl starch	0.1
Urea	0.1
Na_2HPO_4	0.6
KH_2PO_4	0.4
$MgSO_4$	0.08

The inoculum for the fermentation can be prepared in several ways. For example, a soil sample in which the organism is found can be sprinkled on a mixture of paraffinic hydrocarbons and minerals until its prefermentative growth is attained. Alternatively, it can be suspended in water and grown on a conventional carbohydrate-proteinaceous-mineral medium, or it can be grown on a medium such as an agar slant. Such inocula may then be used to inoculate other batches of sterile media in fermentor tanks. A preferred medium for preparing the inoculum is as follows:

	Percent
Cerelose	0.1
Yeast extract S–50	0.1
Urea	0.1
$NaHPO_4$	0.6
KH_2PO_4	0.4
$MgSO_4$	0.08
2,6-dimethylnaphthalene	0.1

The inoculum employed in the practice of this invention may consist not only of the growing cells of the microorganism, but also washed suspensions of these growing cells, either in their growth phase or stationary phase, or as resting cells.

The following is a description of the morphological and cultural characteristics of the previously-undescribed Streptomyces species employed in this process, a culture of which has been deposited in the American Type Culture Collection in Washington, D.C., where it was assigned the above-designated ATCC Number. This culture was isolated from oil-soaked soil in the Marcus Hook Refinery of the Sun Oil Company, Marcus Hook, Pa. It possesses the bacteriological characteristics detailed below, and while it is similar to *Streptomyces achromogenes* described in Bergey's Manual of Determinative Bacteriology, 7th Edition, it cannot be identified as being the same as this organism, and is, therefore, considered to be a new species:

(1) Gram stain—Gram positive, short to long rods
(2) Methylene blue stain—Short to long rods, no granules
(3) Acid fast stain—Not acid fast.
(4) Kliger iron agar—Good growth, white, hard, circular
(5) Methyl red—Negative
(6) Gelatin liquifaction—Negative
(7) Nitrate broth—Nitrites are produced (positive)
(8) Colony description-nutrient agar—Elevated, hard white growth
(9) Litmus milk—White pellicle growth, no change
(10) Loeffler's blood serum—Scant elevated brownish growth
(11) Dorset egg medium—Scant brownish growth, no obvious aerial mycelium
(12) Sim's medium—Indole negative
(13) Glycerol agar—Good growth, colorless colonies
(14) Urea and hexadecane—Good growth, white, circular, dry acid produced.

(15) Carbohydrate test in phenol red broth using 0.5% specific carbohydrate substrate

(a) Mannitol—No acid, no gas
(b) Levulose—Acid, no gas
(c) Lactose—No acid, no gas
(d) Sorbitol—No acid, no gas
(e) Saccharose—No acid, no gas
(f) Arabinose—No acid, no gas
(g) Maltose—No acid, no gas

Example I.—Preparation of 6-methyl-2-naphthoic acid from 2,6-dimethylnaphthalene

A basal nutrient medium prepared from a commercial trace metals solution is combined with the following major components:

	Percent by weight
Glucose	1.0
Urea	0.1
$MgSO_4$	0.08
Phosphate buffer	0.1

The medium is brought to 1000 ml. volume with tap water and then sterilized by autoclaving at 120° C. for 45 minutes. It has a pH of 6.8. The inoculum is prepared by cultivating a viable strain of *S. achromogenes* ATCC No. 15,077 in a commerical beef broth nutrient solution and propagating a loopful of it in the above sterilized media. The pre-growth of microorganism is continued for 18 to 36 hours between 5 and 30° C. on a reciprocating shaker. At the end of this time a suspension of 10% inoculum is added to a sterilized fermentation broth comprising the following substances:

	Percent by weight
Urea	0.1
$MgSO_4$	0.08
Phosphate buffer	0.1
Yeast extract	0.1
2,6-dimethylnaphthalene	0.1

After bringing up the volume of the broth to 1000 ml. with tap water, the fermentation is allowed to proceed at a pH of 7.2 at 30° C. for 120 hours. At the end of this time the pH of the broth is adjusted to 9.0, and the mycelia and unconverted starting material filtered off and discarded. After removal of the mycelia the filtered broth is acidified to pH 2.5 with HCl and a crystalline product precipitates out. Analysis indicates the precipitate is 6-methyl-2-naphthoic acid which can be further purified by a variety of methods well known to the art, as, for example, the following:

The precipitated product is extracted into diethyl ether, back extracted into 1 N NaOH solution and decolorized by treatment with activated carbon. After removal of the carbon, the aqueous solution is treated with dilute HCl until it is neutral or only slightly basic. Then the solution is chilled to about 5° C. overnight and allowed to warm up with vigorous stirring while adding a small quantity of tertiary butyl chloride. The gradual hydrolysis of the butyl chloride to HCl produces the proper degree of acidity to precipitate the product in the form of large crystals. These are washed and the traces of butanol removed under vacuum. Infrared analysis, melting point, and partition chromatography confirm the products identity as 6-methyl-2-naphthoic acid.

Example II.—Preparation of 7-methyl-2-naphthoic acid from 2,7-dimethylnaphthalene

Streptomyces achromogenes (ATCC 15,077) is grown on hexadecane in the same basal mineral medium as is employed in Example I at 30° C. for four days under aerobic conditions. The cells are harvested by centrifugation and washed with M/30 phosphate buffer pH 7.0. They are then resuspended in M/30 buffer to give a concentration of cell material of 12.4 mg./ml. (dry weight). One ml. of this suspension is placed in a War-

burg flask with 20 mg. of 2,7-dimethylnaphthalene in 1 ml. of M/30 phosphate buffer. This mixture is shaken aerobically for 21 hours at 30.3° C. in a water bath. At the conclusion of the experiment, the cells are removed by filtration on a sintered glass filter. The clear filtrate which has a pH of 6.9 is adjusted to pH 3.0 with 10% HCl to yield a precipitate of 7-methyl-2-naphthoic acid.

Example III.—Preparation of other alkyl naphthoic acids from dialkylnaphthalene substrates

Using the same procedures and techniques described in Examples I and II, the following transformations of dialkylnaphthalene substrates to the corresponding alkylnaphthoic acid product are accomplished in good yield. In all cases identity of the products is confirmed by melting point, infrared analysis, and chromatography.

Substrate:

2,6-dipropylnaphthalene
2,7-diethylnaphthalene

Product:

6-propyl-2-naphthoic acid
7-ethyl-2-naphthylacetic acid

Example IV.—Anti-fungal activity of representative compounds of this invention against test organisms

EXPERIMENTAL

Three loopfuls of a viable culture *Fusarium oxysporum*, spores and mycelia are transferred from an agar slant to a 250 ml. portion of the nutrient broth given below:

Component:	Percentage by weight
Bacto-soytone	1.0
Bacto-dextrose	4.0
Deionized water to volume.	

The 250 ml. portions of fungi and broth are placed in sterile trypsinizing flasks (500 ml.) and the flasks placed on a rotary shaker for 72 hours at room temperature. At the end of this incubation time period, 20 ml. aliquots of the liquid are homogenized and an aliquot placed into each one of 4 sterile trypsinizing flasks (300 ml.) containing 90 ml. of the above nutrient broth. To two of the flasks are added 250 p.p.m., respectively, of the alkylnaphthoic acids of Examples I and II being evaluated The other 2 untreated flasks are used as controls for comparison purposes. The 4 flasks (2 treated and 2 untreated) are placed on a rotary shaker operating at 240 r.p.m. at room temperature for 3 days. After the second incubation time the flasks are removed for visible fungal growth.

RESULTS

The two products from Examples I and II give substantially complete inhibition of fungal growth at 250 p.p.m. evidencing their value as anti-fungal agents.

The invention claimed is:

1. A process for the production of alkyl naphthalene monocarboxy acid which comprises subjecting a dialkyl naphthalene having from 1 to 6 carbon atoms in each alkyl group to the oxygenating activity of *Streptomyces achromogenes* ATCC 15,077 in an aqueous nutrient medium under aerobic conditions and recovering the corresponding alkyl naphthalene monocarboxy acid.

2. A process according to claim **1** wherein each of said alkyl groups has 1 to 2 carbon atoms.

3. A process according to claim **2** wherein each of said alkyl groups is methyl.

4. A process according to claim **3** wherein said dialkyl naphthalene is 2,6-dimethylnaphthalene and 6-methyl-2-naphthoic acid is recovered.

5. A process according to claim **3** wherein said dialkyl naphthalene is 2,7-dimethylnaphthalene and 7-methyl-2-naphthoic acid is recovered.

6. A process according to claim **2** wherein each of said alkyl groups is ethyl.

7. A process according to claim **6** wherein said dialkyl naphthalene is 2,7-diethylnaphthalene and 7-ethyl-2-naphthylacetic acid is recovered.

No references cited.

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