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Microbiological Oxidation of Isophorone to 4-Hydroxyisophorone and Chemical Transformation of 4-Hydroxyisophorone to 2,3,5-Trimethyl-*p*-benzoquinone

Young Ae Joe, Yang Mo Goo[§] and Youn Young Lee*
*Department of Pharmacy and *Department of Chemistry,*
Seoul National University, Seoul 151-742, Korea
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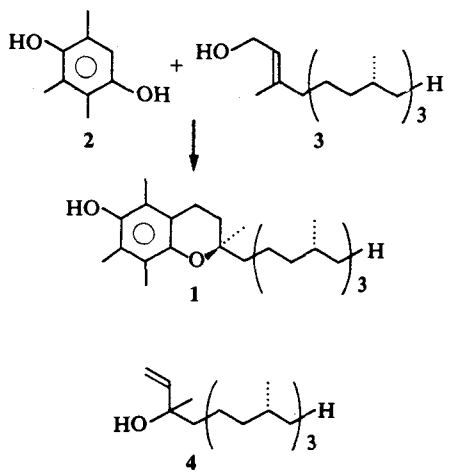
Abstract□Oxidation of isophorone by various fungi was examined. *Aspergillus niger* oxidized isophorone to 4-hydroxyisophorone, 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one and 5-hydroxymethyl-3,5-dimethyl-2-cyclohexen-1-one. 4-Oxoisophorone obtained by chromic acid oxidation of 4-hydroxyisophorone was transformed to 2,3,5-trimethyl-*p*-benzoquinone by acid treatment.

Keywords□isophorone, oxidation by fungi, vitamin E, trimethylbenzoquinone, 4-hydroxyisophorone, 4-oxoisophorone.

For the synthesis of vitamin E (α-tocopherol, **1**) two compounds, 2,3,5-trimethyl-*p*-hydroquinone (**2**) and phytol(**3**) (or isophytol(**4**)) are necessary.[1-3] Currently, production of vitamin E was very much limited by the supply of these two starting materials. 2,3,5-Trimethyl-*p*-hydroquinone can be obtained by rearrangement of 2,6,6-trimethyl-2-cyclohexene-1,4-dione[4], by oxidation from trimethylphenols,[5-7] or rarely by oxidation of 2,5,6-trimethy-2-cyclohexen-1-one.[8] Thus, much attention has been paid to the preparation of 2,6,6-trimethyl-2-cyclohexene-1,4-dione, or trimethyl phenols.[9-13]



[§]To whom all correspondence should be addressed.

Studies on the preparation of 2,6,6-trimethyl-2-cyclohexene-1,4-dione from isophorone have been reported.[14-16]

For the development of a new method for the production of 2,3,5-trimethyl-*p*-hydroquinone, we studied microbial and chemical transformation of isophorone to the 2,3,5-trimethyl-*p*-hydroquinone. We wish to report here our study on the microbial conversion of isophorone to 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one and on the chemical transformation of the latter to 2,3,5-trimethyl-*p*-benzoquinone.

MATERIALS AND METHODS

Materials & instrumentation

^{1}H NMR spectra were obtained with Fourier transformed Bruker WP80SY NMR spectrophotometer(80 MHz) and TMS was used as an internal standard. IR spectra were taken with Perkin-Elmer Model PP-I FT-IR spectrophotometer. UV analyses were carried out by using a LKB ultrospect 4050 spectrophotometer. The optical rotation value and the CD spectrum were obtained by employing Jasco DIP-360 digital polarimeter and Jasco J-20C spectropolarimeter, respectively. Gas liquid chromatographic analysis was performed by using a shimadzu GC RIA equipped with a flame ionization detector by using a glass column (3 mm × 2 m) packed with OV-17 (5%) coated on chromosorb W. The carrier gas (nitrogen) was passed through the col-

umn at the rate of 40 ml/min. The temperature of the oven was programmed from 100 °C to 250 °C by increasing at the rate of 1 °C/min, 5 °C/min, 3 °C/min, and 2 °C/min from 0 to 5 min, from 5 to 10 min, from 10 to 15 min and from 15 to 40 min, respectively. The composition was calculated from the integrated data of each peak. GC-Mass spectra were taken with Shimadzu QP 1000 or JEOL JMS-DX-300. Microorganisms were cultured at 28 °C on a gyrating shaker (180 rpm). TLC analyses were performed on commercial silica gel plates (0.25 mm thickness glass plates) made by Riedel-De Haen Aktiengesellschaft Sleeze-Hannover. The microorganisms were those stocked by step cultures in our laboratory. Isophorone was purchased from Aldrich-Chemie. Yeast extract was purchased from Difco. Silica gel used for purifications was Kieselgel 60 (70-230 mesh) of Merck Co.

Oxidation of isophorone by various fungi

A loop of the fungus (listed in Table I) from agar slants was inoculated in the modified CZ medium (100 ml) consisting of 3% sucrose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$-$7H_2O$ and 0.1% yeast extract in distilled water. The fungi were stocked on agar slants of M or CZ medium which consisted of malt extract (2%), peptone (0.1%), glucose (2%), agar (1.5-2%), in distilled water, or $NaNO_3$ (0.2%), K_2HPO_4 (0.1%) KCl (0.05%), $FeSO_4$(0.001%), sucrose (3%), Bacto-agar (2%) in distilled water, respectively. The inoculated medium was incubated for 2-4 days on a rotary shaker at 28 °C, added with isophorone (0.1 ml), incubated further for 4-5 days and extracted with ethyl acetate. The extract was washed with 5% sodium bicarbonate and with distilled water, dried over sodium sulfate, and evaporated under reduced pressure to give on oily residue. The residue (2.9-9.8 mg) was dissolved in ethyl acetate and 10 ul of the solution was analyzed by gas chromatography.

Oxidation of isophorone by A. niger and isolation of the products

A. niger was inoculated in the modified CZ medium (100 ml × 5) with 0.01% isophorone, cultured on a shaker at 28 °C for 2-3 days. The cultures were transferred to the identical media (700 ml × 5), incubated at 28 °C for 2-3 days, added with isophorone (1 ml), and incubated further for 5-6 days. The cultures were combined and filtered. The mycelia were washed with ethyl acetate. The filtrate was extracted with ethyl acetate. The ethyl acetate extract was washed with 5% sodium bicarbonate and with distilled water, dried over sodium sulfate, and evaporated to give an oily residue (2.87 g, recovery yield; 57.6%).

Oxidation of isophorone with 100% transfer of A. niger

The mycelia obtained by the method given in the previous experiment, which were not washed with ethyl acetate, were suspended in the modified CZ medium (700 ml) and were added with isophorone (1 ml). The broth was incubated at 28 °C for 6 days on a rotary shaker (180 rpm). From the broth, an oil residue (0.74 g, revovery; 80%) was obtained by the same procedure.

Column chromatography of oxidized products

The oily residue (about 3.62 g) was chromatographed (silica gel) to give 4 compounds. 4-hydroxyisophorone: 1.28 g (yield 21%), oil; ^{1}H NMR ($CDCl_3$) δ : 1.02(s, 3H), 1.07(s, 3H), 2.05(s, 3H), 2.21(d, 1H, J = 16 Hz), 2.41(d, 1H, J = 16 Hz), 4.02(s, 1H), 5.82(bs, 1H), and 2.51(s, 1H); IR ν_{max}^{neat} cm^{-1}: 1660 and 3420; MS (*m/z*, relative intensity): 154(M^+, 33), 112(50.3), 98(100), 70(65.1), 69(66.5), 43(33.4), and 41(83.0); UV λ_{max}^{EtOH} nm (ε): 229.3 (10,000); $[\alpha]_D^{26}$ = -50.6 (c, 1.1, methanol); CD [θ] (nm) = + 0.029(310). 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one: 0.64 g (yield 11%); oil; ^{1}H NMR ($CDCl_3$) δ : 1.05(s, 6H), 2.14(s, 2H), 2.26 (s, 2H), 2.77(s,1H), 4.22(s, 2H), and 6.14(bs, 1H); IR ν_{max}^{neat} cm^{-1}: 3400, 1680, and 1710; MS (*m/z*, relative intensity): 154(M^+, 24.1), 125(27.2), 121(9.8), 98(100), 97(39.4), 93(104), 83(19.5), 82(16.2), 70 (32.2), 69(33.9), 67(37.5), 57(29), 56(17.9), 55.5 (49.9), 43(90.9) and 41(61.3); UV λ_{max}^{EtOH} nm(ε): 230.3(13,100). 5-hydroxymethyl-3,5-dimethyl-2-cyclohexen-1-one: 0.06 g(yield 1%); oil; ^{1}H NMR ($CDCl_3$) δ : 0.93(s, 3H), 1.88(s, 5H), 2.08(d, 1H, J = 5.6 Hz), 2.30(d, 1H, J = 5.6 Hz), 3.34(s, 2H), 5.81(s, 1H), and 2.34(s, 1H) or 2.04 (s, 1H); IR ν_{max}^{neat} cm^{-1}: 1650 and 3410; UV λ_{max}^{EtOH} nm(ε): 229.4(12,000); MS (*m/z*, relative intensity): 154 (M^+, 14), 139(6), 123(100), 109(15), 95(46), 82(70), 67(18), 55(23), 43(28).

Large-scale oxidation of isophorone with A. niger

A seed culture (modified CZ medium, 800 ml) of *A. niger* was transferred to the same medium (10 l) containing 1 ml of isophorone in a fermenter, and cultured for 2 days at 28 °C under aeration (5 l/min). Then, the cultured broth was added with isophorone (15 ml) and incubated further. After 10 days' incubation, the broth was extracted and the

Table I. GC analysis of the oxidized products of isophorone by various fungi

organisms[a]	medium[b]	recov.[c] (%)	incub. be.[d]	incub. af.[e]	growth[f]	percent composition(%)[g] A	D	E
A. niger	M	72.8	2	5	+ + +	12.9	58.1	27.2
P. roqueforti	CZ37	52.4	2	5	+ + +	67.9	32.1	—
P. notatum	CZ29	46.9	3	4	+ + +	84.3	13.6	2.1
A. avenaceus	CZ34	66.2	3	4	+ + +	75.7	12.4	1.8
A. alliaceus	CZ10	64.2	3	4	+ +	76.6	12.6	3.0
A. sclerotiorum	CZ18	78.5	3	4	+ +	80.6	14.0	—
A. flaviple	CZ 8	68.3	3	4	+ +	69.9	28.3	—
A. eandidus	CZ 9	81.8	3	4	+ +	75.8	24.2	—
P. camenberti	CZ38	48.9	3	4	+	81.3	7.2	1.5
A. clavatus	CZ56	78.2	3	4	+	77.9	16.9	—
A. panamensis	M31	95.9	3	4	+ + +	24.5	64.7	2.6
P. digitatum	M44	59.5	3	4	+ + +	67.8	24.6	7.6
A. versicolor	CZ 5	31.3	4	4	+ + +	29.8	70.2	—
Cochliobolus sp.	M48	54.8	4	4	+ + +	84.4	3.1	—
Th. elegans	M49	88.8	4	4	+ + +	80.2	18.0	1.8
A. rugulosus	M32	54.3	3	4	+ + +	81.8	15.3	—
By. fulva	CZ28	72.3	3	4	+ + +	72.3	24.2	—
P. purpurogenum	CZ26	43.1	3	4	+ + +	80.3	16.5	—
En. apiculata	M30	52.4	3	4	+ + +	81.8	13.1	—
A. nidulans	CZ57	32.8	3	4	+ + +	22.8	46.3	27.4
Pa. varioti	CZ27	47.1	3	4	+ + +	36.9	51.0	10.4
A. flavipes	CZ 7	61.9	3	4	+ + +	100.0	—	—
Pa. varioti	M46	82.9	3	4	+ + +	30.4	61.8	3.6
A. gigantus	M 1	20.2	3	4	+ + +	35.3	41.6	15.4
A. fumihevola	CZ19	84.2	3	4	+ + +	66.4	32.3	—
A. oryzae	CZ21	108.2	3	4	+ + +	23.3	61.5	14.4
Mu. rammanniamus	M53	41.0	3	4	+ + +	38.8	53.1	3.9

[a] *A: Aspergillus; P: Penicillium; Pa: Paecilomyces; Mu: Mucor;* and *By: Byssochlamyces; Th: Thamnidium* and *En: Entomophthora.*
[b] The numbers refer the stock medium and the strain numbers.
[c] The percentage of the amount recovered from the extract to that of isophorone added.
[d] Incubation time for the growth of the organism before addition of substrate.
[e] Incubation time for the oxidation of isophorone.
[f] Degree of growth prior to the addition of isophorone.
[g] Composition (%) was obtained by area-normalization from the data of GC chromatograms.

oxidized products were purified to give 3.48 g of 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one and 1.42 g of 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one.

Oxidation of 4-hydroxyisophorone to 4-oxoisophorone

The chromic acid solution (5 m*l*, potassium dichromate: 0.97 g (3.3 mmol); sulfuric acid: 1.3 g (13.4 mmol)) was added dropwise to the diethyl ether solution of 4-hydroxyisophorone (1.544 g, 10 mM). The solution was stirred for 3 hours. The aqueous layer separated from ether was extracted with diethyl ether. The combined ether solution was washed with 5% $NaHCO_3$, dried over sodium sulfate, and evaporated to give an oily residue (1.248 g, crude yield: 80.8%). The oily residue was chromatographed (silica gel) to give 4-oxoisophorone

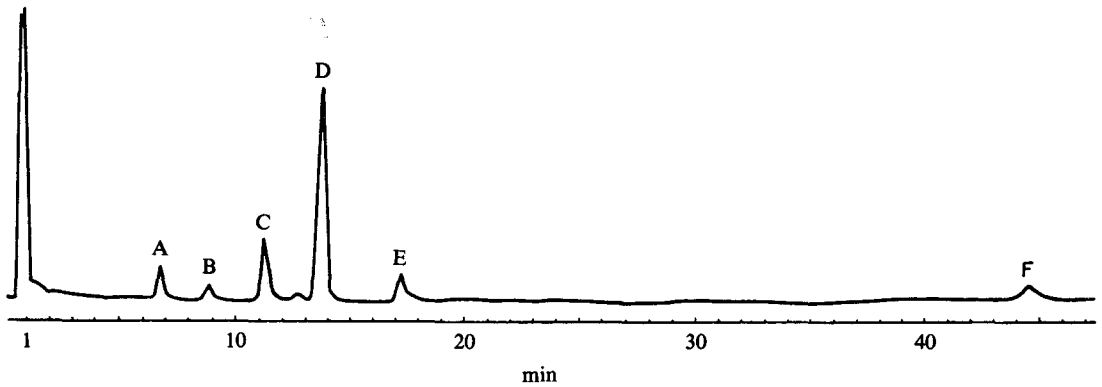


Fig. 1. The GC-trace of the oxidized products of isophorone by *A. niger*

(766.9 mg; yield 50%). Oil; ^{1}H NMR ($CDCl_3$) δ: 1.24(s, 6H), 2.02(d, 3H, J = 1 Hz), 2.71(s, 2H), and 6.55(bs, 1H); IR ν_{max}^{neat} cm^{-1}: 1690 and 1630; UV λ_{max}^{EtOH} (nm): 235.8.

Rearrangement of 4-oxoisophorone to 2,3,5-trimethyl-p-benzoquinone

3,5,5-Trimethyl-2-cyclohexene-1,4-dione (535.7 mg, 3.52 mmol) and sulfuric acid (1.73 g, 17.6 mmol) were dissolved in acetic anhydride (5 m*l*). The solution was allowed to stand for 70 days at room temperature. The reaction mixture was poured into distilled water and the aqueous solution was extracted with ethyl acetate. The extract was dried over sodium sulfate, and evaporated to give a residue (601.6 mg). The residue was chromatographed (silica gel) to give 3 compounds.

2,3,5-trimethyl-*p*-benzoquinone: 196 mg (yield 37%); oil; ^{1}H NMR ($CDCl_3$) δ: 2.02(s, 6H), 2.04(s, 3H), and 6.54(s, 1H); IR ν_{max}^{neat} cm^{-1}: 1660 and 1620; UV λ_{max}^{EtOH} (nm): 253.1; MS (*m/z*): 150(M^+), 122, 107.

4-acetoxy-2,3,5-trimethylphenol[17]: 180 mg (yield 24%); ^{1}H NMR ($CDCl_3$) δ: 2.03(s, 6H), 2.09 (s, 3H), 2.32(s, 3H), 4.91(s, 1H), and 6.40(s, 1H); MS (*m/z*): 194(M^+), 152, 137.

4-acetoxy-2, 3, 6-trimethylphenol: 49 mg (yield 77%); ^{1}H NMR ($CDCl_3$): 2.13(s, 6H), 2.16(s, 3H), 2.29(s, 3H), 4.65(s, 1H) and 6.63(s, 1H); MS (*m/z*): 194(M^+), 152, 137.

RESULTS AND DISCUSSION

Oxidation of isophorone by various fungi

Among 27 species of fungi examined, *A. niger* and *A. panamensis* oxidized isophorone well (Table I). A typical GC chromatogram of the extract obtained from the culture of *A. niger* incubated with isophorone is shown in Fig. 1.

The incubated media were extracted with ethyl acetate and evaporated to give a residue, which was chromatographed to yield three oxidized products. The total amount of isophorone and its oxidized products recovered was 84% of the fed amount. One compound having a R_f value at 0.4 (n-hexane: ethylacetete = 1:2) showed IR bands for a conjugated carbonyl group at 1660 cm^{-1} and for a hydroxyl group at 3420 cm^{-1}, and ^{1}H NMR peaks for methyl groups at 1.02, 1.07, and 2.05 ppm, for methylene group showing a germinal coupling (J = 16 Hz) at 2.21 and 2.41 ppm, for a proton at 4.02 ppm, for a proton attached to double bond at 5.82 ppm, and for a hydroxyl proton at 2.51 ppm. Its mass spectrum showed a molecular ion at *m/z* 154. From these spectroscopic data and optical rotation value, it was identified to be 4(S)-4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one(6). This compound was first reported by Mikami *et al.*[18] during the study of metabolism of isophorone by *A. niger*. The CD spectrum of this compound showed a negative and a positive Cotton effects at 265 and 310 nm, respectively. The CD spectrum of the compound showing n-π^* transition of the chromophore (310 nm) of the conjugated carbonyl group showed a positive Cotton effect.

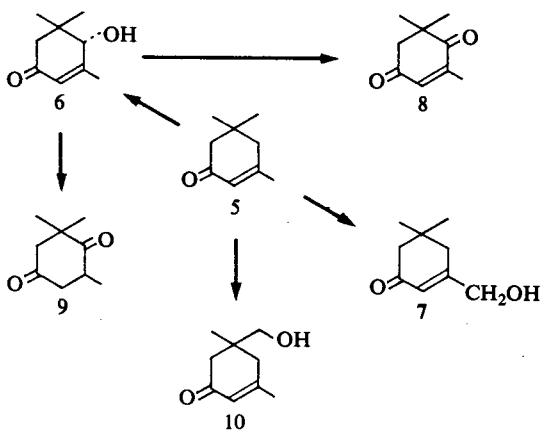
The compound having a R_f value at 0.31 (n-hexane:ethylacetate = 1:2) showed IR bands for a hydroxyl group at 3400 cm^{-1} and for a carbonyl group conjugated to a double bond at 1680 cm^{-1} and ^{1}H NMR peaks for two methyl groups at 1.05 ppm, for three methylene groups at 2.14, 2.26 and 4.22 ppm, for a vinylic proton at 6.14 ppm, and for a hydroxyl proton at 2.77 ppm. The mass spectrum showed a molecular ion at *m/z* 154. The compound

was identified to be 3-hydroxylmethyl-5,5-dimethyl-2-cyclohexen-1-one (7). 3,5,5-Trimethyl-2-cyclohexene-1,4-dione (4-oxoisophorone, 8) was also identified as a minor product by cospotting of the residue of the extract with 4-oxoisophorone obtained from chromic acid oxidation of 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one.

A new compound, 5-hydroxylmethyl-3,5-dimethyl-2-cyclohexen-1-one(10), was also identified as an oxidized product of isophorone by *A. niger*. This compound showed IR band for a hydroxyl group at 3410 cm^{-1} and for a carbonyl group conjugated to a double bond at 1650 cm^{-1} and ^1H NMR peaks for a methyl group at 0.93 ppm, for a methylene group and a methyl group at 1.88 ppm, for a methylene group showing a germinal coupling (J = 5.6 Hz) at 2.08 and 2.30 ppm, for a vinylic proton at 5.81 ppm, and for a hydroxyl group of racemic mixture at 2.34 or 2.04 ppm. No optical rotation was observed. The mass spectrum showed a molecular ion at m/z 154.

The GC/MS analysis of the extract indicated that the compounds of the peaks of A, D, and E were isophorone (5), 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one(6), and 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one(7), respectively. The other minor products at peaks of B and C were identified to be 3,5,5-trimethylcyclohexane-1,4-dione(9) and 3,5,5-trimethyl-2-cyclohexene-1,4-dione (8), which showed molecular ions at m/z 154 and 152, respectively.

When isophorone was incubated with the mycelia of *A. niger*, about 23.5% of the fed isophorone was recovered as the oxidized product, 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one. The ratio of 4-hydroxyl-3,5,5-trimethyl-2-cyclohexen-1-one to 3-hydroxylmethyl-5,5-dimethyl-2-cyclohexen-1-one was about 2:1.



A proposed pathway for transformation of isophorone by *A. niger*.

An attempt was made to increase the amount of isophorone fed to the culture medium. Tween 80 or DMF (less than 0.1%) was used to dissolve isophorone and to solubilize isophorone in the broth. However, addition of isophorone more than 0.3% (w/v) to the culture medium showed inhibition of the oxidation of isophorone. Too much amount of isophorone in the cultured media of *A. panamensis* caused the mycelia lysed. Isophorone was found to be cytotoxic to fungi.

Chemical transformation of 4-hydroxyisophorone to 2,3,5-trimethyl-p-benzoquinone

When 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one was oxidized with chromic acid, a new compound showing IR bands at 1690 and 1630 cm^{-1} and ^1H NMR peaks at 1.24 ppm for two methyl groups, at 2.02 ppm for a methyl group coupled to a vinyl proton, at 2.71 ppm for a methylene group, and at 6.55 ppm for a vinyl proton was obtained in 50% yield. These spectroscopic data indicated the compound to be 3,5,5-trimethyl-2-cyclohexene-1,4-dione. The starting material (4-hydroxyisophorone) and other minor products were recovered in 21% and 4.5% yields, respectively. When 4-oxoisophorone was kept in acetic anhydride with sulfuric acid, 2,3,5-trimethyl-*p*-benzoquinone and 2,3,5-trimethyl-*p*-hydroquinone monoacetates were obtained in the yield of about 70%. Formation of 2,3,5-trimethyl-*p*-hydroquinone 1,4-diacetate was not confirmed.

2,3,5-Trimethyl-*p*-benzoquinone showed IR bands at 1660 and 1620 cm^{-1} for a carbonyl group and ^1H NMR peaks at 2.02(6H) and 2.04(3H) ppm for three methyl groups, and at 6.54 ppm for a vinylic proton. 4-Acetoxy-2,3,5-trimethylphenol showed ^1H NMR peaks at 2.03(6H) and 2.09(3H) ppm for three methyl groups, at 2.32 ppm for an acetoxy group, at 4.91 ppm for a hydroxyl proton, and at 6.40 ppm for a benzene proton and 4-acetoxyl-2,3,6-trimethylphenol showed ^1H NMR peaks at 2.13(6H) and 2.16(3H) ppm for three methyl groups, at 2.29 ppm for an acetoxy group, at 4.65 ppm for a hydroxyl proton, and at 6.63 ppm for a benzene proton. These two compounds were not succeeded to isolate in pure forms.

Investigation of the time necessary for the complete rearrangement of the starting material to the benzoquinone and hydroquinone acetates was not

carefully examined. Treatment of 4-oxoisophorone with polyphosphoric acid instead of sulfuric acid also gave complete conversion of 4-oxoisophorone to 2,3,5-trimethyl-*p*-benzoquinone as a major product.

The overall yield of 2,3,5-trimethyl-*p*-benzoquinone from isophorone was 7.4%. Oxidation of isophorone to 4-hydroxyisophorone by *A. niger* was accompanied by difficulties in extraction of the culture medium with a large volume of ethyl acetate and in elimination of the organism. Thus, investigation of direct conversion of isophorone to 4-oxoisophorone or 2,3,5-trimethyl-*p*-benzoquinone is necessary.

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