Microbial Degradation of Chloroaromatics: Use of the meta-Cleavage Pathway for Mineralization of Chlorobenzene

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Pseudomonas putida GJ31 is able to simultaneously grow on toluene and chlorobenzene. When cultures of this strain were inhibited with 3-fluorocatechol while growing on toluene or chlorobenzene, 3-methylcatechol or 3-chlorocatechol, respectively, accumulated in the medium. To establish the catabolic routes for these catechols, activities of enzymes of the (modified) ortho- and meta-cleavage pathways were measured in crude extracts of cells of P. putida GJ31 grown on various aromatic substrates, including chlorobenzene. The enzymes of the modified ortho-cleavage pathway were never present, while the enzymes of the meta-cleavage pathway were detected in all cultures. This indicated that chloroaromatics and methylaromatics are both converted via the meta-cleavage pathway. Meta cleavage of 3-chlorocatechol usually leads to the formation of a reactive acylchloride, which inactivates the catechol 2,3-dioxygenase and blocks further degradation of catechols. However, partially purified catechol 2,3-dioxygenase of P. putida GJ31 converted 3-chlorocatechol to 2-hydroxycis,cis-muconic acid. Apparently, P. putida GJ31 has a meta-cleavage enzyme which is resistant to inactivation by the acylchloride, providing this strain with the exceptional ability to degrade both toluene and chlorobenzene via the meta-cleavage pathway.

Various bacterial cultures which can use chloroaromatics as the single source of carbon and energy for growth, resulting in the formation of carbon dioxide, chloride, and biomass, have been described. These organisms can be differentiated on the basis of the catabolic pathways dealing with the substrates. Chlorosubstituents can be removed by initial oxygenolytic, reductive, or hydrolytic reactions. Further mineralization can then occur via classical pathways such as the 3-oxoadipate and the meta-cleavage pathways. However, the majority of the organisms able to mineralize chlorinated aromatics do not possess enzyme systems capable of initial dechlorination. They transform chlorinated aromatics to chlorocatechols, which are further metabolized by the enzymes of the modified ortho-cleavage pathway, and dechlorination occurs after ring cleavage (32).

It is generally accepted that degradation of chloroaromatics does not proceed via the meta-cleavage pathway (20, 26, 29). An explanation for this has been found in the production of an acylchloride from 3-chlorocatechol by the catechol 2,3-dioxygenase of the meta-cleavage pathway, which leads to rapid suicide inactivation of the enzyme (3). Therefore, meta cleavage is considered to be unsuitable for the mineralization of halocatechols that are degraded via halocatechols.

Whereas chlorocatechols are mineralized via ortho-cleavage pathways, methylicaromatics are commonly mineralized via meta-cleavage routes. Simultaneous metabolism of chloro- and methylcatechols often creates biochemical anarchy, meta cleavage leads to substrate misrouting in the case of 4-chlorocatechol or formation of a suicide product in the case of 3-chlorocatechol. Formation of dead-end methylketones can occur when the ortho-cleavage pathway is dealing with methylcatechols.

Consequently, only a few strains which can grow on mixtures of methylated and chlorinated aromatics are known (9, 26, 37). They all use a modified ortho-cleavage pathway for the conversion of the chlorinated substrate. Pseudomonas putida GJ31 (24) and Pseudomonas sp. strains JS6 (26) and JS150 (9) are the only strains known to grow on a mixture of chlorobenzene and toluene. In Pseudomonas sp. strain JS6, the presence of toluene slightly decreased the yield on chlorobenzene. The organism degraded both substrates primarily via a modified ortho-cleavage pathway, which is less efficient for methylated substrates than the meta-cleavage pathway. In P. putida GJ31, the presence of toluene enhanced the degradation of chlorobenzene (18). Here, we show that this organism grows on chlorobenzene via a meta-cleavage pathway which allows the simultaneous utilization of toluene. In addition, 3-chlorocatechol was found to be the ring cleavage substrate formed from chlorobenzene, which was dehalogenated during ring cleavage to produce 2-hydroxymuconic acid.

MATERIALS AND METHODS

Bacterial strains and culture conditions. P. putida GJ31 was isolated from sediment of the river Rhine, with chlorobenzene as the sole carbon and energy source (24). The organism was grown in the mineral medium described by Dorn et al. (5). Cells were grown in 500-ml cultures in 3-liter sealed Erlenmeyer flasks with baffles; the flasks were shaken on a rotary shaker at 100 rpm at 30°C.

Preparation of cell extracts. Cells were harvested by centrifugation (15 min, 8,000 × g, 4°C), washed twice with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 mM 1,4-dithiothreitol, and disrupted by one passage through a French pressure cell (Aminco, Silver Spring, Md.; 140 MPa, 0°C) before centrifugation (60 min, 100,000 × g, 4°C) to remove cell debris. The clear supernatant solution was used as a source of crude cell extract.

Enzyme assays. All enzyme assays were done at 25°C. Purified enzymes of Pseudomonas sp. strain B13 were taken as a positive control of the assays of the modified ortho-cleavage pathway.

Benzene dihydrodiol dehydrogenase was measured by determining NAD reduction at 340 nm (ε_{NADH} = 6,300 liters mol⁻¹ cm⁻¹). Reactionmixtures
Vol. 179, 1997 Degradation of chlorobenzene by a meta-cleavage route

Enzymes used in the degradation of monoaromatic compounds. To investigate which degradation pathway is used by P. putida G31 for growth on aromatic compounds, the presence of enzymes involved in the meta-, ortho-, and modified ortho-cleavage pathways was determined in crude extracts prepared from cells grown on toluene, chlorobenzene, benzene, benzoate, a mixture of toluene and chlorobenzene, succinate, or acetate (Table 1). When measuring catechol 1,2-dioxigenase enzymes of the ortho- and modified ortho-cleavage pathways, putative interfering catechol 2,3-dioxigenase activity in the extract was inhibited with hydrogen peroxide. Catechol 2,3-dioxigenases usually contain ferrous iron as a cofactor, which makes them sensitive to hydrogen peroxide, in contrast to catechol 1,2-dioxigenases, which use ferric iron (22).

Surprisingly, activities of the enzymes of the modified ortho-cleavage pathway, which are usually involved in the degradation of chlorinated aromatics, were not detectable in crude extracts of cells grown on chlorobenzene or the other substrates.

Some activity of a catechol 1,2-dioxigenase towards catechol was found in extracts made of cells pregrown on benzene and benzoate, showing that this enzyme indeed resisted the hydrogen peroxide treatment. A muconate cycloisomerase, which is the second enzyme of the ortho-cleavage pathway, was also active in these extracts, suggesting that P. putida G31 can induce an ortho-cleavage pathway to degrade catechol.

For all the extracts made of cells grown on the aromatic substrates including chlorobenzene, the activities of the enzymes of the meta-cleavage pathway were high, with a maximal specific activity of 934 U g of protein  \(^{-1}\) for the catechol 2,3,5-dioxigenase activity.
dioxygenase in crude extracts of cells which were grown on a mixture of toluene and chlorobenzene. This indicates that both methylaromatics and chloroaromatics were converted by a meta-cleavage pathway. Some enzymes of the meta-cleavage pathway were also active in extracts made of cells grown on succinate or acetate, indicating that they are constitutively expressed.

The key enzyme in the meta-cleavage pathway is a catechol 2,3-dioxygenase, which converts catechol to 2-hydroxymuconic semialdehyde. The activity of this enzyme was unstable in crude extracts but could be partially restored by addition of a Fe^{2+} solution (50 mM). After incubation with hydrogen peroxide (0.009%), the conversion of catechol was inhibited and no yellow product was formed (Fig. 1A). These data indicate that the catechol 2,3-dioxygenase uses Fe^{2+} for its activity.

Also, 3-chlorocatechol was no longer converted after hydrogen peroxide treatment (Fig. 1A), indicating that the same enzyme was responsible for the conversion of both compounds.

A putative product of the conversion of 3-chlorocatechol by a catechol 2,3-dioxygenase is 2-pyrone-6-carboxylate. However, spectrophotometric analysis of absorbance spectra from 250 to 400 nm did not reveal any detectable turnover of 2-pyrone-6-carboxylate (20 mM) in 50 mM Tris-HCl buffer (pH 7.5) by crude extract (0.4 mg ml\(^{-1}\)) of cells of P. putida GJ31 grown on chlorobenzene.

**Conversion of toluene by chlorobenzene-grown cells.** When chlorobenzene-grown bacteria cooxidize toluene, methylactones are formed if degradation proceeds via the modified ortho-cleavage pathway (28). Accumulation of 2- or 4-methylactone would indicate that chlorobenzene degradation proceeds via 3- or 4-chlorocatechol, respectively, as ring cleavage substrates. In experiments with cells of P. putida GJ31, no formation of 2-methyl- or 4-methylactone was observed in HPLC chromatograms obtained at the highest sensitivity setting of the instrument. This confirms the enzyme data, suggesting that chlorobenzene is degraded via a meta-cleavage pathway.

**Conversion of several catechols.** Crude extracts prepared from toluene-grown cells were tested for their capacity to degrade substituted catechols. HPLC measurements showed that the extract (0.26 mg ml\(^{-1}\)) converted 0.5 mM catechol and 3-chloro-, 4-chloro-, 3-methyl-, and 4-methylcatechol within 30 min. These activities were lost after hydrogen peroxide treatment, indicating that a catechol 2,3-dioxygenase was responsible for the conversion of all the catechols. A yellow compound which could be detected at 375 nm accumulated when the extract was converting catechol, 4-methylcatechol, or 4-chlorocatechol. However, very little product could be detected when the extract was converting 3-methylcatechol. The same was observed at 388 nm, at which wavelength the meta-cleavage product of 3-methylcatechol possessed maximum absorption (30). This indicates that enzymes further downstream in the

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sp act (U g of protein(^{-1})) of enzyme in crude extracts of P. putida GJ31 pregrown on:</th>
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<tr>
<td></td>
<td>Acetate</td>
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<tr>
<td>Benzene dihydrodiol dehydrogenase</td>
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<td>2-Hydroxymuconic semialdehyde dehydrogenase</td>
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<td>0</td>
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<td>Maleylacetate reductase</td>
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\(^a\) n.d., not determined.

**FIG. 1.** Effect of hydrogen peroxide on the conversion of several catechols by crude extract (0.2 mg ml\(^{-1}\)) of P. putida GJ31 grown on chlorobenzene. The conversion of catechol (A; circles), chlorocatechol (A; triangles), and 3-fluorocatechol (3-FC; B; squares) was monitored over time. Solid symbols, no H\(_2\)O\(_2\) was added; open symbols, conversion was inhibited with 0.009% H\(_2\)O\(_2\), time at which extra crude extract (0.2 mg ml\(^{-1}\)) was added to the incubation mixture containing 3-fluorocatechol.
pathway converted the degradation product of 3-methylcatechol at a rate which does not allow significant accumulation of the yellow product. During conversion of 3-chlorocatechol, a small increase and subsequent decrease of the absorption at 260 nm were observed. This could indicate the formation of a (substituted) muconic acid. When the extract was inactivated with hydrogen peroxide, this pattern was no longer observed, indicating that it was not due to some catechol 1,2-dioxygenase activity.

Only a small amount of 3-fluorocatechol was converted by the extract of chlorobenzene-grown cells; after conversion, the activity stopped. This small amount of activity was lost after treatment with hydrogen peroxide (Fig. 1B).

Inhibition of catechol 2,3-dioxygenase in whole cells of *P. putida* GJ31 and PaW1. The catechol 2,3-dioxygenase of *P. putida* PaW1 is rapidly inhibited by several 3-halocatechols (3). To study the putative inhibition of the catechol 2,3-dioxygenase of *P. putida* GJ31 by these compounds, chlorobenzene-grown cells were incubated for 10 min in the presence of various concentrations of 3-chlorocatechol and 3-fluorocatechol. The relative activity of the catechol 2,3-dioxygenase was calculated from the amounts of 5-chloro-2-hydroxymuconic semialdehyde formation at 375 nm as a measure of the residual activity of the same enzyme with 3-fluorocatechol as the inhibitor.

![FIG. 2. Inactivation of catechol 2,3-dioxygenase in whole cells of *P. putida* PaW1 (A) and *P. putida* GJ31 (B).](image)

The enzyme which initially oxidizes these aromatics is probably a dioxygenase, because high activities of a benzene dihydrodiol dehydrogenase were detected in crude extracts made of cells pregrown on toluene or chlorobenzene (Table 1).

Since 3-chlorocatechol is the sole metabolite from chlorobenzene, initial dechlorination, for example by formation of an unstable *gem*-alcohol, can be ruled out as the strategy to degrade chlorobenzene.

**Growth of *P. putida* GJ31 and PaW1 with 3-chlorocatechol, 4-chlorocatechol, and catechol.** Since catechols and their autoxidation products are known to be toxic at high concentrations, which makes it difficult to use them as growth substrates in liquid cultures, growth on catechols was tested on solid medium by using a simple gradient plate technique. Suspensions of strains PaW1 and GJ31 were spread on the surfaces of mineral medium plates. Different catechols were dissolved in acetone and applied on paper disks. After evaporation of the solvent, the disks were applied on the plates. Figure 4 shows plates with *P. putida* PaW1 and GJ31 after an incubation period of 5 days. Since dark-brown coloration originated from the autoxidation of the catechols, the plates were sprayed with a solution of fluorescein diacetate in *N*,*N*-dimethylformamide.

![FIG. 3. Accumulation of metabolites in the supernatants of 3-fluorocatechol-inhibited cultures of *P. putida* GJ31 as a function of time.](image)
for better visualization of active cells, which are able to hydrolyze fluorescein diacetate to fluorescein. Cells which were spread on mineral medium plates and incubated for 5 days without catechols as the potential growth substrate did not give a significant coloration after the plates were sprayed with fluorescein diacetate, showing that growth is necessary for a positive response. *P. putida* PaW1 was shown to grow only with catechol. Even after 14 days no growth was observed in the presence of 3-chloro- and 4-chlorocatechol. Instead, diffusion of 3-chloro- and 4-chlorocatechol led to an inhibition of the growth of strain PaW1 with catechol so that the growth halo around the disk with catechol was incomplete. In contrast, *P. putida* GJ31 was able to use catechol and 3-chloro- and 4-chlorocatechol as the sole source of carbon and energy. The fastest growth was observed with catechol, resulting in only a small clear zone around the disk without living cells. The zone of growth inhibition was larger with 3-chlorocatechol and reached a maximum with 4-chlorocatechol. A yellow halo was observed during growth of *P. putida* GJ31 with 4-chlorocatechol in the first 24 h, indicating the accumulation of the meta-cleavage product. This yellow coloration disappeared during the following days.

**Production of pyruvate from catechol and 3-chlorocatechol.**

To prove that 3-chlorocatechol was degraded by the meta-cleavage pathway, the amount of pyruvate, which is one of the products of the meta-cleavage pathway, was determined in assay mixtures where crude extract of chlorobenzene-grown cells was converting 3-chlorocatechol or catechol. For this, NADH and lactate dehydrogenase were added to a sample of the assay mixture, and the decrease in the concentration of NADH was determined spectrophotometrically as a measure of the concentration of pyruvate. During conversion of catechol or 3-chlorocatechol by the crude extract, an increasing amount of NADH was consumed after addition of lactate dehydrogenase, showing that pyruvate was formed (Fig. 5). The rate at which pyruvate was formed was much lower than the rate at which 3-chlorocatechol was degraded, indicating accumulation of intermediates.

**Conversion of 3-chlorocatechol to 2-hydroxyisooconic acid by a catechol 2,3-dioxygenase preparation free of 4-oxalocrotonate decarboxylase.** Although 3-chlorocatechol is an intermediate in the degradation of chlorobenzene, it was not possible to identify the product of conversion of 3-chlorocatechol by the catechol 2,3-dioxygenase. Enzymes further downstream in the catabolic pathway are probably converting the conversion product of 3-chlorocatechol at a rate which does not allow significant accumulation of it. The same holds for the conversion product of 3-methylcatechol. One of the possible enzymes which could be responsible for this is a decarboxylase involved in the degradation of 4-oxalocrotonate to 3-oxopentenoate. This enzyme could be detected in crude extract of chlorobenzene-grown cells by measuring the decrease of the keto form of oxalocrotonate at 235 nm.

To determine the product of 3-chlorocatechol conversion by the catechol 2,3-dioxygenase, the decarboxylase was separated from the catechol 2,3-dioxygenase by anionic exchange chromatography. When the fraction containing the catechol 2,3-dioxygenase was incubated with 3-chlorocatechol, a product accumulated; this product was identified as 2-hydroxy-cis,cis-muconic acid by HPLC with standards of 2-chloro-cis,cis-muconic acid, cis,cis-muconic acid, 2-hydroxy-cis,cis-muconic acid, and 2-pyrene-6-carboxylic acid (Fig. 6). After approximately 80 µM 3-chlorocatechol was converted to 2-hydroxy-cis,cis-muconic acid, the conversion stopped. After the addition of new protein, the 3-chlorocatechol conversion resumed and then stopped again (Fig. 6).

**DISCUSSION**

All pure cultures of *Alcaligenes, Pseudomonas, Xanthobacter,* and other bacteria which can use chlorobenzenes as their sole source of carbon and energy degrade them via the modified ortho-cleavage pathway (4, 8, 25, 28, 31, 33, 35, 36, 38), and it
is generally accepted that the meta-cleavage pathway is not suitable for the degradation of haloaromatic compounds. In fact, halocatechols usually are strong inhibitors of the meta-cleavage pathway. 3-Halocatechols can be suicide substrates for the meta-fission enzyme catechol 2,3-dioxygenase of *P. putida* PaW1 (3) or can inactivate the enzyme by chelating the ferrous iron, as in *P. putida* F1 (19). In the first case, an acylhalide is formed which probably acylates the protein and destroys its enzymatic activity. 4-Halocatechols usually yield dead-end products, although there are indications that some organisms are capable of mineralizing catechols chlorinated at the 4 position via the meta-cleavage pathway (1, 11–13, 21). However, information about the type of dechlorination is missing, while in some cases, dechlorination occurred very slowly.

Since toluene is usually metabolized via the meta-cleavage pathway and since chloroaromatics inhibit this route, we decided to study the type of ring cleavage in *P. putida* GJ31. This organism is capable of growth both on chlorobenzene and on toluene, either separately or added in mixtures (24).

Surprisingly, no activity of enzymes of the modified ortho-cleavage pathway could be detected in cells of strain GJ31 grown on chlorobenzene or on any other aromatic substrate. However, a high level of catechol 2,3-dioxygenase was detected in extracts of cells grown on aromatic substrates, including chlorobenzene. This suggested that *P. putida* GJ31 degrades chlorobenzene by a meta-cleavage pathway.

Direct evidence for the degradation of chlorobenzene via meta cleavage of 3-chlorocatechol was obtained by testing the induction of several enzymes of the meta-cleavage pathway in crude extract and by demonstrating the accumulation of catechols in 3-fluorocatechol-inhibited cells. Hydrogen peroxide inhibited the conversion of 3-chlorocatechol in crude extract, which also indicates that a catechol 2,3-dioxygenase is converting 3-chlorocatechol. The product of the meta cleavage was identified to be 2-hydroxy-cis,cis-muconic acid, which is an intermediate of the meta-cleavage pathway, and complete conversion of 3-chlorocatechol to pyruvate was shown to occur in the crude extract. Apparently, *P. putida* GJ31 contains a special catechol 2,3-dioxygenase which is able to convert 3-chlorocatechol to 2-hydroxy-cis,cis-muconic acid and which resists rapid inactivation by the acylchloride, although some inactivation occurs. This allows the organism to use the more efficient meta-cleavage pathway for toluene degradation (26) while chlorobenzene is present. Figure 7 summarizes the data on the pathway used by *P. putida* GJ31 for the degradation of chlorobenzene.

Besides the meta-cleavage pathway, *P. putida* GJ31 probably also possesses an ortho-cleavage pathway for the degradation of catechol, since some activity of enzymes of this route was found in crude extracts of cells grown on benzene or benzoate. The combination of a meta- and an ortho-cleavage pathway has also been observed in *P. putida* mt-2. In this strain, the genes of the meta-cleavage pathway are located on a 117-kb TOL plasmid (pWW0), while the ortho-cleavage pathway is encoded on the chromosome (2). *P. putida* GJ31 also possesses a large plasmid, and mutants of this strain which are unable to grow on chlorobenzene and toluene but which could still grow on benzoate and benzene were isolated (unpublished data). This phenotype could be explained by the loss of meta-cleavage genes, which are possibly encoded on the plasmid.

3-Fluorocatechol was found to be a much stronger inhibitor of the catechol 2,3-dioxygenase of *P. putida* GJ31 than 3-chlorocatechol, although acylfluorides are expected to be less reactive than acylchlorides. Bartels et al. (3) also observed that 3-fluorocatechol is a stronger inhibitor than 3-chlorocatechol for the catechol 2,3-dioxygenase of *P. putida* PaW1. Their results indicated that reactive oxygen species causing additional inactivation are produced during conversion of 3-fluorocatechol, but not during 3-chlorocatechol conversion.

Although it has long been thought that simultaneous conversion of halo- and methylcatechols is not possible and can only be obtained in constructed strains (29), *P. putida* GJ31 is not unique in its ability to simultaneously metabolize chlorobenzene and toluene. *Pseudomonas* sp. strain JS6 has the same capacity but degrades toluene and chlorobenzene via the modified ortho-cleavage pathway (26). The presence of toluene reduced the efficiency of chlorobenzene degradation because of induction of a catechol 2,3-dioxygenase. In contrast, toluene stimulated chlorobenzene degradation by *P. putida* GJ31 in continuous culture, and it was suggested that toluene might be a better inducer of catabolic enzymes (18). The specific activities of the enzymes of the meta-cleavage pathway in crude extracts of batch-grown cultures on chlorobenzene and toluene are comparable, which does not indicate that there are differences in induction levels of these enzymes. However, when *P. putida* GJ31 was pregrown on a mixture of chlorobenzene and toluene, the specific activity of the catechol 2,3-dioxygenase increased approximately threefold, suggesting that a combina-
tion of both substrates leads to a higher induction of the extradiol cleavage enzyme. 

*P. putida* GJ31 appears to dechlorinate 3-chlorocatechol during the extradiol cleavage reaction without cyclization. An alternative would be the formation of a cyclic product from the acylchloride, yielding 2-pyrene-6-carboxylic acid. However, since we did not detect any activity towards 2-pyrene-6-carboxylic acid in crude extract of chlorobenzene-grown cells and since the only metabolite which was detected after conversion of 3-chlorocatechol by partially purified catechol 2,3-dioxygenase was 2-hydroxy-cis,cis-muconic acid, this cyclization probably did not occur. This is in contrast to what was found for an organism described by Kersten et al. (17), who examined bacteria which could grow on 5-chloroanilinate by using a *meta*-cleavage pathway. After conversion of 5-chloroanilinate to 5-chloroprotocatechuate, the latter compound was converted to 2-pyrene-4,6-dicarboxylate by extradiol-cleav-
ing protocatechuate 4,5-dioxygenase. This indicates that cy-
clization of the generated acylhalide can provide an effective alternative to the enzyme suicide which occurs when a nucleo-
philic group of the enzyme undergoes acylation. Degradation then continued via a 2-pyrene-4,6-dicarboxylate hydrolase, fi-
nally resulting in pyruvate and oxaloacetate.

In conclusion, we have shown that *P. putida* GJ31 degrades chlorobenzene via 3-chlorocatechol by using a *meta*-cleavage pathway which contains a catechol 2,3-dioxygenase that is able to convert 3-chlorocatechol to 2-hydroxy-cis,cis-muconic acid. It has been assumed for a long time that *meta* cleavage of halocatechols is not a feasible route (20). Although this may be true for most of the isolates that have been studied, the work described here illustrates that the diversity of organisms and routes makes it difficult to develop such biochemical general-
izations based on the study of individual pathways and organ-
isms. Currently, research focuses on differences between the catechol 2,3-dioxygenase of *P. putida* PaW1 and that of *P. putida* GJ31 which enables the latter to prevent suicide inac-
tivation by the acylchloride that is expected to be formed from 3-chlorocatechol.

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