Microbial degradation of aromatic organic pollutants – A study of dead-end metabolites including the stereochemical mechanistic studies of cycloisomerase enzyme and their products using deuterium labelled precursors

Or

Microbes and the mechanism of degradation of organic pollutants-Deuterium labeling of phenols to unravel the enzyme specificity in the beta-ketoadipate pathway

The microbial degradation of aromatic compounds has tremendous practical significance. Both aerobic and anaerobic microorganisms have been isolated that degrade aromatic compounds, but much more is known about aerobic pathways. In general, degradation proceeds in two phases. First, an aromatic compound is prepared for ring cleavage by a variety of ring modification reactions. Of the many diverse pathways that have been identified, all have in common a mono- or dioxygenation step that results in the formation of a dihydroxylated benzene ring. The second phase of degradation includes ring fission and subsequent reactions leading to the generation of tricarboxylic acid cycle intermediates. Ring fission is catalyzed by dioxygenases and is termed ortho-cleavage when it occurs between the hydroxyl groups (intradiol cleavage) and meta-cleavage when it occurs adjacent to one of the hydroxyls (extradiol cleavage). A third aerobic ring cleavage pathway, the gentisate pathway, is followed when the two hydroxyl groups on the aromatic ring are para to each other, and cleavage occurs between the carboxyl-substituted carbon and the adjacent hydroxylated carbon. Of the three pathways, the best studied are the meta-cleavage pathway and the ortho-cleavage or, as it is commonly known, the [Beta]-ketoadipate pathway. The latter name derives from the fact that [Beta]-ketoadipate is a key intermediate of the ortho-cleavage pathway.

The meta-cleavage and the [Beta]-ketoadipate pathways each catalyze the dissimilation of the archetypal ring cleavage substrates, catechol and protocatechuate. Meta-fission pathway enzymes differ from those of the ortho-pathway in their ability to also catalyze the degradation of methylated catecholic substrates, and thus they have been well studied in connection with the degradation of methylated aromatic hydrocarbons such as toluene and xylene. Modified ortho-cleavage pathways include enzymes that are closely related to those of the [Beta]-ketoadipate pathway but that have evolved to handle chlorinated substrates. These pathways appear to be used primarily for the dissimilation of chlorinated catechols generated from the metabolism of chlorobenzoates, chlorobenzenes, and chlorophenoxyacetate. The modified ortho-cleavage pathways are encoded on catabolic plasmids. Meta-cleavage pathways specifying the degradation of phenol, toluene, and naphthalene have been described that are plasmid encoded. Because they contribute to the degradation of environmental pollutants, the meta-cleavage pathway and modified ortho-pathways have been the subject of our research.

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Prokaryotes

Bacterial representation includes species of the gram-positive organisms of Bacillus, Rhodococcus, Arthrobacter, and Nocardia (133), also the [Beta]-ketoadipate pathway has been examined in most detail in the following gram-negative genera: Acinetobacter (15), Alcaligenes etc.

Eukaryotes

A eukaryotic version of the [Beta]-ketoadipate pathway has been identified in members of the ascomycetous and basidiomycetous yeasts and fungi. The fungal pathway has been studied in Rhodotorula spp., Trichosporon cutaneum, Aspergillus spp., and Neurospora crassa. The catechol branch of the eukaryotic pathway is present in some but not all eukaryotes and, when present, appears to match that of the prokaryotic pathway. The protocatechuate branch differs from its prokaryotic counterpart in that [Beta]-carboxy-cis,cis-muconate, the product of protocatechuate ring cleavage, is cyclized to give [Beta]-carboxymuconolactone rather than y-carboxymuconolactone. The two branches of the fungal [Beta]-ketoadipate pathway converge at [Beta]-ketoadipate rather than at [Beta]-ketoadipate enol-lactone, as in the bacterial pathway

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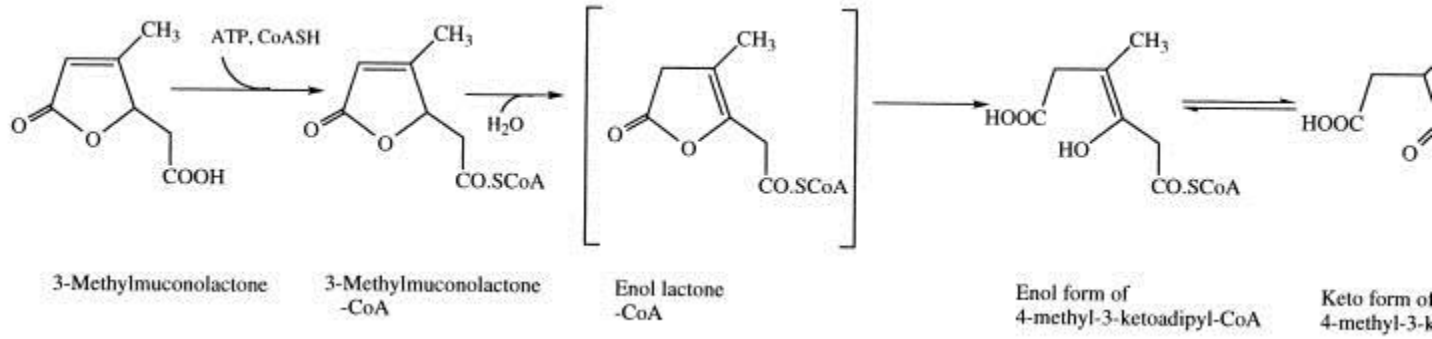
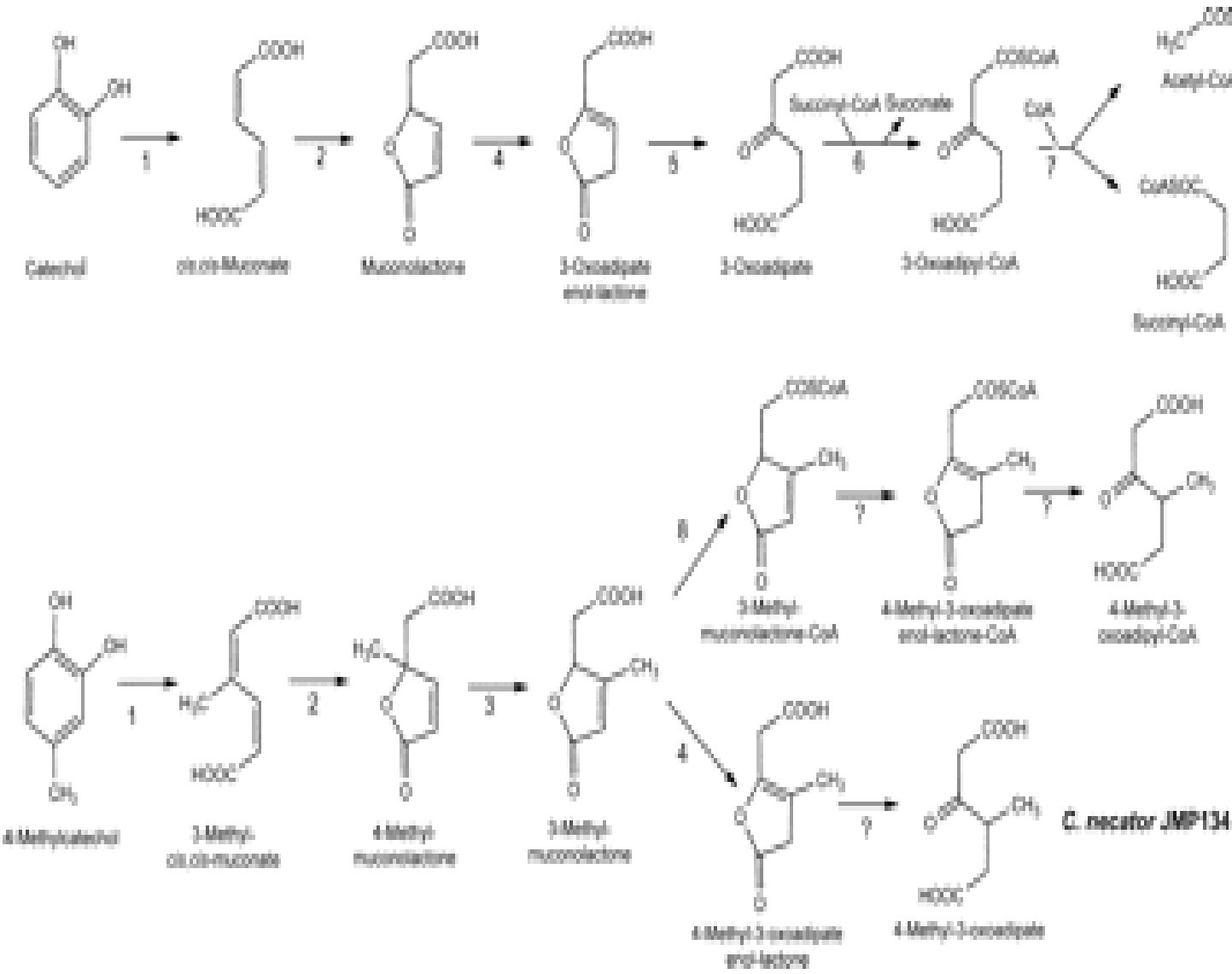


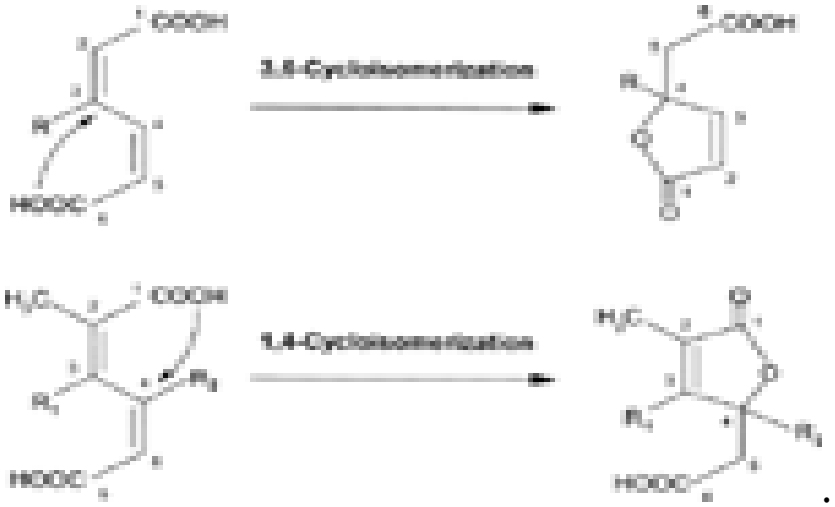
Biological synthesis of 3-methylmuconolactone.
3-Methylmuconolactone was prepared biologically. The yield was 0.413 g (53%) of long white crystals. The ^{1}H nuclear magnetic resonance spectrum of the crystals coincided well with the reference data for 3-methylmuconolactone. Mass spectra indicated a molecular ion with an *m*/*e* of 157, which corresponds to the formula $C_7H_8O_4$ for 3-methylmuconolactone. Purified 3-methylmuconolactone was found to be optically active; the specific rotation of sodium D line at 20°C in methanol was $-28.6 \pm 0.9°$.



Regiospecificity of enzymatic cycloisomerization

The course of enzymatic cycloisomerization of muconates to muconolactones was monitored by HPLC and the resulting products were identified by comparison of their retention times with those of authentic standards. Enzymically prepared muconates were shown to be cycloisomerized to their corresponding muconolactones by the purified cycloisomerase illustrates the types of cycloisomerization which are mediated by the cycloisomerase purified from *R. rhodochrous* N75. HPLC analysis showed that 3-methyl-*cis,cis*-muconate was lactonized to 4-methylmuconolactone by 3,6-intramolecular cycloisomerization, as previously shown with partially purified enzyme. Since 3-ethyl-*cis,cis*-muconate was converted to a lactonic compound which did not co-elute with authentic 3-ethylmuconolactone, it was assumed to be 4-ethylmuconolactone. Therefore, a general reaction formula can be deduced that 3-alkyl-substituted *cis,cis*-muconates are cycloisomerized to 4-alkyl-substituted muconolactones. In contrast, 2-methyl-*cis,cis*-muconate was lactonized to 2-methylmuconolactone by 1,4-intramolecular cycloisomerization. 2,3-Dimethyl-*cis,cis*-muconate and 2,4-dimethyl-*cis,cis*-muconate were also found to undergo 1,4-intramolecular cycloisomerization to yield 2,3-dimethylmuconolactone and 2,4-dimethylmuconolactone, respectively. Schmidt et al. showed that these dimethyllactones were accumulated as dead-end metabolites in the metabolism of 3,4-

dimethylbenzoate and 3,5-dimethylbenzoate, respectively, by *p*-toluate-grown cells of *R. rhodochrous* N75. Likewise, the Gram-negative strain *Ralstonia eutropha* JMP 134 was reported to produce these dimethyllactones via 3,4-dimethylcatechol and 3,5-dimethylcatechol during the metabolism of dimethylphenols, suggesting the 1,4-type cycloisomerization. These results, therefore, suggest that the methyl substitution at the C-2 position of muconate directs the cycloisomerization by 1,4-addition rather than 3,6-addition. The steric influence caused by the methyl substitution may encourage the 1,4-cycloisomerization.



Different regiochemistry of cycloisomerization. R=CH_3, CH_2CH_3, $CH_2(CH_3)_2$, or $CH(CH_3)_3$; R_1=CH_3 and R_2=H, or R_1=H and R_2=CH_3.

R. rhodochrous N75 was known to metabolize 4-methylcatechol via a modified β-ketoadipate pathway. Like catechol 1,2-dioxygenase, the cycloisomerase from *R. rhodochrous* N75 displayed a broad substrate specificity against a range of alkyl-substituted *cis,cis*-muconates though the relative activities could not be quantified due to the instability of those substrates. 3-methyl-*cis,cis*-muconate was non-enzymically cycloisomerized to racemic 4-methylmuconolactone at pH 6.5, whereas Cain et al. discovered that the compound was rapidly transformed to 3-methyl-*cis,trans*-muconate and 3-methylmuconolactone, followed by a slower conversion of the former into the latter. When examined in this study, non-enzymic conversion of 3-methyl-*cis,cis*-muconate to 3-methylmuconolactone was observed. HPLC analysis showed that the product from non-enzymic conversion co-eluted with authentic 3-methylmuconolactone, whereas the product from the enzyme-mediated conversion was shown to be 4-methylmuconolactone. 3-Ethyl-*cis,cis*-muconate also appeared to undergo non-enzymic conversion to 3-ethylmuconolactone.

Bacterial cycloisomerases have been known to catalyze the cycloisomerization of 3-methyl-*cis,cis*-muconate to 4-methylmuconolactone, which resulted in a metabolic block in pseudomonads when metabolizing methyl-substituted aromatics by the *ortho*-cleavage pathway . In contrast, eukaryotic cycloisomerases produce 3-methylmuconolactone from 3-methyl-*cis,cis*-muconate . The cycloisomerase from *R. rhodochrous* N75 was found to display a strict regiospecificity; 3-alkyl-substituted *cis,cis*-muconates were lactonized to form 4-alkyl-substituted muconolactones by 3,6-cycloisomerization, whereas 1,4-cycloisomerization was observed when a methyl group was substituted at the C-2 of muconate.

Muconolactone produced by the purified cycloisomerase from *R. rhodochrous* N75 was characterized by ^{1}H nuclear magnetic resonance studies using chiral reagents (A.G. Sutherland, personal communication). The product was (4*S*)-muconolactone with an enantiomeric excess of 98%. Although the absolute stereochemistry of cycloisomerization in *R. rhodochrous* N75 was not presented in these studies, it can be predicted in an indirect manner based on the known stereochemistry that muconolactone produced from *cis,cis*-muconate has 4*S* absolute configuration muconolactone isomerase from *P. putida* has been known to act only on the (4*S*)-lactone. The product resulting from cycloisomerization of *cis,cis*-muconate by the cycloisomerase from *R. rhodochrous* N75 was observed to be degraded when incubated with muconolactone isomerase and enol lactone hydrolase prepared from recombinant strains of *Escherichia coli* which contained genes encoding the pseudomonad enzymes. The details will be presented in the talk.

We have also prepared deuterium labelled 4-methypyrocatechols as precursors to study the enzyme mechanisms. In doing so, biosynthetically we made deuterium labeled optically active methyl muconolactones using several bacteria and fungi. The results will be presented in the talk. We have purified isomerase enzyme and used the deuterium muconolactones to study its stereospecificity in isomerisation. The results are very interesting and forms the main topic of this presentation