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Degradation of Coniferyl Alcohol and Other Lignin-Related Aromatic Compounds by *Nocardia* sp. DSM 1069

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Abstract. *Nocardia* sp. DSM 1069 was grown on mineral salt media with coniferyl alcohol, 4-methoxybenzoic acid, 3-methoxybenzoic acid or veratric acid as sole sources of carbon and energy. During incubation on coniferyl alcohol, the formation of coniferyl aldehyde, ferulic acid and quantitative accumulation of vanillic acid and protocatechuic acid could be achieved with mutants. Washed cell suspensions of *N.* sp. grown on 4-methoxybenzoic acid, oxidized 4-hydroxybenzoic acid and protocatechuic acid. Cells grown on veratric acid, oxidized vanillic acid, isovanillic acid, and protocatechuic acid. Cell extracts were shown to cleave protocatechuic acid by "ortho-fission".

A mutant without protocatechuate 3,4-dioxygenase activity was not influenced in its growth on 3 methoxybenzoic acid. Cell free extracts of cells grown on 3-methoxybenzoic acid were shown to catalyze the oxidation of gentisic acid (2,5-dihydroxybenzoic acid). The resulting ring cleavage product was further metabolized by a glutathione dependent reaction.

The specificity of the demethylation reactions has been investigated with a mutant unable to grow on vanillic acid. This mutant was not impaired in the degradation of isovanillic acid, 4-methoxy-, or 3-methoxybenzoic acid, whereas growth of this mutant on veratric acid (3,4-dimethoxybenzoic acid) was only half as much as that of the wild type. Concomitantly with growth on veratric acid this mutant accumulated vanillic acid with a yield of about 50%.

A pathway for the catabolism of coniferyl alcohol, involving oxidation and shortening of the side chain, and of 4-methoxybenzoic acid and veratric acid with protocatechuic acid as intermediate is being proposed. A second one is proposed for the degradation of 3-methoxybenzoic acid with gentisic acid as intermediate.

Key words: Coniferyl alcohol – Veratric acid – Demethylation – β-Ketoadipate pathway – Gentisate pathway – *Nocardia.*

Much information is available on the degradation of aromatic carboxylic acids by soil inhabiting pseudomonads and to a lesser extent by Gram-positive bacteria (for reviews see Stanier et al., 1966; Dagley, 1971). Despite the fact that methoxylated aromatic compounds are abundant in nature in the form of lignin and lignin fragments (Freudenberg and Neish, 1968), there are only a few investigations on the degradation of such compounds.

The degradation of two methoxylated benzoic acids has been investigated by Crawford et al. (1973) with *Nocardia corallina.* In its metabolism by this organism veratric acid is demethylated to a mixture of vanillic and isovanillic acid. Both these isomers are demethylated to yield the ring fission substrate protocatechuic acid, which is also an intermediate during the metabolism of 4-methoxybenzoic acid. Similarly, during growth of *N.* sp. DSM 43251 on vanillic acid and 4-methoxybenzoic acid, protocatechuic acid was found to be transiently formed (Engelhardt et al., 1979). Like the strain of *N. corallina*, the *N.* sp. DSM 43251 cleaved protocatechuic acid by "ortho-fission".

With a *Pseudomonas acidovorans* the degradation of a more complex methoxylated aromatic compound containing the propanoid-side chain characteristic for lignin precursors has been investigated. Toms and Wood (1970) found that the organism catabolized ferulic acid by removing part of the side chain to yield vanillic acid. The demethylation of this acid resulted in the formation of protocatechuic acid, which was cleaved by "meta-fission".

Since Gram-positive bacteria are able to attack lignin (Trojanowski et al., 1977; Haider et al., 1978), there is a need to extend investigations on the degradation of lignin-related methoxylated compounds by these organisms. The present paper describes the catabolism of some methoxylated aromatic compounds by *N.* sp. DSM 1069. These compounds include the phenyl-propanoid-like structure coniferyl alcohol, whose degradation has not been investigated so far.

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Since the demethylation reactions might be of importance for lignin degradation, special attention is paid to the specificity of these reactions.

Materials and Methods

Organism and Isolation of Mutants. Isolation and characterization of *Nocardia* sp. DSM 1069 have been described previously by Trojanowski et al. (1977). Mutants were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG): log phase cells of the wild type were suspended at a titer of 2×10^6 cells per ml in complete medium (Difco 0711), pH 6.2. NTG was added to a final concentration of 1 mg/ml and cultures incubated for 20 min at 27° C. The mutagenized cells were washed, spread on complete medium and mutants selected by replica-plating onto mineral salt plates containing either ferulic acid, vanillic acid, protocatechuic acid or glucose as substrate. All mutants isolated were characterized by their growth in liquid cultures on various substrates and substrate mixtures.

Culture Conditions and Preparation of Cell-free Extracts. Shake flask cultures were grown at 27° C in a double baffled Erlenmeyer flask (500 ml) containing 100 ml medium. The mineral salt medium contained (per liter): 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.5 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.015 g $FeCl_3$, 0.5 mg $MnSO_4 \cdot H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $CaCl_2$, 0.1 mg KCL, 0.5 mg H_3BO_3, adjusted to pH 7.0 with 1 N HCl and 300 mg of the filter sterilized carbon and energy source. Cultures were inoculated with about 10^8 cells derived from a preculture grown on complete medium. Growth was followed by nephelometry, measuring the optical density at 600 nm. For preparation of cell-free extracts, cells of the early stationary phase were harvested by centrifugation, washed twice with 100 mM potassium phosphate buffer, pH 7.5, and resuspended in 3 ml of the same buffer. The cell suspension thus obtained was frozen to −20° C and the cells were disrupted using an X-press (0.8 mm orifice). After centrifugation of the resulting homogenate (40.000 × g, 30 min, 4° C), the supernatant was used for enzyme determinations.

Enzyme Assays. Gentisate 1,2-dioxygenase (EC 1.13.1.4) was assayed essentially as described by Cain (1968). Cuvettes contained in a total volume of 3 ml: 250 µmol potassium phosphate buffer, pH 7.0, 3 µmol sodium gentisate and cell-free extract. The oxidation was followed by the increase in absorbance at 330 nm due to the formation of maleylpyruvate using a molar extinction coefficient of 13.370 $M^{-1} \cdot cm^{-1}$ (Lack, 1959). Protocatechuate 3,4-dioxygenase activity (EC 1.13.1.3) was determined spectrophotometrically by the increase in absorbance at 290 nm ($\varepsilon = 2.300\ M^{-1} \cdot cm^{-1}$) (Fujisawa and Hayaishi, 1968). The assay system contained, in a total volume of 3 ml: 150 µmol Tris-acetate buffer, pH 7.5; 1.2 µmol protocatechuic acid and extract. Protocatechuate 4,5-dioxygenase was assayed at pH 9.0 according to Nozaki et al., 1970, protocatechuate 2,3-dioxygenase according to Crawford (1975a). All assays were carried out at 30° C.

Protein was determined by the Folin-Ciocalteau method (Lowry et al., 1951), after precipitation of the protein as described by Bensadoun and Weinstein (1976). Specific activity is expressed as µmol substrate converted per min per mg protein.

Oxygen-uptake Measurements. O_2 consumption by whole-cell suspensions was determined with a Clark-type O_2 electrode. The total volume in the reaction vessel was 3 ml, and contained 200 µmol potassium phosphate buffer, pH 7.5, and about $2 \cdot 10^8$ washed cells of the late exponential growth phase. After equilibration at 30° C and determination of the endogenous rate of O_2 uptake, reactions were started by injection of 5 µmol of substrates.

$^{14}CO_2$ Measurements. The following device was used to aerate shake flask cultures under sterile conditions and to trap the CO_2 evolved. An open bottle filled with soda lime was connected via silicone tubing, with built-in gas-sterilizing filter, to a glass tube inserted in a rubber stopper which fitted the culture flask. A second glass tube inserted – the air outlet – was connected to a gas washing flask filled with 50 ml 1 N NaOH to trap the CO_2 evolved. This flask was connected to a pump, which was continuously sucking air through the whole system at a rate of 100 l/h. At appropriate time intervals 5 ml samples of the NaOH solution were taken and the washing flask refilled. The samples were mixed with 10 ml Insta-Gel and counted in a liquid scintillation counter.

Chromatography. For qualitative analyses, culture filtrates were acidified to pH 2.0 with H_2SO_4 and extracted with chloroform/acetone (1/1) or di-butyl ether. The extracts were washed with water and dried over anhydrous Na_2SO_4. Solvents were removed at 50° C in a vacuum evaporator and residues redissolved in a small volume of methanol. Chromatograms were run on thin layer plates (Merck, silica gel 60, F_{254}) in either benzol/methanol/acetic acid solvent (90/16/8) or di-ethyl ether/acetic acid solvent (90/9). Compounds were visualized under UV-light ($\lambda_{max} = 254$ nm or 366 nm).

Quantitative analysis was performed by liquid chromatography without prior extraction, using a Hewlett Packard HPLC, model 1084b chromatograph. Compounds were separated by reversed phase chromatography (RP-8, stationary phase) with various ratios of methanol/0.005 M KH_2PO_4 pH 3.2 as eluant. Separation of coniferyl alcohol, coniferyl aldehyde, *cis*-, and *trans*-ferulic acid was achieved by isocratic elution with 34% methanol at a flow rate of 1.5 ml/min.

Results

Oxygen-Uptake Experiments with Nocardia Grown on Different Methoxylated Compounds

Coniferyl alcohol and 4-methoxybenzoic acid were used for growth by *N.* sp. DSM 1069, allowing doubling times of about 4.5 h. Only slow growth was obtained with 3-methoxybenzoic acid, which supported a doubling time of 7 h. Freshly harvested cells of the organism grown with coniferyl alcohol or 4-methoxybenzoic acid accomplished the oxidation of their growth substrate and of protocatechuic acid, suggesting this compound to be an intermediate during degradation (Table 1). In contrast, when 3-methoxybenzoic acid was employed as a growth substrate, the cells had no capacity to oxidize protocatechuic acid, although oxygen uptake with 3-hydroxybenzoic acid was observed.

It has been found that several *Pseudomonas* species utilized their growth substrate 3-hydroxybenzoic acid by hydroxylation in the 6-position to give 2,5-dihydroxybenzoic acid (gentisic acid) (Tanaka et al., 1957; Hareland et al., 1975). Similarly, gentisic acid has been identified in culture fluids of some *Actinomycetes* during growth on 3-hydroxybenzoic acid (Hammann, 1977). The data in Table 1 show that cells of *N.* sp. DSM 1069 grown on 3-methoxybenzoic acid accomplished the rapid oxidation of 2,5-dihydroxybenzoic acid. On the basis of this observation the *Nocardia* metabolizes 3-methoxybenzoic acid most likely by

Table 1. Oxygen uptake by freshly harvested cells of *Nocardia* grown on various substrates

Cells grown on	nmol O_2 uptake/min with				
	Growth-substrate	Protocatechuic acid	4-Hydroxy-benzoic acid	3-Hydroxy-benzoic acid	2,5-Dihydroxy-benzoic acid
Coniferyl alcohol	4	36	0	0	0
Protocatechuic acid	–	25	0	0	0
3-Methoxybenzoic acid	4	0	0	6	20
4-Methoxybenzoic acid	25	8	16	0	0

Table 2. Specific activities (μmol O_2/min · mg) of protocatechuate 3,4- and gentisate 1,2-dioxygenase in extracts prepared from cells of *Nocardia* grown on various substrates

Substrate	Proto-catechuate 3,4-dioxygenase	Gentisate 1,2-dioxygenase
Coniferyl alcohol	0.46	0.00
4-Methoxybenzoic acid	0.35	0.00
3-Methoxybenzoic acid	0.00	1.20
3,6-Dihydroxybenzoic acid	0.00	0.52
Glucose	0.00	0.00
Protocatechuic acid	0.38	0.00

demethylation to yield 3-hydroxybenzoic acid, which is hydroxylated to give gentisic acid.

Protocatechuate- and Gentisate-Dioxygenase Activities

Further confirmation on the metabolism of the methoxylated compounds was obtained by the determination of key enzymes. Consistent with the oxygen-uptake experiments protocatechuate 3,4-dioxygenase activity was observed in cell-free extracts prepared from cells grown on coniferyl alcohol or 4-methoxybenzoic acid (Table 2).

These extracts were also proved for 2,3- and 4,5-cleaving activity which was shown to occur in some Gram-negative bacteria (Stanier et al., 1966), but no "meta-fission" activity could be detected.

Extracts of cells grown on 3-methoxy- or 2,5-dihydroxybenzoic acid contained gentisate 1,2-dioxygenase activity, that was virtually absent from cells grown on protocatechuic acid or glucose (Table 2). These activities were calculated from the initial increase in extinction at 330 nm, due to the formation of maleylpyruvate (Lack, 1959). When reaction mixtures were incubated for longer periods, however, a slow decrease in absorbance could be observed. This was not found when dialyzed extracts were used (Fig. 1).

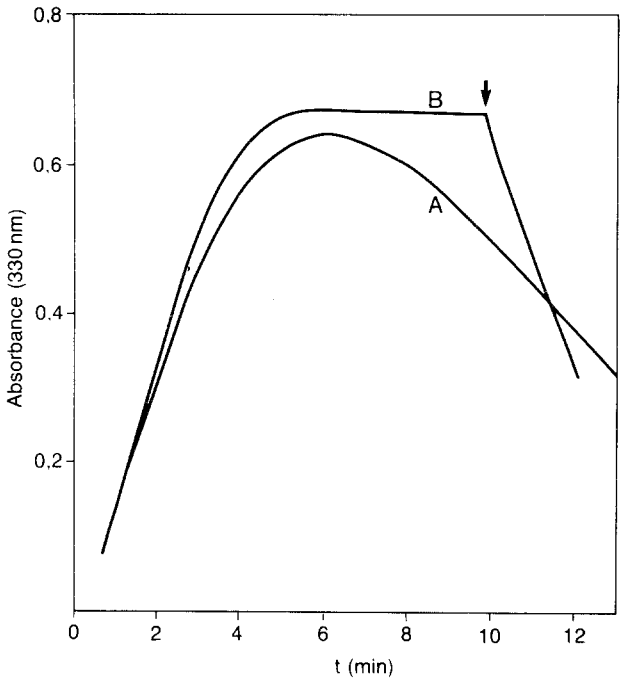

Fig. 1. Formation of maleylpyruvate by extracts (*A*) and dialyzed extracts (*B*) of 3-methoxybenzoic acid-grown *Nocardia*, followed spectrophotometrically. At arrow reduced glutathione was added to a final concentration of 1 mM

Moreover, the decrease could be partially inhibited when reaction mixtures were supplemented with 1 mM of the sulfhydryl-binding agent N-ethylmaleimide, which was shown by Crawford and Frick (1977) to prevent the reduced glutathione dependent conversion of maleylpyruvate to fumarylpyruvate. Addition of excess glutathione to assay mixtures (Fig. 1) resulted in a rapid decrease in absorbance, thus indicating the *cis, trans* isomerization of maleylpyruvate to fumarylpyruvate during degradation of gentisic acid.

Accumulation of Intermediates by Mutants

It has been shown that in *Pseudomonas* species some enzymes for the degradation of aromatic compounds are subject to gratious induction under various growth

Table 3. Growth of mutants in liquid cultures on different substrates. (+) growth like wild type, (−) no growth

Mutant	Coniferyl alcohol	Protocatechuic acid	4-Methoxy-benzoic acid	3-Methoxy-benzoic acid	Vanillic acid	Isovanillic acid
580	−	−	−	+	−	−
37/1	−	+	+	+	+	+
V_5	−	+	+	+	−	+

Table 4. Oxygen uptake by cells of *Nocardia* with different methoxylated benzoic acids

Cells grown on	O_2-uptake (nmol/min)			
	Vanillic acid	Isovanillic acid	Veratric acid	3-Methoxybenzoic acid
Vanillic acid	18	0	1	0
Isovanillic acid	0	22	12	0
Veratric acid	12	14	17	0
3-Methoxybenzoic acid	0	0	1	4

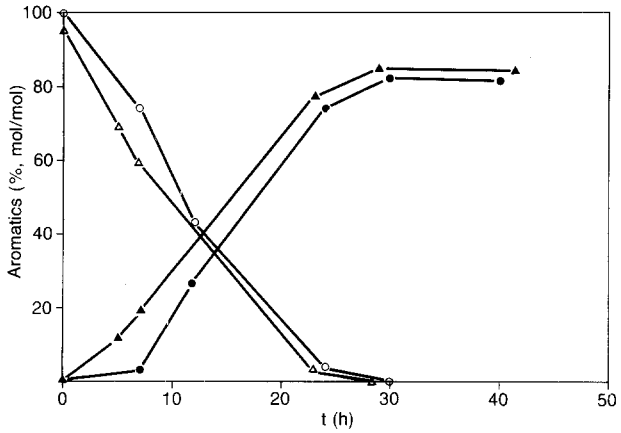

Fig. 2. Conversion of ferulic acid (△——△) to protocatechuic acid (▲——▲) by mutant 580 and formation of vanillic acid (●——●) at the expense of ferulic acid (○——○) by mutant V_5

conditions (Ornston and Stanier, 1966; Kemp and Hegeman, 1952). This phenomenon is also known for *N. opaca* (Rann and Cain, 1973), so that the presence of protocatechuate 3,4-dioxygenase activity is no complete evidence that protocatechuic acid is an intermediate of the presumed pathway. A transient accumulation of protocatechuic acid (more than 20% of the growth substrate) has been reported during growth of *N.* sp. DSM 43251 on phtalic and terephtalic acid (Engelhardt et al., 1979). With the *Nocardia* species DSM 1069, however, a formation of intermediates could not be observed during growth on the methoxylated compounds investigated. Therefore, three different types of mutants have been isolated to substantiate the suggested degradation pathways (Table 3).

Mutants lacking protocatechuate3,4-dioxygenase activity (represented by mutant 580) were found to be unable to grow on coniferyl alcohol, ferulic acid, or 4-methoxybenzoic acid, whereas growth on 3-methoxybenzoic acid was not affected. When cells of these mutants were incubated on one of these methoxylated compounds (except 3-methoxybenzoic acid) the culture fluid turned blue-violet, due to the accumulation of protocatechuic acid. As shown in Fig. 2, nearly quantitative conversion of ferulic acid to protocatechuic acid could be achieved with the mutant 580. In culture filtrates of this mutant, incubated with 4-methoxybenzoic acid, also 4-hydroxybenzoic acid could be identified besides protocatechuic acid. 4-Methoxy-3-hydroxybenzoic acid, found with *N. corallina* during incubation with 4-methoxybenzoic acid (Crawford et al., 1973), could not be found.

To investigate the degradation of coniferyl alcohol to protocatechuic acid, a mutant has been isolated (mutant 37/1) which was unable to grow on coniferyl alcohol. When cells of this mutant were incubated with the alcohol for 20 h, analysis of the culture fluid revealed the formation of ferulic acid, as identified by cochromatography on thin layers. Besides residual coniferyl alcohol and formed ferulic acid with R_F of 0.15 and 0.30, respectively, in the di-ethyl ether/acetic acid solvent system, a third intense spot with intermediate R_F of 0.20 could be observed. This compound, which showed fluorescence after excitation with UV-light of 366 nm, gave positive reaction when sprayed with 2,4-dinitrophenylhydrazine, and was therefore most likely coniferyl aldehyde.

The third type of mutant (mutant V_5), unable to grow on coniferyl alcohol or ferulic acid, was also unable to grow on vanillic acid, but was not affected in its growth on protocatechuic acid. This blocked mutant

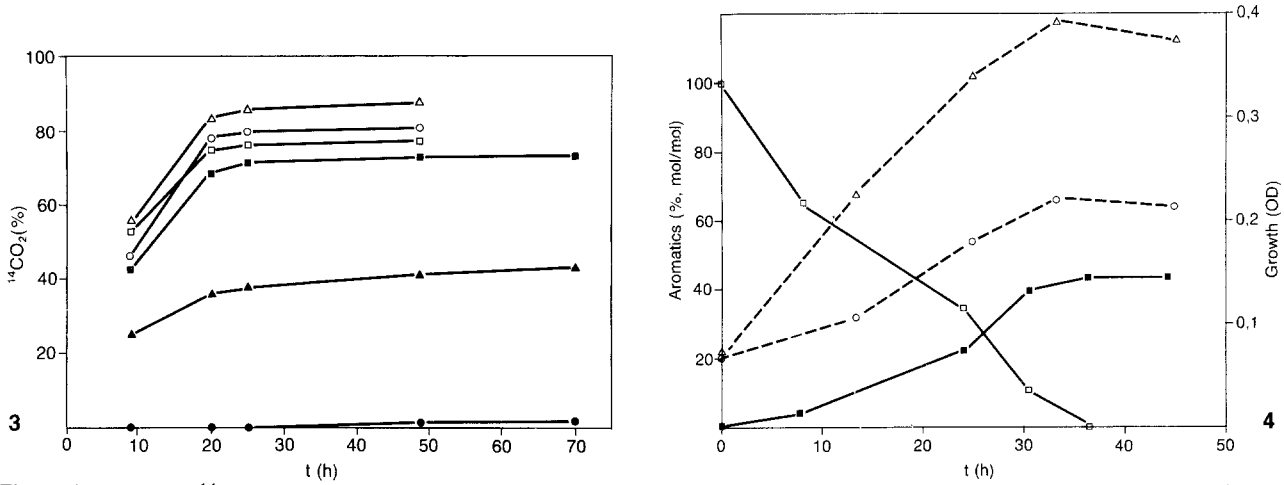

Fig. 3. Time course of $^{14}CO_2$ evolution by the wild type (*open symbols*) and the vanillic acid negative mutant V_5 (*filled symbols*) from methoxyl-labelled vanillic acid (○/●), 3-methoxyl-labelled veratric acid (△/▲), and 4-methoxyl-labelled veratric acid (□/■)

Fig. 4. Accumulation of vanillic acid (■——■) at the expense of veratric acid (□——□) by mutant V_5, with concomitant growth (○- - - -○). Growth of the wild type on veratric acid (△- - - -△)

converted ferulic acid to vanillic acid, which accumulated with a molar yield of about 80% (Fig. 2).

In the culture fluid of this mutant no remaining ferulic acid could be detected, although the remaining amounts calculated stayed within the limits of detection. Consistent with the complete disappearance of ferulic acid, various unidentified spots have been found on thin layers. In order to study whether or not *Nocardia* is capable of attacking ferulic acid by removing the side chain as well as by demethylation of the 3-methoxyl-group, attempts were made to demonstrate the formation of caffeic acid, which is the direct demethylation product of ferulic acid. As was revealed by cochromatography, however, caffeic acid could not be detected in culture fluids of the wild type and mutants incubated with ferulic acid, even if the wild type was pregrown on vanillic acid. Accordingly, attack on the methoxyl-group is of limited importance for the first step in ferulic acid degradation.

Specificity of Demethylation and Degradation of Veratric Acid

Whole cells of *Nocardia* sp. DSM 1069 grown with vanillic acid have no capacity to oxidize immediately isovanillic acid (Table 4). Similarly, isovanillic acid-grown cells were incapable of oxidizing vanillic acid. Consequently, the growth of mutant V_5, being unable to grow on vanillic acid, was not affected on 4-methoxybenzoic acid or isovanillic acid, which shows that in *N.* sp. different demethylases may be responsible for methoxyl-groups located in either the 3- or 4-position of the corresponding benzoic acids. With the purified enzyme system of *Pseudomonas putida* grown on 4-methoxybenzoic acid, Bernhardt et al. (1970) demonstrated that the demethylase reacted only with methoxyl-groups in the 4-position, but not with those of the 3- or 2-position. Moreover, in *N.* sp. the demethylation, at least that of the 3-methoxyl-group seems to be dependent on the adjacent substituent, since vanillic acid grown cells were unable to oxidize 3-methoxybenzoic acid or veratric acid.

This hypothesis was supported when studying the CO_2 evolution from vanillic acid and veratric acid, ^{14}C-labelled in the methoxyl-groups. Cells of the mutant V_5, incubated with vanillic acid, were unable to convert the methoxyl-group to CO_2 (Fig. 3), which is indicative of a defect in the vanillic acid demethylase. However, when cells of this mutant were incubated with 3-methoxyl-labelled veratric acid, about 40% of the methoxyl-group was converted to CO_2, which reflects the influence of the substituent in the 4-position on the 3-methoxyl-group. In contrast, the wild type converted even 80% of the 3-methoxyl-group to CO_2, whereas the 4-methoxyl-group was converted to CO_2 by both the wild type and mutant to about 80%. Therefore, 40% of the veratric acid employed was demethylated to protocatechuic acid. Consistent with these data, growth of the mutant was possible on veratric acid to a limited extend (Fig. 4). In stationary cultures no veratric acid could be detected in the culture fluid, but along with the disappearance of veratric acid, vanillic acid was formed, which accumulated with a yield of about 40%, based on the amount of veratric acid used. This observation is consistent with the assumption that different enzyme systems may be responsible for the demethylation of vanillic acid and the 3-methoxyl-group of veratric acid. From these results, together with those of the oxygen uptake experiments, it is further concluded that veratric acid is degraded to yield an equimolar mixture of vanillic and isovanillic acid.

Discussion

The lignin degrading bacteria *Nocardia* sp. DSM 1069 used in this study has been shown by Trojanowski et al. (1977) to evolve CO_2 from both the methoxyl-group of coniferyl alcohol and the side chain. This is in conformity with two possible degradation mechanisms of coniferyl alcohol, i.e. initial attack from either the methoxyl-group or the side chain. Attack on the methoxyl-group would result in the formation of a C-4 substituted catechol, which might then be degraded by ring splitting. Such a mechanism has been found with *Pseudomonas fluorescens* (Seidman et al., 1969). This organism degrades caffeic acid by "ortho-fission" to give a *cis, cis*-muconic acid, bearing the intact side chain of caffeic acid. Attack on the nucleus of coniferyl alcohol by such reaction might be of interest for the degradation of coniferyl alcohol integrated within the lignin polymer. *Nocardia*, however, initially attacks the alcohol by successive oxidation of the side chain via the aldehyde to yield ferulic acid. This acid is not demethylated either, but further degraded by removing part of the side chain to yield vanillic acid (Fig. 5). After these initial steps of degradation, the ring is prepared for fission by demethylation to give protocatechuic acid. A similar reaction sequence for the degradation of ferulic acid was proposed by Toms and Wood (1970). These authors found that extracts of *P. acidovorans* catalyzed the formation of acetate and vanillic acid from ferulic acid. Similarly, *P. testosteroni* was found to attack 4-hydroxycinnamic acid first by shortening the side chain, which is then followed by hydroxylation of the nucleus to give protocatechuic acid (Karanth and Reber, 1979). Protocatechuic acid is oxidized by *N.* sp. DSM 1069 by 3,4-cleavage as was also observed with *N. opaca* and other *Nocardia* species investigated by Rann and Cain (1973).

The β-ketoadipate pathway with protocatechuic acid as a central intermediate is also used during growth of the organism on 4-methoxybenzoic acid and veratric acid. Degradation of 4-methoxybenzoic acid is initiated by demethylation and subsequent hydroxylation to yield protocatechuic acid. (Fig. 5). In contrast, Crawford (1973) found that *N. corallina* simultaneously hydroxylates and demethylates 4-methoxybenzoic acid to give a mixture of isovanillic and 4-hydroxybenzoic acid. Assuming high substrate specificities of hydroxylases, this would mean different enzymic compositions of the two Nocardiae during growth on 4-methoxybenzoic acid. Interestingly, the degradation of veratric acid proceeds via vanillic and isovanillic acid. Both isomers have also been found in culture fluids of *N. corallina* grown on veratric acid (Crawford, 1973). Hence, *Nocardia* could be capable of initiating the degradation of veratric acid in either the 4- or 3-position. However, the organism is not able to use both ways independently of each other, since the vanillic acid negative mutant converted veratric acid in a constant amount of nearly 50% to vanillic acid which accumulated. This fact remains unexplained, but is indicative of a coupled process which results in the formation of equimolar amounts of vanillic and isovanillic acid.

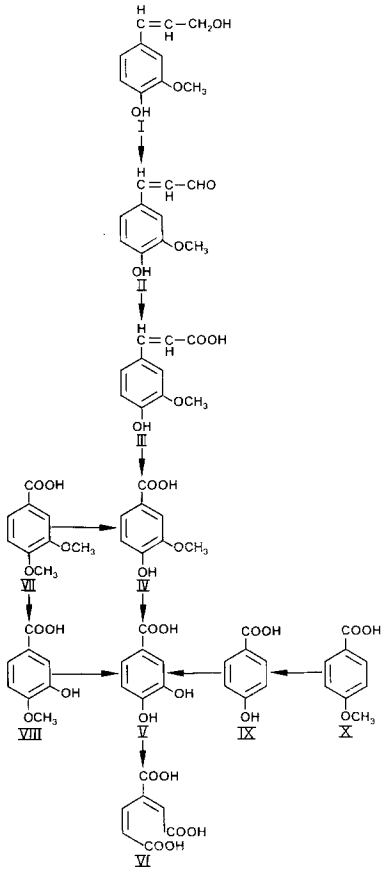

Fig. 5. Proposed pathways for the degradation of coniferyl alcohol, veratric acid and 4-methoxybenzoic acid. *I*, coniferyl alcohol; *II*, coniferyl aldehyde; *III*, ferulic acid; *IV*, vanillic acid; *V*, protocatechuic acid; *VI*, β-carboxy-*cis*, *cis*-muconic acid; *VII*, veratric acid; *VIII*, isovanillic acid; *IX*, 4-hydroxybenzoic acid; *X*, 4-methoxybenzoic acid

The degradation of 3-methoxybenzoic acid is effected by a pathway entirely different from that of 4-methoxybenzoic acid (Fig. 6). The first step is again demethylation, but then *N.* sp. DSM 1069 hydroxylates the 6-position of the nucleus to give gentisic acid. This dihydroxybenzoic acid was also found as an intermediate during the metabolism of 3-hydroxybenzoic acid by certain *Pseudomonas* and *Bacillus* strains (Hopper and Chapman, 1970; Hareland et al., 1975; Crawford, 1975b). Furthermore, *N. opaca* was shown by Cain (1968) to metabolize, at least partially by a side reaction, 2-aminobenzoic acid via gentisic acid.

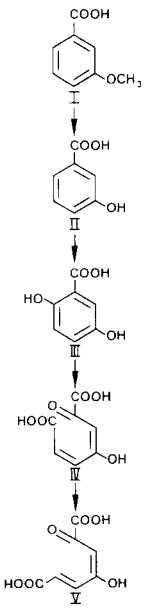

Fig. 6. Proposed pathway of 3-methoxybenzoic acid degradation by *Nocardia* sp. *I*, 3-methoxybenzoic acid; *II*, 3-hydrobenzoic acid; *III*, 2,5-dihydroxybenzoic acid (gentisic acid); *IV*, maleylpyruvate; *V*, fumarylpyruvate

According to Cain the organism uses this gentisate pathway or the alternative catechol pathway depending upon the culture conditions. The further sequence of reactions involves ring cleavage to yield maleylpyruvate and most likely the subsequent reduced glutathione-dependent isomerization to fumarylpyruvate. Thus, *N.* sp. DSM 1069 catabolizes gentisic acid by mechanisms similar to those acting in *N. opaca* and *P. acidovorans*, whereas most Bacilli strains investigated catabolized maleyl-pyruvate by glutathione-independent mechanisms (Crawford, 1975b; Clark and Buswell, 1979).

The present work shows the multiple metabolic activities of *N.* sp. DSM 1069 during growth on different aromatic compounds structural related to lignin. Further work will show the relevance of these activities for attack of the organism on the aromatic polymer.

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On the basis of the analysis of lipid and cell wall components, and auxanographic investigations the *Nocardia* sp. under investigation has now been classified as a *Rhodococcus erythropolis*. Rhodococcus is a new genus within the Actinomycetales which includes former members of the Nocardiaceae, Mycobacteriaceae and Corynebacteriaceae. We are grateful to Prof. K. P. Schaal from the Hygiene Institut, Köln for identification of the organism.