

Biodegradation of *n*-Alkylcycloalkanes and *n*-Alkylbenzenes via New Pathways in *Alcanivorax* sp. Strain MBIC 4326

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The degradation of long-chain *n*-alkylbenzenes and *n*-alkylcyclohexanes by *Alcanivorax* sp. strain MBIC 4326 was investigated. The alkyl side chain of these compounds was mainly processed by β -oxidation. In the degradation of *n*-alkylcyclohexanes, cyclohexanecarboxylic acid was formed as an intermediate. This compound was further transformed to benzoic acid via 1-cyclohexene-1-carboxylic acid.

Crude oil is a complex mixture containing a homologous series of alkylated hydrocarbons, such as *n*-alkylcyclohexanes, *n*-alkylcyclopentanes, and *n*-alkylbenzenes (7, 10, 14, 16–19, 22, 24–28, 30). These hydrocarbons are degraded by a number of fungi and bacteria (1–3, 6, 8, 11–13, 20, 21, 23, 29). Generally, the terminal methyl group of a long *n*-alkyl side chain of these compounds is initially oxidized to a carboxylic group, which is followed by classical β -oxidation to form carboxylic or acetic acid derivatives, depending on whether there is an odd or even number of carbons in the alkyl side chain (3, 29). In *Acinetobacter lwoffii*, for example, *n*-dodecylbenzene was completely degraded via phenylacetic acid and homogentisic acid, while *n*-tridecylbenzene was transformed via 3-phenylpropionic acid to *trans*-cinnamic acid, which was the dead-end product (1).

We investigated the degradation of *n*-alkylbenzenes and *n*-alkylcyclohexanes in *Alcanivorax* sp. strain MBIC 4326. This strain was isolated from Kamaishi Bay seawater and closely related to *Alcanivorax borkumensis*, the type strain of the genus *Alcanivorax* (31). This strain grew on BSM medium (8) supplemented with 1 g of *n*-octadecylcyclohexane (compound 1 in Fig. 1), *n*-nonadecylcyclohexane (compound 2), *n*-undecylbenzene (compound b in Fig. 2), or *n*-hexadecylbenzene (compound a) per liter as the sole source of carbon and energy. These cultures were acidified to pH 2 with concentrated hydrochloric acid and extracted three times with an equal volume of dichloromethane. The combined extracts were evaporated in a rotary evaporator to dryness under reduced pressure. The residues of each dichloromethane extract were methylated with a boron trifluoride-methanol solution (Supelco) prior to an analysis by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was performed as described previously (8, 9).

Both cyclohexanecarboxylic acid (compound 10) and cyclohexaneacetic acid (compound 9) accumulated in the cultures grown on *n*-octadecylcyclohexane (compound 1) and *n*-nonadecylcyclohexane (compound 2), indicating that these *n*-alkylcyclohexanes were transformed by following more than a single pathway (Table 1). One pathway might be involved in the oxidation of the terminal methyl group of the alkyl side chain

to a carboxylic group, with subsequent β -oxidation yielding cyclohexaneacetic acid (compound 9) from *n*-octadecylcyclohexane and cyclohexanecarboxylic acid (compound 10) from *n*-nonadecylcyclohexane (compound 2). The formation of cyclohexaneacetic acid (compound 9) from *n*-nonadecylcyclohexane (compound 2) and of cyclohexanecarboxylic acid (compound 10) from *n*-octadecylcyclohexane (compound 1) (Table 1) cannot be explained by β -oxidation. This point is discussed later in this paper.

Trace amounts of 1-cyclohexene-1-carboxylic acid (compound 12) and benzoic acid (compound 13) were also detected in the cultures grown on *n*-octadecylcyclohexane (compound 1) and *n*-nonadecylcyclohexane (compound 2) (Table 1), indicating that cyclohexanecarboxylic acid (compound 10) was further metabolized.

Alcanivorax sp. strain MBIC 4326 did not grow on 4-cyclohexylbutanoic acid (compound 3) and 3-cyclohexylpentanoic acid (compound 4) as the sole sources of carbon and energy. Thus, the strain was grown on *n*-hexadecane (0.5 g/liter) in BSM medium for 5 days. One gram of 4-cyclohexylbutanoic acid (compound 3) or 3-cyclohexylpentanoic acid (compound 4) per liter was subsequently added, and the cultivation was continued for different periods of time. The major product of the 4-cyclohexylbutanoic acid degradation was cyclohexaneacetic acid (compound 9), which could have been formed by β -oxidation (Table 2). 4-Cyclohexyl-2-butenic acid (compound 5) detected in a trace amount could also have been formed by β -oxidation, while the formation of other intermediates, 4-cyclohexyl-3-butenic acid (compound 6) and cyclohexanecarboxylic acid (compound 10), suggested the low probability of another type of degradation. The same conclusion was obtained by examining the biodegradation of 5-cyclohexylpentanoic acid (compound 4). The detection of cyclohexanecarboxylic acid (compound 10) as the major product and 3-cyclohexylpropionic acid (compound 11) and 5-cyclohexyl-2-pentenoic acid (compound 7) as minor products supported the β -oxidation pathway, while the formation of cyclohexaneacetic acid (compound 9) and 5-cyclohexyl-3-pentenoic acid (compound 8) suggested another type of degradation.

Besides these observations, the formation of 1-cyclohexene-1-carboxylic acid (compound 12) and benzoic acid (compound 13) from 5-cyclohexylpentanoic acid (compound 4) was observed, suggesting the transformation of cyclohexanecarboxylic acid (compound 10) to these products. This was confirmed by

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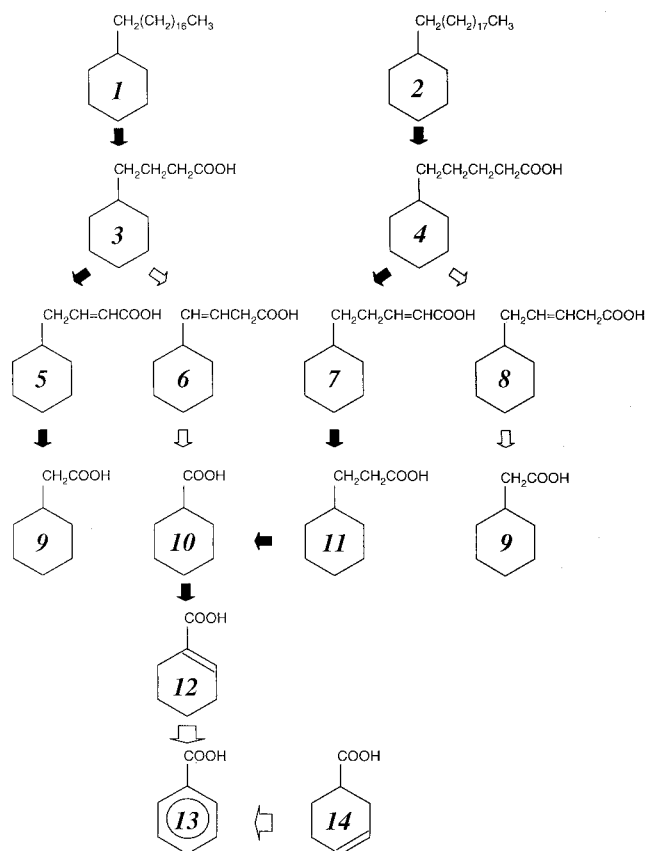


FIG. 1. Proposed pathways for the biodegradation of *n*-alkylcyclohexanes by *Alcanivorax* sp. strain MBIC 4326. β -Oxidation routes shown to be major metabolic routes are indicated by solid arrows, while minor routes are indicated by open arrows. Novel metabolic routes are indicated by larger open arrows. Compound numbers are defined in the text.

cultivating *Alcanivorax* sp. strain MBIC 4326 on 0.5 g of *n*-hexadecane per liter in the presence of 1 g of cyclohexanecarboxylic acid (compound 10) per liter. Cyclohexanecarboxylic acid (compound 10) was converted to 1-cyclohexene-1-carboxylic acid (compound 12) and benzoic acid (compound 13) (Table 2). When benzoic acid (compound 13) or cyclohexanecarboxylic acid (compound 9) was used as a cosubstrate for the culture of *Alcanivorax* sp. strain MBIC 4326 grown on *n*-hexadecane, the transformation of these cosubstrates was not observed.

The metabolism of cyclohexanecarboxylic acid (compound 10) to 1-cyclohexene-1-carboxylic acid (compound 12) and pimelic acid has been observed (4, 20). In some bacteria, cyclohexanecarboxylic acid (compound 10) is transformed to 4-hydroxybenzoic acid via 4-oxocyclohexanecarboxylic acid (29). In contrast to these previous findings, the formation of benzoic acid (compound 13) from cyclohexanecarboxylic acid (compound 10) via 1-cyclohexene-1-carboxylic acid (compound 12) was demonstrated in the present study. In addition, *n*-hexadecane-grown cells could also transform 3-cyclohexene-1-carboxylic acid (compound 14) to benzoic acid (compound 13) (Table 2). To our knowledge, this is the first report of a pathway involving the conversion of cyclohexanecarboxylic acid (compound 10) to benzoic acid (compound 13).

In the cultures grown on *n*-undecylbenzene (compound b) and *n*-hexadecylbenzene (compound a), both benzoic acid (compound j) and phenylacetic acid (compound i) were detected (Table 1). Benzoic acid (compound j) was the major product from the *n*-undecylbenzene (compound b) culture, while *n*-hexadecylbenzene (compound a) mainly yielded phenylacetic acid (compound i). Thus, these compounds were mainly degraded by β -oxidation. Apart from these, small amounts of 3-phenylpropanoic acid (compound k) and *trans*-cinnamic acid (compound l) were also formed in both the *n*-undecylbenzene (compound b) and *n*-hexadecylbenzene (compound a) cultures. In addition, 4-phenylbutanoic acid (compound c) and two isomers of 4-phenylbutenoic acid (compounds e and f) were detected in the *n*-hexadecylbenzene-grown culture.

To investigate further, the transformation of 4-phenylbutanoic acid (compound c) and 5-phenylpentanoic acid (compound d) was examined with cultures of *Alcanivorax* sp. strain MBIC 4326 grown on *n*-hexadecane. 5-Phenylpentanoic acid (compound d) was transformed by *Alcanivorax* sp. strain MBIC 4326, mainly via β -oxidation, with 5-phenyl-2-pentenoic acid

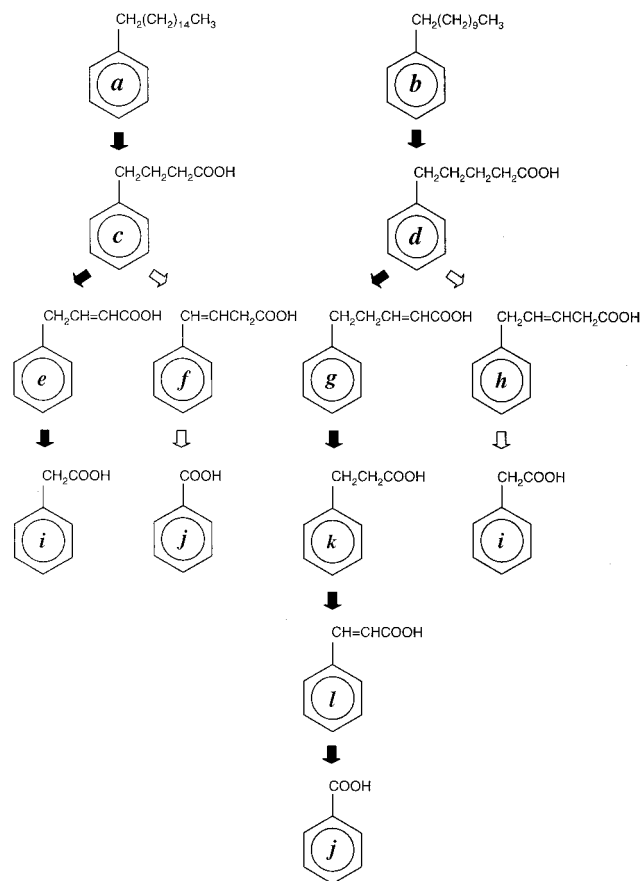


FIG. 2. Proposed pathway(s) for the biodegradation of *n*-alkylbenzenes by *Alcanivorax* sp. strain MBIC 4326. β -Oxidation routes shown to be major metabolic routes are indicated by solid arrows, while minor routes are indicated by open arrows. Trace amounts of 3-phenylpropanoic acid (k) and *trans*-cinnamic acid (l) were formed from *n*-hexadecylbenzene. This route is not indicated by an arrow. Compound letters are defined in the text.

TABLE 1. GC-MS data for the metabolites^a formed from *n*-alkylcyclohexanes and *n*-alkylbenzenes by *Alcanivorax* sp. strain MBIC 4326

Compound source ^b and metabolite no.	Amt (μmol) ^c	Retention time (min)	<i>m/z</i> of major ion peaks (%) ^d	Suggested structure ^e	
<i>n</i> -Octadecylcyclohexane	51	8.9	142 (M ⁺) (30), 127 (9), 113 (22), 111 (20), 110 (26), 101 (16), 100 (7), 88 (8), 87 (100), 84 (5), 83 (73), 82 (31), 81 (18), 79 (6), 75 (9), 74 (55), 69 (17), 68 (28)	Methyl cyclohexanecarboxylate	
	83	11.2	156 (M ⁺) (2), 125 (20), 113 (5), 97 (13), 83 (9), 82 (11), 81 (17), 79 (7), 76 (7), 75 (68), 74 (100), 68 (5)	Methyl cyclohexaneacetate	
	3.1	10.8	140 (M ⁺) (31), 109 (22), 108 (24), 82 (8), 81 (100), 80 (73), 79 (64), 77 (16), 67 (5)	Methyl 1-cyclohexene-1-carboxylate	
	0.02	9.7	136 (M ⁺) (58), 135 (6), 106 (14), 105 (100), 92 (6), 78 (9), 77 (81), 75 (4), 74 (6)	Methyl benzoate	
<i>n</i> -Nonadecylcyclohexane	86	8.9	Same as the results described above	Methyl cyclohexanecarboxylate	
	42	11.2	Same as the results described above	Methyl cyclohexaneacetate	
	4.7	10.8	Same as the results described above	Methyl 1-cyclohexene-1-carboxylate	
	1.1	9.7	Same as the results described above	Methyl benzoate	
<i>n</i> -Undecylbenzene	190	9.7	Same as the results described above	Methyl benzoate	
	4.6	11.9	150 (M ⁺) (71), 119 (6), 118 (5), 92 (22), 91 (100), 89 (14), 65 (41), 64 (5)	Phenylacetic acid, methyl ester	
	0.9	14.2	164 (M ⁺) (24), 133 (8), 107 (4), 105 (33), 104 (100), 103 (13), 92 (5), 91 (69), 79 (12), 78 (13), 77 (18), 65 (12)	3-Phenylpropanoic acid, methyl ester	
	5.0	16.7	162 (M ⁺) (43), 161 (24), 132 (9), 131 (100), 104 (7), 103 (60), 102 (14), 78 (4), 77 (40), 76 (7), 75 (4), 74 (4), 65 (4)	<i>trans</i> -Cinnamic acid, methyl ester	
<i>n</i> -Hexadecylbenzene	4	14	9.7	Same as the results described above	
	5	130	11.8	Same as the results described above	
	6	0.7	14.2	Same as the results described above	
	7	0.4	16.7	Same as the results described above	
	8	3.2	16.8	178 (M ⁺) (16), 162 (7), 161 (5), 147 (21), 146 (25), 131 (17), 117 (5), 115 (5), 105 (30), 104 (100), 103 (17), 92 (6), 91 (64), 89 (4), 78 (8), 77 (15), 75 (5), 74 (68), 65 (23)	4-Phenylbutanoic acid, methyl ester
	9	0.8	17.2	176 (M ⁺) (28), 145 (5), 134 (9), 118 (9), 117 (100), 116 (13), 115 (51), 91 (17), 65 (7)	4-Phenyl-2-butenic acid, methyl ester ^f
10	9.4	18.6	176 (M ⁺) (32), 161 (4), 144 (5), 134 (10), 118 (11), 117 (100), 116 (14), 115 (49), 91 (17), 89 (4), 65 (6)	4-Phenyl-3-butenic acid, methyl ester	

^a Analyses were performed after methylation with trifluoro boron-methanol.

^b Substrate concentration was as follows: 1 g/liter in 50 ml of medium contains 149, 143, 215, and 165 μmol of *n*-octadecylcyclohexane, *n*-nonadecylcyclohexane, *n*-undecylbenzene, and *n*-hexadecylbenzene, respectively.

^c Based on the total ion current response of the mass-selective detector relative to the authentic standards.

^d The ion abundance percentages are shown in parentheses.

^e Identification was based on the match of mass spectra (fragmentation and peak intensity) and capillary GC retention times with data for authentic samples, except for data described in footnote *f*, which indicated a fragmentation match higher than 85%.

^f High degree of match to the library (NIST and WILEY installed in Shimadzu GCMS-QP5050A9).

(compound g), 3-phenylpropionic acid (compound k), *trans*-cinnamic acid (compound l), and benzoic acid (compound j) detected as the β-oxidation intermediates. While *trans*-cinnamic acid (compound l) has been reported as the dead-end metabolite in the degradation of tridecanylbenzene by *Acinetobacter lwoffii* (1), this compound was found to be efficiently degraded by *Alcanivorax* sp. strain MBIC 4326 to benzoic acid (compound j) by classical β-oxidation pathway enzymes (Table 2). The detection of 5-phenyl-3-pentenoic acid (compound h) and phenylacetic acid (compound i), however, indicated the existence of another minor pathway for the degradation of 5-phenylpentanoic acid.

The degradation of the *n*-alkyl side chain by mechanisms other than β-oxidation has been suggested by a number of

observations. In fungi (*Beauveria*, *Penicillium*, and *Paecilomyces* spp.), both carboxylic acid and acetic acid derivatives accumulated from 2-*n*-dodecyltetrahydrothiophene (12). Similar phenomena have been observed in various microbial degradation processes of *n*-alkylcyclohexanes, *n*-alkylbenzenes, and *n*-alkylbenzene sulfonic acids (3, 11, 20, 23). In the degradation of branched-chain dodecylbenzene sulfonic acid by *Pseudomonas aeruginosa* W51D, desulfonation was followed by complete oxidation of the alkyl side chain via 3-(4-hydroxyphenyl)propionic acid, 4-hydroxycinnamic acid, 4-hydroxyphenylacetic acid, and 4-hydroxybenzoic acid (5). *n*-Undecylcyclohexane and *n*-dodecylcyclohexane were both transformed to cyclohexaneacetic acid and cyclohexanecarboxylic acid in a marine bacterium. To explain the formation of these metabolic intermediates, the

TABLE 2. Biodegradation of cyclohexyl- and phenylalkanoic acids by *Alcanivorax* sp. strain MBIC 4326

Source compound ^a	Metabolite(s)	Amt of metabolites (μmol) with time:			
		8 h	1 day	3 days	5 days
4-Cyclohexanebutanoic acid	Cyclohexanecarboxylic acid	2.6	Trace	0.13	0.38
	Cyclohexaneacetic acid		4.1	15	16
	1-Cyclohexene-1-carboxylic acid			0.03	0.12
	4-Cyclohexane-2-butenic acid		Trace	Trace	Trace
	4-Cyclohexane-3-butenic acid			Trace	Trace
4-Phenylbutanoic acid	Benzoic acid	0.05	0.11	0.25	0.42
	Phenylacetic acid	4.3	10	26	28
	4-Phenyl-2-butenic acid	0.60	1.7	1.9	2.3
	4-Phenyl-3-butenic acid	8.9	17	25	29
5-Cyclohexanepentanoic acid	Cyclohexanecarboxylic acid	0.17	0.23	1.4	44
	Cyclohexaneacetic acid			Trace	Trace
	3-Cyclohexanepropionic acid		Trace	0.14	Trace
	1-Cyclohexene-1-carboxylic acid			0.21	3.6
	Benzoic acid				0.08
	5-Cyclohexane-2-pentenoic acid		Trace	Trace	Trace
5-Cyclohexane-3-pentenoic acid			Trace	Trace	
5-Phenylpentanoic acid	Benzoic acid	1.2	7.5	31	43
	Phenylacetic acid		Trace	Trace	Trace
	3-Phenylpropionic acid	5.2	21	4.5	0.25
	<i>trans</i> -Cinnamic acid	3.2	14	2.9	0.08
	5-Phenyl-2-pentenoic acid		Trace	Trace	Trace
	5-Phenyl-3-pentenoic acid		Trace	Trace	Trace
4-Phenyl-3-butenic acid	Benzoic acid	ND ^b	ND	ND	1.8
<i>trans</i> -Cinnamic acid	Benzoic acid	ND	ND	ND	2.5
Cyclohexanecarboxylic acid	1-Cyclohexene-1-carboxylic acid	ND	ND	ND	0.77
	Benzoic acid	ND	ND	ND	0.06
1-Cyclohexene-1-carboxylic acid	Benzoic acid	ND	ND	ND	0.39
3-Cyclohexene-1-carboxylic acid	Benzoic acid	ND	ND	ND	0.09

^a Substrate concentration was as follows: 1 g/liter in 10 ml of medium contains 59, 61, 54, 56, 62, 67, 78, 79, and 79 μmol of 4-cyclohexanebutanoic acid, 4-phenylbutanoic acid, 5-cyclohexanepentanoic acid, 5-phenylpentanoic acid, 4-phenyl-3-butenic acid, *trans*-cinnamic acid, cyclohexanecarboxylic acid, 1-cyclohexene-1-carboxylic acid, and 3-cyclohexene-1-carboxylic acid, respectively.

^b ND, not determined.

simultaneous occurrence of α - and β -oxidation has been proposed as a possible mechanism (20).

The major degradation products of 4-phenylbutanoic acid (compound c) were phenylacetic acid (compound i) and 4-phenyl-2-butenic acid (compound e), which had certainly been formed by β -oxidation. In addition, the accumulation of the Δ^3 isomer of 4-phenylbutenoic acid (compound f) was exceptionally high when 4-phenylbutanoic acid (compound c) was used as the substrate. It has been reported that one of the β -oxidation enzymes, butyryl coenzyme A (CoA) dehydrogenase, was found to possess low affinity towards 4-phenylbutyryl-CoA, resulting in the accumulation of 4-phenylbutyric acid (compound c) in the degradation of 1-phenyl-dodecane by *Nocardia salmonicolor* (23). In the present study, the accumulation of 4-phenyl-3-butenic acid at a relatively high concentration indicates that 4-phenyl-3-butenic acid (compound f) might have been formed either by direct Δ^3 -dehydrogenation of 4-phenylbutanoic acid (compound c) or from 4-phenyl-2-butenic acid (compound l) by the action of enoyl CoA isomerase (15).

4-Phenyl-3-butenic acid (compound f) was degraded by *Alcanivorax* sp. strain MBIC 4326 to benzoic acid (Table 2).

Considering the structures of the characterized metabolites, the pathways for the degradation of *n*-alkylcyclohexanes (Fig. 1) and *n*-alkylbenzenes (Fig. 2) in *Alcanivorax* sp. strain MBIC 4326 are proposed. The transformation may proceed in vivo in the form of CoA derivatives. Further genetic and biochemical studies are required to clarify the enzymes involved in these steps.

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