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Biosynthesis of a Cyclic Tautomer[1] of (3-Methylmaleyl)acetone from 4-Hydroxy-3,5-dimethylbenzoate by *Pseudomonas* sp. HH35 but Not by *Rhodococcus rhodochrous* N75

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Here we report that the bacterial catabolism of 4-hydroxy-3,5-dimethylbenzoic acid 1 takes a different course in *Rhodococcus rhodochrous* N75 and *Pseudomonas* sp. strain HH35. The former organism accumulates a degradation metabolite of the acid which we isolated and identified as 2,6-dimethylhydroquinone 2. The latter bacterial strain converts the acid and the hydroquinone into a dead-end metabolite. This novel compound was characterised unequivocally by mass spectrometry and ^{1}H and ^{13}C NMR and UV spectroscopy as 4-acetonyl-4-hydroxy-2-methylbut-2-en-1,4-olide 4, a cyclic tautomer of (3-methylmaleyl)acetone, which exists as the enol carboxylate form 3 in aqueous solution. © 1997 Academic Press

4-Hydroxy-3,5-dimethylbenzoic acid **1** has been described as a dead-end metabolite accumulated from the environmental pollutant 2,4,6-trimethylphenol by a cresol-degrading *Comamonas* sp. [1] and a *Pseudomonas* sp. [R. B. Cain, P. J. Sage, and S. Schmidt; unpublished observations]. We were therefore interested to find out whether this compound would be further catabolised by selected bacterial strains able to degrade substituted benzoic acids. So far, the bacterial conversion of 3,5-dimethylbenzoic acid into 3,5-dimethylpyrocatechol and thence 2,4-dimethylmuconolactone has been reported for both gram-negative [2, 3] as well as gram-positive organisms [4]. Similarly, 3,4-dimethylbenzoic acid and 3,4-dimethylpyrocatechol gave 2,3-dimethylmuconolactone in *Rhodococcus rhodochrous* N75 [4], which also converted 3,4,5-trimethylpyrocatechol into 2,3,4-trimethylmuconolactone [R. B. Cain, G. W. Kirby, H. J. S. McLenaghan, and S. Schmidt; unpublished observation]. The strain *Pseudomonas* sp. HH35 was originally isolated for its ability to degrade among others, 3,5-dichloro- and 3,5-dibromo-4-hydroxybenzoic acid [5]. To help us to understand the fate of pollutants in the environment and predict their probable environmental impact, it is essential to elucidate the possible fate of known ‘dead-end’ metabolites derived thereof by microbial activity.

MATERIALS AND METHODS

Organisms and growth conditions. Cultivation of the nocardioform actinomycete *Rhodococcus rhodochrous* N75 was done as described [4]. *Pseudomonas* sp. HH35 was routinely grown in known mineral salts medium [6] supplemented with either 3,5-dichloro-4-hydroxybenzoate (5 mM) or acetate (5mM) as sole source of carbon and energy. For co-oxidation experiments cells were grown in 2.5 l Erlenmeyer flasks containing 500 ml of the required growth medium on a shaking waterbath at 28°C to the late-exponential growth phase. Cells were harvested, washed twice and resuspended in phosphate buffer (pH 7.2, 20 mM) to an OD_{578nm} of about 2.5 and then incubated with the appropriate substrate.

Preparation of crude extracts. Crude extracts were produced from exponentially growing cultures by a described procedure [7]. Soluble protein was determined according to Bradford [8].

Oxygen consumption. Specific oxygen uptake rates of resting cells or crude extracts were determined with a Clark-type oxygen-electrode as described [7].

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[2] Alternative names for the butenolide **4** are 4-acetonyl-4-hydroxy-2-methylbut-2-en-1,4-olide (used in this report), 1-(2-hydroxy-4-methyl-5-oxo-2,5-dihydro-2-furyl)propan-2-one (IUPAC recommended), and 5-acetonyl-5-hydroxy-3-methyl-2(5H)-furanone (Chemical Abstracts usage).

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TABLE 1

Specific Oxygen Uptake Rates of Resting Cells of *Pseudomonas* sp. HH35 and *Rhodococcus rhodochrous* N75

	Specific oxygen uptake[a] after growth on			
	3,5-Dichloro-4-hydroxybenzoate	Acetate	4-Methylbenzoate	Acetate
Substrate	*Pseudomonas* sp. HH35		*R. rhodochrous* N75	
Acetate	78	147	28	37
4-Methylbenzoate	n.d.	n.d.	74	<1
3,5-Dichloro-4-hydroxybenzoate	185	<1	n.d.	n.d.
4-Hydroxy-3,5-dimethylbenzoate **1**	21	<1	9	<1
2,6-Dimethylhydroquinone **2**	204	<1	<1	<1
2,6-Dimethylbenzoquinone	<1	<1	<1	<1

[a] All activities are given in nmoles O_2 per min per mg of protein and are corrected for endogenous respiration and autoxidation. n.d., not determined.

Analytical methods. Optical densities at 578nm and UV spectra were recorded with a Uvikon 922 spectrophotometer. Analytical and preparative HPLC was done as described [4,7]. ^1H NMR Spectra at 200 MHz and ^{13}C NMR spectra at 50.3 MHz were obtained using a Bruker WP 200 spectrometer. Mass spectra were obtained, by direct insertion on a probe, with an AEI MS9 spectrometer in the EI mode at 70 eV. IR Spectra were recorded with a Perkin Elmer Paragon 1000 FT-IR spectrometer.

Enzyme activities. Activities of catabolic enzymes in crude extracts were determined at 25°C photometrically using potassium phosphate buffer (20mM, pH 7.0). Oxidation of 3,5-substituted 4-hydroxybenzoic acid derivatives was measured by following the oxidation of NADPH at 340nm (ϵ 6220). 2,6-Dimethylhydroquinone oxygenation was followed by measuring the formation of (3-methylmaleyl)acetone **3** (4-acetonyl-4-hydroxy-2-methylbut-2-en-1,4-olide **4**) at 316 nm, using ϵ 4225 for calculation of activity. All assays were performed using a final volume of 1ml, containing 20μmoles buffer, 5 - 30μg of protein, 0.5μmoles of substrate, and, if required, 0.5μmoles of NADPH.

Chemicals. The 2,6-dimethylhydroquinone **2** was obtained by hydrogenation of 2,6-dimethyl-*p*-benzoquinone (obtained from Lancaster Synthesis Ltd.) in ethanol at ambient pressure and temperature with a 10% palladium-carbon catalyst. The product was freed from traces of the quinone, formed by autoxidation during work-up, by sublimation onto a cold-finger at 0.05 mbar. At 50°C a black film of the quinhydrone (the 1:1 complex of the quinone and hydroquinone) formed on the cold-finger. This was removed and then sublimation at 95-100°C and 0.06-0.20 mbar gave the hydroquinone **2** (86%) as cream-coloured crystals, m.p. 150-152°C (lit.,[9] 148°C); δ_H $[(CD_3)_2CO]$ 7.57 and 6.65 (2 x br s, OH), 6.45 (s, 3- and 5-H) and 2.15 (s, Me); δ_C $[(CD_3)_2CO]$ 150.9 (C-1), 146.8 (C-4), 125.9 (C-2 and -6), 115.5 (C-3 and -5) and 16.7 (Me); *m/z* (the compositions of ions were determined by accurate mass measurements) 138 ($C_8H_{10}O_2$, $M+$, 100%), 137 ($C_8H_9O_2$, 27.2), 136 ($C_8H_8O_2$, 5.9), 123 ($C_7H_7O_2$, 25.0), 121 (C_8H_9O, 6.9), 109 (C_7H_9O, 5.3), 95 (C_6H_7O, 6.5) and 91 (C_7H_7, 5.9). All other compounds used were of the highest purity commercially available.

TABLE 2

Specific Catabolic Activities in Crude Extracts of *Pseudomonas* sp. HH35

	Specific activity[a] after growth on	
Substrate	3,5-Dichloro-4-hydroxybenzoate	Acetate
4-Hydroxy-3,5-dimethylbenzoate **1**		
−NADPH	<1	<1
+NADPH	27	<1
2,6-Dimethylhydroquinone **2**		
−NADH	121	<1
+NADH	120	<1

[a] All activities are given in nmoles per min per mg of protein and were measured spectrophotometrically as described.

RESULTS AND DISCUSSION

Growth of the strains employed in this study on selected benzoates (*p*-toluate and 3,5-dichloro-4-hydroxybenzoate for *R. rhodochrous* N75 and *Pseudomonas* sp. HH35, respectively) was essential to induce the activities required to oxidise 4-hydroxy-3,5-dimethylbenzoate **1** which, however, did not serve as a carbon source for the organisms. Acetate-grown cells of both strains exhibited no significant activity for the oxidation of 4-hydroxy-3,5-dimethylbenzoate (table 1). The 4-hydroxy-3,5-dimethylbenzoic acid **1** was incubated as described with cell-suspensions of *Rhodococcus rhodochrous* N75 and *Pseudomonas* sp. strain HH35 until the co-oxidation substrate had been completely transformed into a more polar metabolite. The cells were separated by centrifugation and the cell-free supernatant was extracted at pH 7.2 with ethyl acetate, to remove non-acidic material, then was acidified to pH *ca.* 2 with phosphoric acid and again extracted with ethyl acetate. Both the extract obtained prior to acidification in the case of *Rhodococcus rhodochrous* N75 and the extract obtained after acidification in the case of *Pseudomonas* sp. HH35 contained a new, major metabolite. The metabolite produced from 4-hydroxy-3,5-dimethylbenzoic acid by *Rhodococcus rhodochrous* N75

TABLE 3

Mass Spectrum (EI Mode at 70 eV) of the Acetonylbutenolide **4** (Peak Intensities Given as Percentages of the Base Peak; Ion Compositions Determined by Accurate Mass Measurements)

m/z	%	Composition
170	1.4	$C_8H_{10}O_4$ ($M+$)
153	3.5	$C_8H_9O_3$
152	23.6	$C_8H_8O_3$
137	100.0	$C_7H_5O_3$
128	8.2	$C_6H_8O_3$
125	6.6	$C_7H_9O_2$
113	17.4	$C_5H_5O_3$
111	4.6	$C_6H_7O_2$
110	10.5	$C_6H_6O_2$
109	7.9	$C_6H_5O_2$
85	14.9	$C_4H_5O_2$
81	8.5	C_5H_5O
68	10.7	C_4H_4O

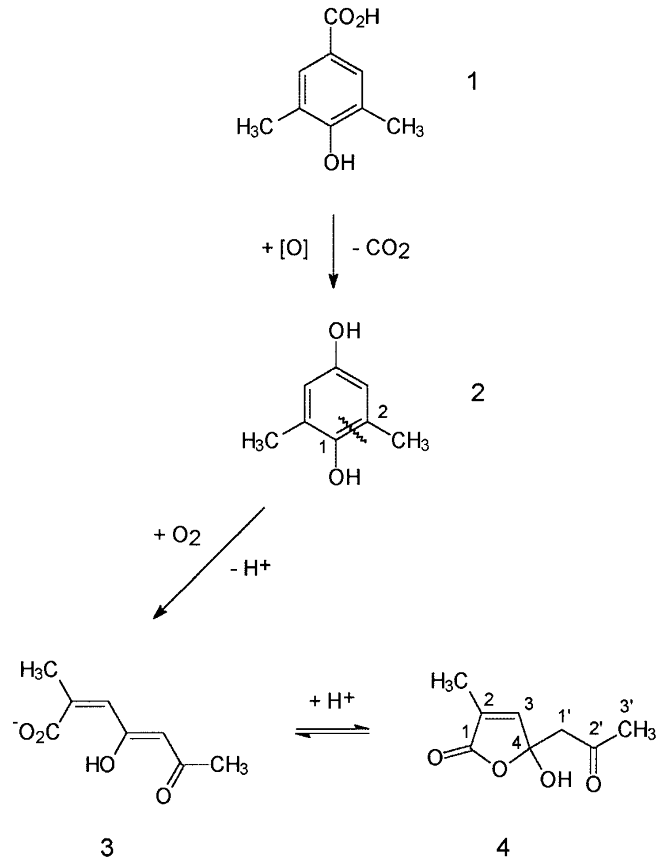

FIG. 1. Proposed pathway for the catabolism of 4-hydroxy-3,5-dimethylbenzoate **1** by *Rhodococcus rhodochrous* N75 and *Pseudomonas* sp. HH35.

cells had ^1H and ^{13}C NMR and mass spectra indistinguishable from those of the synthetic 2,6-dimethylhydroquinone **2.** However, the co-oxidation of 4-hydroxy-3,5-dimethylbenzoate by *Pseudomonas* sp. HH35 yielded a single metabolite which was identified as the 4-acetonyl-4-hydroxy-2-methylbut-2-en-1,4-olide* **4** by the following analytical data. After initial purification by HPLC, the butenolide crystallised from diethyl ether-hexane as plates, m.p. 59-61°C. The composition $C_8H_{10}O_4$, (Found: C, 56.25; H, 5.8%; m/z 170.0589. $C_8H_{10}O_4$ requires C, 56.5; H, 5.9%; M, 170.0579) determined by combustion analysis and mass spectrometrically (table 3), corresponded to that of an isomer of 2,4-dimethylmuconolactone. Moreover, many features of the ^1H NMR spectrum (table 4) resembled those of this muconolactone. Thus, a vinyl methyl group with an adjacent vinyl proton was revealed by signals at δ 1.92 and 6.87, respectively, showing the appropriate, allylic coupling (J 1.6 Hz). Again, an AB quartet, J 16.6 Hz, for a methylene group attached to a carbonyl group in a chiral molecule, was evident. However, a methyl singlet appeared at much lower field, δ 2.29, than that for the 4-methyl group of the muconolactone. The ^{13}C NMR spectrum, especially the signals from non-protonated carbons, was decisive. Signals at δ 171.1 and 208.2 showed that the butenolide ring in the new metabolite was attached to a ketonic rather than a carboxylic side-chain. Further, the low-field signal at δ 103.6 indicated the presence of an acetal carbon (C-4). The structure accounts for all the NMR data and is supported by the mass spectrometric fragmentation with loss, separately, of H_2O and CH_2COCH_3 from the quaternary centre C-4. The acidity of the butenolide is understandable since cyclic hemiacetals of carboxylic acids readily undergo ring-opening to give the corresponding ketonic (or aldehydic) acids. At or above pH 7, ionisation of the ring-opened tautomer will give the carboxylate anion and displace the equilibrium **4** ⇆ **3** in favour of the latter species, which probably exists mainly as the enolic form shown in figure 1 because of the extended con-

TABLE 4

^{13}C and ^1H NMR Spectra of the Acetonylbutenolide **4** (in $CDCl_3$; J Values in Hz)

Position	δ_C[a]	δ_H
1	171.1	
2	133.0	
3	145.4	6.87 (q, J 1.6)
4	103.6	
1′	47.8	2.87 and 3.06 (ABq, J 16.6)
2′	208.2	
3′	31.9	2.29 (s)
2-Me	10.4	1.92 (d, J 1.6)
5-OH		6.12 (br s)

[a] The number of protons attached to each carbon was verified by the standard DEPT technique.

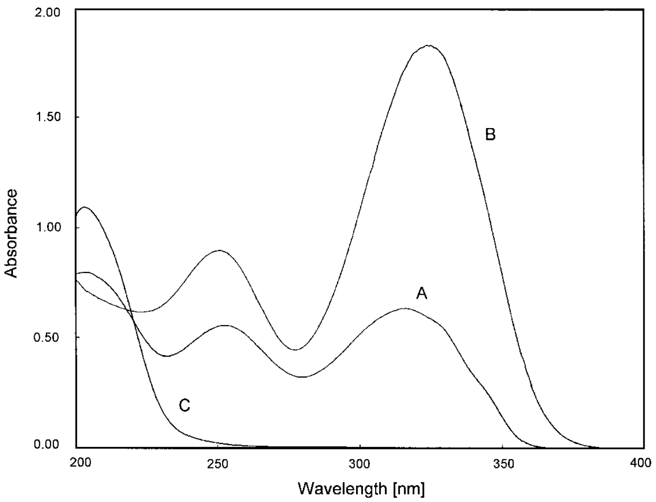

FIG. 2. UV spectra of (3-methylmaleyl)acetone enol **3** and its butenolide tautomer **4** in aqueous solution: *A* in H_2O (pH *ca.* 5); *B* in $H_2O + NaHCO_3$ (pH *ca.* 8); and *C* in $H_2O + HCl$ (pH *ca.* 3).

jugation. In $CDC1_3$ or $(CD_3)_2CO$, only the cyclic tautomer was detected by NMR spectroscopy. The equilibrium **3** ⇆ **4** was confirmed in aqueous solution by UV spectroscopy (figure 2). In the presence of sodium hydrogen carbonate (pH *ca.* 8) the metabolite showed intense absorption at λ_{max} 251 (ϵ 5950) and 325 nm (12100)(figure 2, curve B), attributable to the enol carboxylate **3** and possibly some of the corresponding enolate dianion. In water alone (pH *ca.* 5), absorption at λ_{max} 204.5 (ϵ 5280), 253 (3690) and 316 nm (4225)(figure 2, curve A) suggested the presence of both species **3** and **4,** whereas in the presence of hydrochloric acid (pH *ca.* 3) only the intense band at λ_{max} 204 nm (ϵ 7270)(figure 2, curve C), expected for the methylbutenolide, was apparent (a very weak absorption near 290 nm may arise from the n-π^* transition of the methyl ketone). The IR spectrum in $CHCl_3$ showed bands at 3400, 1771 and 1706 cm^{-1} attributable to hydroxy, lactonic carbonyl and ketonic carbonyl groups, respectively. Intramolecular hydrogen bonding between the hydroxy and ketone groups would account for the low frequency of the 3400 and 1706 cm^{-1} bands and the broad character of the former.

Supporting evidence for the structure was fortuitously obtained at an early stage of the investigation, although its significance at the time was not clear. Initially, the ^1H NMR spectrum of the metabolite **4** (δ_H 7.11 (q, *J* 1.6, 3-H), 2.92 and 3.24 (ABq, *J* 15.8, 1′-H_2), 2.23 (s, 3′-H_3) and 1.83 (d, *J* 1.6, 2-Me) was obtained for solutions in $(CD_3)_2CO$ (later, $CDCl_3$ was used), the solvent usually chosen for muconolactones. However, when solutions in $(CD_3)_2CO$ were stored at ambient temperature, partial dehydration of the butenolide **4** occurred to give what is now believed to be a mixture of the *cis* and *trans* diene lactones **5** and **6** (figure 3), respectively, in approximately equal amounts. The diene lactones were identified by the following ^1H NMR data: *cis* isomer **5,** δ 7.97 (m, *J ca.* 1, 3-H), 6.29 (s, 5-H), 2.33 (s, 7-H_3) and *ca.* 2.1 (2-Me, partly obscured by the CHD_2COCD_3 signal); *trans* isomer **6,** δ 7.52 (m, *J ca.* 1, 3-H), 5.63 (s, 5-H), 2.45 (s, 7-H_3) and *ca.* 2.1 (2-Me, partly obscured by the CHD_2COCD_3 signal). Dehydration of the butenolide **4,** possibly catalysed by an adventitious trace of acid, is consistent with the β-hydroxy ketone structure. The ^1H NMR signals for the vinyl protons were especially informative; δ 7.97 (3-H) and 6.29 (5-H) for the *cis* isomer **5,** and δ 7.52 (3-H) and 5.63 (5-H) for the *trans* isomer **6.** Similar values have been reported [10,11] for the related pair of geometrical isomers lacking the 2-methyl group. Further, a significant change was observed in the ^1H spectrum of the butenolide that remained in mixtures containing its dehydration products. The integral for the AB quartet of the methylene group had diminished relatively to those of other signals. Also, broad singlets (unresolved, deuterium-coupled triplets) were apparent with the chemical shifts expected for epimeric CHD groups. Thus, exchange of the 1′-methylene protons had occurred with the HOD present in the $(CD_3)_2CO$ solvent. In our experience, no similar exchange had ever been observed for the methylene groups of muconolactones, *e.g.* 4-carboxymethyl-2,4-dimethylbut-2-en-1,4-olide [4], in this solvent. However, deuterium exchange in the ketone would be the natural consequence of slow interconversion with the enolic form of its acyclic, (3-methylmaleyl)acetone tautomer. The overall conversion **1** → **4** (figure 1) implies an initial monooxygenation and decarboxylation to yield 2,6-dimethylhydroquinone **2.** Although this metabolite was quantitatively accumulated

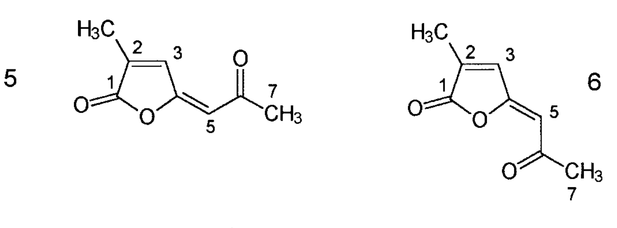

FIG. 3. Dehydration of the 4-acetonyl-4-hydroxy-2-methylbut-2-en-1,4-olide **4** yielding the corresponding *cis*- and *trans*-diene lactones, **5** and **6.**

by *Rhodococcus rhodochrous* N75, it was found to be an excellent substrate for cells of *Pseudomonas* HH35 (table 1), giving the butenolide **4** in quantitative yield. As it was oxidised with about a 10-fold higher specific activity than the benzoic acid **1** by resting cells of *Pseudomonas* sp. HH35 (table 1) and still with about a 5-fold higher specific activity by crude extracts of the same strain (table 2), we were so far not able to detect it directly as an intermediate. In contrast, the corresponding quinone was not oxidised by the cells or crude extracts. In *Pseudomonas* sp. HH35 the transformation **2** → **4** requires formally a dioxygenative cleavage of the benzene ring. We propose that the 2,6-dimethylhydroquinone is directly subjected to a dioxygenolytic ring-cleavage between C-1 and C-2 by analogy with the cleavage of homogentisate by homogentisate-1,2-dioxygenase [12] or the 1,6-cleavage of 3,6-dihydroxy-2-methoxyphenylacetic acid reported recently [13]. An NADH-dependent hydroxylation of the 2,6-dimethylhydroquinone to yield 2,6-dimethyl-3-hydroxyhydroquinone as the substrate for a subsequent 1,2-cleavage has been described for *Mycobacterium* sp. strain DM1 [14]. In contrast, the 2,6-dimethylhydroquinone-converting enzyme in crude extracts of *Pseudomonas* sp. HH35 did not require NADH for its activity (table 2). In addition, only 1 mole of O_2 and no NADH were consumed per mole of 4-hydroxy-2,6-dimethylhydroquinone catabolised. Thus, with crude extracts, for each mole of the hydroquinone **2** oxidised, 0.98 mole O_2 was consumed and 0.97 mole of the butenolide **4** was formed, in agreement with the catabolic sequence shown in figure 1.

Natural products that exist, like the butenolide **4,** as the cyclic tautomers of ketonic carbonyl compounds have been known for some time. An early example is penicillic acid, which is derived biosynthetically from orsellinic acid in several *Penicillium* species [15]. Again, the transformation of homogentisic acid into maleylacetoacetic acid occurs widely in bacteria, as does the related transformation of gentisic acid into maleylpyruvic acid [16]. To our knowledge the butenolide **4** has not previously been described, either as a natural or a synthetic product. However, the chemistry of maleylacetone and its cyclic tautomer has been studied in some detail, especially by Seltzer and co-workers [11], who have investigated the mechanism of the enzymic conversion of maleylacetone into fumarylacetone [17]. The ^{1}H NMR data published [11] for the cyclic form of maleylacetone agree well, *mutatis mutandis,* with those for the 2-methyl derivative **4.** Recently, Prucha *et al.* [18] have shown that 4-hydroxy-3-methylmuconolactone exists in the cyclic form in acidic solution. Our findings clearly demonstrate the importance of research into the fate of dead-end metabolites derived from environmental pollutants. Although both of the strains employed in our experiments were able further to degrade the 4-hydroxy-3,5-dimethylbenzoate, they did indeed produce "new" metabolites instead of mineralising this 'dead-end' metabolite of 2,4,6-trimethylphenol. In view of recent reports on new dead-end metabolites derived from known pollutants such as protoanemonin produced from 4-chlorobiphenyl [19] or chloro- and bromopicolinic acid from 4-chloro- and 4-bromobiphenyl [20], it becomes clear that we still need more information on the fate of individual compounds or the corresponding catabolites in the environment.

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