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Metabolism of Nitrodiphenyl Ether Herbicides by Dioxin-Degrading Bacterium *Sphingomonas wittichii* RW1

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Nitrodiphenyl ether herbicides, including chlomethoxyfen, nitrofen, and oxyfluorfen are potent herbicides. Some metabolites and parent compounds are considered as possible mutagens and endocrine disruptors. Both properties pose serious hygienic and environmental risks. *Sphingomonas wittichii* RW1 is a well-known degrader of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and diphenyl ethers. However, no detailed research of its metabolic activity has been performed against pesticides with a diphenyl ether scaffold. In this study, we report *S. wittichii* RW1 as a very potent diphenyl ether herbicide-metabolizing bacterium with broad substrate specificity. The structures of metabolites were determined by instrumental analysis and synthetic standards. Most pesticides were rapidly removed from the culture medium in the order of nitrofen > oxyfluorfen > chlomethoxyfen. In general, herbicides were degraded through the initial reduction and *N*-acetylation of nitro groups, followed by ether bond cleavage. Relatively low concentrations of phenolic and catecholic metabolites throughout the study suggested that these metabolites were rapidly metabolized and incorporated into primary metabolism. These results indicate that strain RW1 has very versatile metabolic activities over a wide range of environmental contaminants.

KEYWORDS: Diphenyl ether herbicide; mutagen; metabolism; *Sphingomonas wittichii*; reduction; acetylation

INTRODUCTION

Nitrodiphenyl ether herbicides (e.g., nitrofen, oxyfluorfen, and acifluorfen) are very potent herbicides (*1, 2*). From a toxicological point of view, they can induce various physiological effects, including master cell activation, organ morphogenesis, and several endocrine disruptor activities (*3*−*5*). In addition, some metabolites were known as possible mutagens (*6, 7*). Because of these harmful effects, productions and use of them are of regulatory concern in Korea and other countries.

Environmental dissipation of nitrodiphenyl ether herbicide occurred through physical removal or photochemical and biological degradation (e.g., refs *8*−*11*). Among the environmental transformation procedures of diphenyl ethers, photochemical cyclization to dibenzofurans or dioxins is of particular interest since the products are highly toxic to animals (*12*).

As structural constituents, diaryl ether groups are common in numerous pesticides (e.g., aryloxyphenoxypropionates, benzoylureas with aryloxy appendage, nitrodiphenyl ethers, pyrethroids, and pyrimidinyloxybenzoates, strobilurins), flame retardants (e.g., polybrominated diphenyl ethers), and in-house hygienic materials (e.g., triclosan).

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Although diaryl ethers are relatively resistant to biological degradation, several microorganisms, including some Sphingomonad are able to metabolize or mineralize various substituted diaryl ethers (*13*−*17*).

Sphingomonad bacteria are commonly found in highly contaminated natural environments with pesticides and polyhalogenated aromatic compounds (*13, 18*−*21*). *Sphingomonas wittichii* RW1 is a well-known polychlorinated dibenzo-*p*-dioxins (PCDD) degrading bacterium (*22, 23*). Because of its promising xenobiotic degradation potential, genetic engineering has been performed to improve environmental adaptation (*24*). According to previous degradation studies, various xenobiotics (PCDDs, dibenzofurans, hydroxylated and brominated diphenyl ethers, and aromatic heterocycles), with a common structural building block, namely, diaryl ether groups (*20, 22, 23*), were readily metabolized by strain RW1. These findings indicate a possible application of strain RW1 for bioremediation of toxic pesticides with similar structural groups (e.g., nitrodiphenyl ethers and aryloxyphenoxylpropionates). However, no related research has been published so far.

In this study, metabolism of selected nitrodiphenyl ether herbicides has been investigated with strain RW1. Metabolites were determined with instrumental analysis and synthetic standards. Comparative analysis of metabolite profiles from structurally diverse herbicides gave several interesting aspects of metabolisms.

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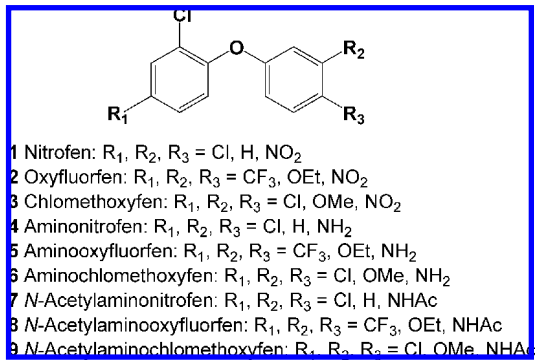

Figure 1. Structures of nitrodiphenyl ether herbicides and selected metabolites in this study.

MATERIALS AND METHODS

Chemical. Chemical structures of pesticides and selected metabolites were described in **Figure 1**. Nitrofen and oxyfluorfen were obtained from Sigma-Aldrich (Sigma-Aldrich Korea, Korea). Chlomethoxyfen was kindly provided from Dongbang Agro Inc. (Korea). Other reagents for chemical synthesis and bacterial cultures were obtained from Aldrich or TCI (Tokyo Chemical Industries Inc., Japan). Ethyl acetate and other solvents were of HPLC or higher grade. Concentrated hydrochloric acid was from Duksan Chemical Co (Korea). Aminonitrofen, aminochlomethoxyfen, and aminooxyfluorfen were prepared by literature method with modification (*6*). Briefly, pesticides were reduced with aqueous suspension zinc powder for 4 days under room temperature. After extraction with ethyl acetate, the organic residue was purified with silica gel column chromatography. *N*-Acetyl derivatives of aminonitrofen, aminochlomethoxyfen, and aminooxyfluorfen were prepared by acetylation with acetyl chloride. Acetyl chloride (150 μmole) was added to a solution of amino metabolite (100 μmole) and triethylamine (40 μmole) in chloroform (10 mL) with stirring. The mixture was further stirred for 2 h at 50 °C. The reaction mixture was diluted with distilled water (100 mL) and extracted with diethyl ether (100 mL). The organic layer was washed with distilled water (100 mL × 2). The organic residue was further purified with silica gel TLC.

Culture of Bacterium. *Sphingomonas wittichii* RW1 obtained from Korean Agricultural Culture Collection (KACC, #12172) and was maintained in nutrient agar. The seed culture was prepared in M9 medium (*25*), supplemented with dibenzo-*p*-dioxin (100 mg/L) at 150 rpm and 30 °C for 3 days. For the kinetic experiment, the seed culture (1 mL) was inoculated to M9 medium (100 mL) for nitrofen or M9 medium, supplemented with nutrient broth (NBM, 100 mL, 100 mg nutrient broth/100 mL of M9 medium) for nitrofen, oxyfluorfen, and chlomethoxyfen. Sterilized control was prepared from 2-day old cultures in nutrient broth and sterilized for 30 min at 120 °C. Pesticides (1000 mg/L, 100 μL in dimethyl sulfoxide) were fortified to the above medium. Cultures were maintained at 150 rpm and 30 °C. Three replicate experiments were performed for each treatment. For the preparation of metabolites, the strain was cultured in NBM (1 L) with crystalline pesticide (100 mg) at the same conditions.

Extraction and Derivatization of Metabolites. For degradation kinetics, aliquot amount of culture (50 mL) was diluted with saturated NaCl solution (50 mL) and extracted with ethyl acetate (50 mL × 2) without the separation of the bacterial cell. The organic layer was dried over anhydrous sodium sulfate. After the removal of solvent, the residue was dissolved in ethyl acetate (1 mL) and analyzed with gas chromatography−mass spectrometry (GC-MS).

Extraction efficiencies of pesticides and metabolites (**1**−**9**) were determined as follows: To a heat-sterilized control culture (100 mL, precultured for 3 days in NBM), stock solutions (100 μL, 1000 mg/L in dimethyl sulfoxide) were added and extracted as described above.

For structural identification of metabolites, batch cultures (1 L) were centrifuged (4000*g*, 30 min). The supernatant was saturated with NaCl and extracted with ethyl acetate (250 mL × 2). After adjusting the pH to 2.0 with concentrated hydrochloric acid, the supernatant was extracted with additional ethyl acetate (200 mL × 2). The combined organic layer was dried over anhydrous sodium sulfate. After the removal of solvent, the residue was dissolved in ethyl acetate (2 mL).

To determine the chemical structures of metabolites (**4**−**9**), an aliquot amount of extracts from the batch culture was dried under nitrogen atmosphere, and the residue was derivatized with BSTFA-TMCS (200 μL) in dry pyridine (800 μL) for 2 h at 80 °C. Derivatized samples were analyzed with GC-MS, and the results were compared with those of nonderivatized extracts. Phenolic metabolites (**10**−**14**) from kinetic experiments were analyzed after the same derivatization, while **1**−**9** were analyzed without derivatization.

Instrumental Analysis. Pesticides and their metabolite profiles were analyzed with GC-MS (Shimadzu GCMS QP-2000 and GC-2010), equipped with a DB-1 column (60 m, 0.25 μm film thickness, 0.2 mm i.d.; Agilent Technologies, USA). Helium was a carrier gas at a flow rate of 1 mL/min. The column temperature was programmed as follows: 95 °C (10 min) and raised to 295 °C at a rate of 2 °C/min, and held for 20 min. Temperatures of injection port and interface were set at 270 and 254 °C, respectively. The mass spectrometer was operated at electron impact (EI) mode at 70 eV.

Table 1. Extraction Efficiencies of Parent Pesticides and Selected Metabolites

ID[a]	compounds	recovery ± SD[b]
1	nitrofen	95.5 ± 7.2
2	oxyfluorfen	99.3 ± 4.3
3	chlomethoxyfen	97.2 ± 9.5
4	aminonitrofen	78.2 ± 10.4
5	aminooxyfluorfen	82.5 ± 5.9
6	aminochlomethoxyfen	75.2 ± 10.1
7	*N*-acetylaminonitrofen	89.2 ± 5.9
8	*N*-acetylaminooxyfluorfen	94.3 ± 5.2
9	*N*-acetylaminochlomethoxyfen	90.4 ± 9.5

[a] Structures were described in **Figure 1**. [b] Standard deviations of three replicates.

RESULTS

Chemical Syntheses of Metabolites. Among several degradation products of nitrodiphenyl ether herbicides, six metabolites (**4**−**9**, **Figure 1**), commonly found in soil, plants, or animals, were prepared by literature methods with some modification (*6*). Although reduced metabolites (**4**−**6**, **Figure 1**) can be easily prepared by literature methods, substituted chlorine was also labile to reduction. For example, only trace amounts of **4** and **6** were obtained after prolonged reflux (2 h), where monochlorinated aminodiphenyl ethers were dominant products (data not shown). In comparison with the literature method, reduction under room temperature proceeded slowly. However, the reaction yield was reasonable (35−65%), and no trace of monochlorinated byproduct was observed. Acetamide metabolites (**7**−**9**) were prepared in almost quantitative yield without any trace of byproduct.

Extraction Efficiencies of Pesticides and Selected Metabolites. Efficiencies of extraction method were determined with **1**−**9**. Overall, the recoveries were in the range of 75.2−97.2% (**Table 1**). Parent pesticide and *N*-acetylamino metabolites gave better recoveries than those of amino metabolites (89.2−97.2 vs 75.2−82.5%).

Growth of Bacterium and Kinetics of Herbicide Degradation. Both nitrofen and nutrient broth can support bacterial growth, where strain RW1 grew much faster in NB than in the mineral medium with nitrofen (**Figure 2**). In NB-supplimented M9 medium (NB), bacterial growth was initiated without the lag phase and reached the stationary phase after 4 days. However, strain RW1 grew more slowly in nitrofen-supplimented M9 medium and did not reach the stationary phase even after 7 days. The presence of nitrofen did not produce any difference in bacterial growth, cultured in NB alone and in nitrofen-supplimented NB.

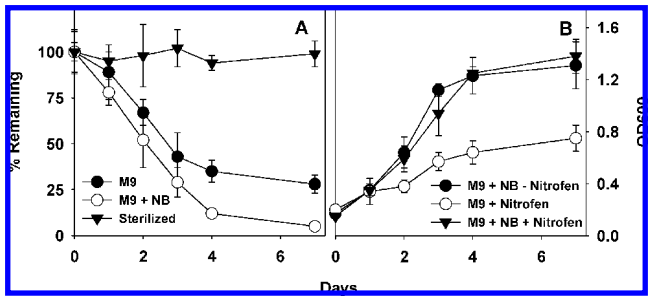

Figure 2. Degradation of nitrofen in sterilized control, viable cells in M9, and nutrient broth-supplimented M9 medium by *Sphingomonas wittichii* RW1 (**A**) and the growth of the bacterium (**B**). Error bar indicates the standard deviations of three replicate experiments.

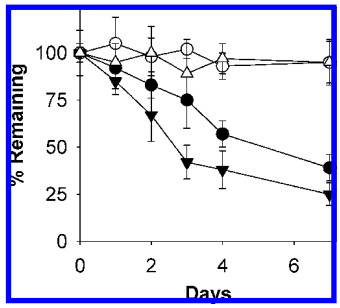

Figure 3. Degradation of oxyfluorfen and chlomethoxyfen by *Sphingomonas wittichii* RW1, grown in nutrient broth-supplimented M9 medium. Symbols: circles for chlomethoxyfen, triangles for oxyfluorfen, blank symbol for the control, and filled symbol for viable culture. Error bar indicates the standard deviations of three replicate experiments.

All three herbicides were rapidly degraded by strain RW1 (**Figures 2** and **3**). After 7 days, complete degradation of nitrofen was observed in NB, while 26% of pesticide remained in M9 medium (**Figure 2**). Oxyfluorfen and chlomethoxyfen were much more persistent than nitrofen. Approximately, 25 and 42% of residual oxyfluorfen and chlomethoxyfen still persisted in the culture medium (**Figure 3**).

Metabolisms of Herbicides. Several metabolites were found in the culture supernatant. Approximately, 3−5 metabolites were found from culture supernatants for each pesticide (**Figure 4** and **Table 2**). At the initial incubation period (2−3 days), the highly retained metabolites (retention times (*Rt*s), 59−74 min) were rapidly accumulated. Mass/charge ratio of molecular ions of nonderivatized **4**, **5**, and **6** were lower than those of parent pesticides (*m*/*z* of parent-30), and all of them can give mono-TMS derivatives (*m*/*z* of parent −30 + 72). Their mass spectral fragmentations and retention times were exactly the same as those of synthetic amino pesticides, of which the nitro group in parents was reduced to amine group (**Figure 5**). These metabolites were identified as aminonitrofen (**4**), aminooxyfluorfen (**5**), and aminochlomethoxyfen (**6**). Metabolites **7**, **8**, and **9** retained much longer than parent pesticides (**Table 2**). Their molecular weights were higher than parents (*m*/*z* of parent + 12) and could not be derivatized with BSTFA-TMCS. Mass spectral patterns and retention times were well-correlated with synthetic *N*-acetyl derivatives of **4**, **5**, and **6** (**Figure 5**). These metabolites were confirmed as *N*-acetyl derivative of **4**, **5**, and **6**. 2,4-Dichlorophenol (**13**) was detected in culture supernatants of nitrofen and chlomethoxyfen. *N*-acetylaminophenol (**12**) metabolite was found only in nitrofen-treated cultures. No traces of corresponding analogues were found in oxyfluorfen and chlomethoxyfen cultures. During the metabolism of oxyfluorfen, additional metabolites were observed. Metabolites **10** and **14** (*Rt*s, 66.367 and 48.117 min) were tentatively identified as

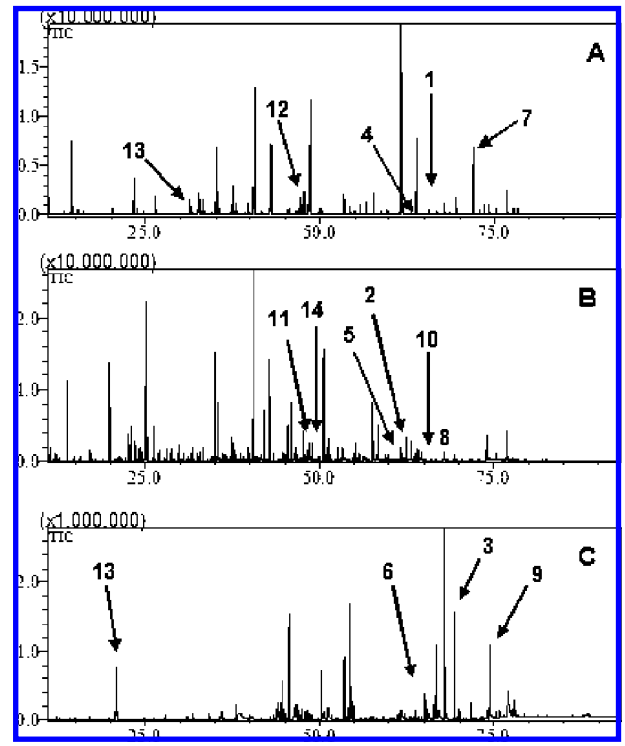

Figure 4. GC-MS total ion chromatogram of ethyl acetate extracts of culture supernatants, grown for 4 days with nitrofen (**A**), oxyfluorfen (**B**), and chlomethoxyfen (**C**). Samples for chlomethoxyfen was not derivatized.

Table 2. List of Metabolites of Nitrofen, Oxyfluorfen, and Chlomethoxyfen by *Sphingomonas wittichii* RW1

ID	name	*Rt*, min	MS fragmentation
4	aminonitrofen	65.350	325(M^+,99), 327(64), 310(41) 312(28), 149(100), 134(25)
		60.592[a]	253(M^+,92), 255(60), 183(11), 108(100)
5	aminooxyfluorfen	62.092	403(M^+,100), 405(42), 374(53), 376(21), 359(83), 361(31)
		59.217[a]	331(M^+,100), 333(33), 302(76), 304(30), 274(56)
6	aminochlomethoxyfen	65.183[a]	283(M^+,100), 285(63), 268(64), 270(39), 240(30), 242(5)
7	*N*-acetylaminonitrofen	72.033	295(M^+,51), 297(36), 253(100), 255(67), 183(9), 108(68)
8	*N*-acetylaminooxyfluorfen	67.892	373(M^+,100), 375(40), 331(94), 302(79), 274(34)
9	*N*-acetylaminochlomethoxyfen	74.525	325(M^+,73), 327(49), 283(100), 285(61), 268(52) 270(32), 240(25), 242(16)
10	desethyl *N*-acetyl-aminooxyfluorfen	66.367	417(M^+,100), 419(40), 402(57), 375(87), 360(37)
11	4-trifluoromethylcatechol[b]	47.017	322(M^+,95), 307(64), 276(10), 249 (7), 232(100), 217(49), 143(15)
12	4-acetamidophenol	47.658	223(M^+,84), 208(25), 192(4), 181(100), 166(79)
13	2,4-dichlorophenol	31.400	234(M^+,43), 236(29), 219(100), 221(71), 183(46), 185(19)
14	3-ethoxy-4-nitrophenol	48.117	255(M^+,53), 240(25), 227(7), 212(100), 194(41)

[a] Retention times of non-derivatized metabolite. [b] Tentative identification.

desethyl *N*-acetylaminooxyfluorfen and 3-ethoxy-4-nitrophenol, respectively. The mass spectrum of metabolite **11** (*Rt*, 47.107 min; M^+, 322) showed the presence of trifluoromethylphenyl (*m*/*z* 143), TMS-derivatized hydroxy trifluoromethylphenyl (*m*/*z*

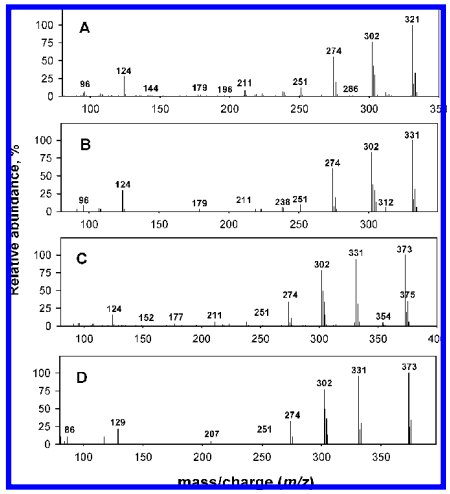

Figure 5. Mass spectrum of selected metabolites of oxyfluorfen from culture supernatant and synthetic metabolite standards. Aminooxyfluorfen from culture supernatant (**A**) and synthetic standard (**B**); *N*-acetylaminooxyfluorfen from supernatant (**C**) and standard (**D**).

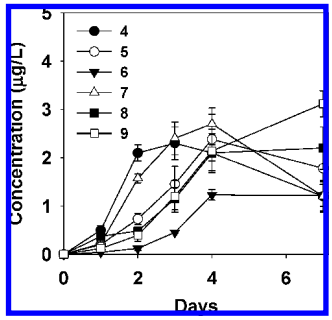

Figure 6. Kinetics of metabolites with the diphenyl ether group during the culture period. Error bar indicates the standard deviations of three replicate experiments.

232), and TMS-derivatized dihydroxy trifluoromethylphenyl (*m*/*z* 249) groups, while no characteristic isotope patterns of chlorine atoms were observed. This metabolite was tentatively identified as 4-trifluoromethylcatechol.

In general, the concentration of metabolites **4** to **9** accounts for 40−90% of the parent pesticide degradation (**Figure 6**). Two initial metabolites (**6** and **9**) of chlomethoxyfen were gradually accumulated throughout the experimental period with concomitant increase of other metabolites (e.g., 2,4-dichlorophenol). However, similar types of metabolites (**4** and **7**) in the nitrofen-treated culture were accumulated up to 4 days and then rapidly decreased (**Figure 6**). The amounts of aminonitrofen and aminooxyfluorfen (**4** and **5**) were higher at initial incubation period (2−3 days) than those of *N*-acetyl derivatives (**7** and **8**), but the latter were more persistent to degradation; larger amount of metabolite were found at 4 to 7 days.

DISCUSSION

In relation to biomass production, nitrofen showed no difference with that of nutrient broth alone, indicating no appreciable effects of this pesticide on bacterial growth (**Figure 2**). However, slow growth in nitrofen-supplimented M9 medium suggested that the pesticide is a less favorable carbon source than natural nutrients. It is noteworthy that both biomass production and nitrofen degradation were enhanced by NB supplementation. In some Gram-negative bacteria, the degradation of xenobiotics is repressed by the presence of readily metabolized natural carbon sources (*26*). Because there are various easy-to-use substrates in the natural environment, other than pesticides, this phenomenon is one of the most serious drawbacks of the corresponding bacterium for use in bioremediation. However, the experimental results indicate that at least in selected conditions, nitrofen degradation by strain RW1 was not suppressed through such a phenomenon.

Among the selected herbicides, nitrofen was the most labile to biodegradation, followed by oxyfluorfen and chlomethoxyfen. Various factors of substrates and microorganisms can change the biodegradation rate of xenobiotics. For example, hydrophobicities (e.g., log *P*) of pesticides and their structural fitness on degradative enzymes may determine the degradation rate of selected pesticides. However, a detailed explanation of the different metabolic rates in this study is yet to be studied.

The most interesting metabolic aspects of nitrodiphenyl ethers by this strain were the initial reduction of the nitro group, followed by *N*-acetylation. These reactions have never been reported with this strain. Rapid accumulation of **4**−**9** revealed that reduction of the nitro group is a much more dominant reaction. For example, **6** and **9** were attributable to most fractions of chlomethoxyfen metabolites. Although specific enzymes for these reductions were not identified from this organism, various redox enzymes have been reported from other bacterial species (*27*). *N*-Acetylation of metabolites **4**−**6** was also enzymatic since no trace of **7**−**9** was observed in amino metabolite-supplemented sterilized culture (data not shown).

As in other Sphingomonads (*13, 20, 30*), dissociation of the diaryl ether bond was also found in strain RW1 during nitrodiphenyl ether metabolism (**Figure 7**). Catecholic metabolite **14** implies the dissociation of diaryl ether groups. Although ether bond cleavage is a common metabolic pathway of diaryl ethers in animal, plant, fungi, and bacteria (*24, 28*−*31*), corresponding enzymes of eukaryotes and prokaryotes are different. Bacterial degradation of diphenyl ethers and related environmental contaminants are usually catalyzed by multicomponent dioxygenase system (*14, 18, 28*), while eukaryotic biodegradation is generally catalyzed by monooxygenase or peroxidase (e.g., refs *10, 15, 17, and 32*. These differences result in the differential metabolite profiles. For example, both catechol and phenol are common metabolites from bacteria, while no catechol is usually observed in eukaryotic metabolism (*10, 15, 17, 30*−*32*).

In addition to diaryl ether, other types of ether (aryl alkyl ether) may also be labile to degradation in strain RW1. Oxyfluorfen (**2**) contains two different ethers, namely, diaryl ether and aryl alkyl ether. Metabolite **10** is an example of aryl alkyl ether cleavage (**Figure 7**). Although several bacteria can catalyze this reaction (e.g., refs *13 and 33*, similar types of cleavage and corresponding enzymes have not been reported from this strain. Close inspection of the recently released draft genome of this strain will give more detailed information.

The presence of **12** and **13** during nitrofen metabolism indicates that the initial reaction (dioxygen addition, **Figure 7**) can occur both in the halogenated phenyl and nitrophenyl sides. However, only the phenolic metabolite from the halogenated phenyl side (**13**) was observed during chlomethoxyfen metabolism. These findings suggest that the initial reaction by dioxygenase or other equivalent enzymes in strain RW1 may have very complex regioselectivity.

Slow but definitive growth of strain RW1 in nitrofen-supplimented minimal salt medium suggested that organic carbons from nitrodiphenyl ethers can be incorporated into primary metabolism to support bacterial growth. In consideration of mass balance, metabolites **4**−**14** can only account for

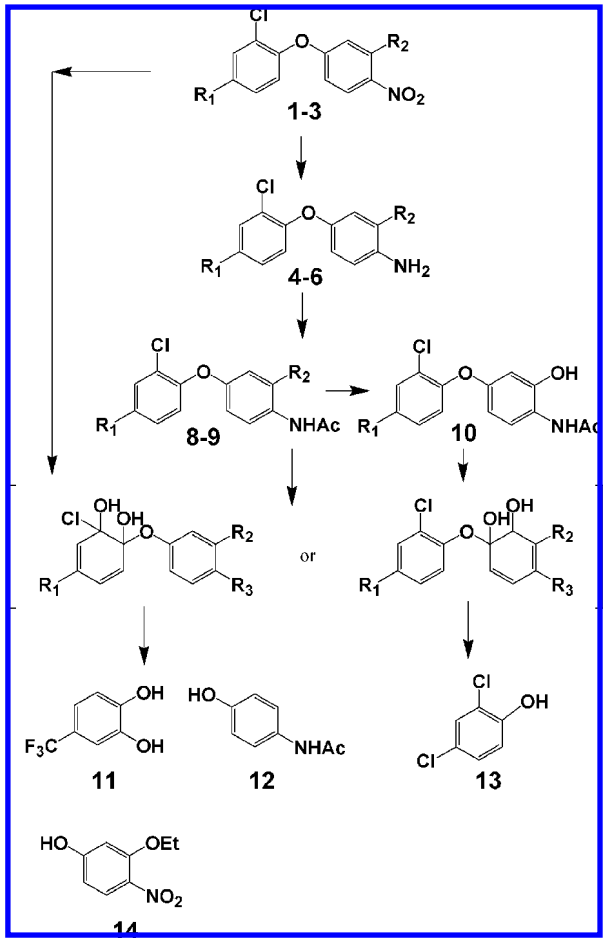

Figure 7. Metabolic pathways of nitrodiphenyl ether herbicides by *Sphingomonas wittichii* RW1. Metabolites within brackets are threoretical intermediates, not detected in this study.

30−75% of parent pesticide degradation, which reveals further degradation or incorporation into primary metabolism. It is well known that bacterial degradation of substituted phenols proceeds to muconate (*34, 35*). Because catechols and phenols from nitrodiphenyl ether herbicide can also be metabolized into muconate or its analogues, it can be postulated that strain RW1 may be able to assimilate selected pesticides through similar pathways.

In summary, strain RW1 can utilize not only PCDD/Fs but also several nitrodiphenyl ether herbicides as a sole carbon source for growth. Reduction of the nitro group with consecutive *N*-acetylation was the most dominant initial reaction, followed by diaryl ether bond cleavage. Detailed analysis of metabolite profiles suggests that degradative enzyme(s) in RW1 may have complex regio-selectivities. Kinetic analysis indicates that identified metabolites may be further degraded to support bacterial growth. These findings suggest extremely versatile metabolic activities of this strain in relation to possible application in bioremediation.

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