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Biodegradation of alachlor by soil streptomycetes

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Abstract Streptomycetes resistant to the herbicide alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide] were used in degradation assays to characterize the products of alachlor biodegradation. Of six strains tested, *Streptomyces* sp. LS166, LS177, and LS182 were able to grow at an alachlor concentration of 144 mg l^{-1} and degraded approximately 60–75% of the alachlor in 14 days, as evaluated by high performance liquid chromatography. The alachlor biodegradation products were identified by gas chromatography-mass spectrometry based on mass spectral data and fragmentation patterns. All compounds detected in these assays were similar for all streptomycetes strains tested, and involved dechlorination with subsequent *N*-dealkylation and cyclization of the remaining *N*-substituent with one of the ethyl groups to produce indole and quinoline derivatives. The enzymatic pathway used by *Streptomyces* sp. LS182 did not generate DEA (2′,6′-diethylaniline), a carcinogenic derivative of alachlor reported in other studies. Given the high degradation rates observed here, the *Streptomyces* strains tested may be useful in the degradation/detoxification processes of alachlor.

Introduction

The chloroacetanilides, including alachlor (**1**, in Fig. 1) [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide], are selective herbicides widely used for pre-emergent weed control in the cultivation of corn, soybeans, and other commercially important crops. Alachlor is classified as extremely toxic, with a moderate residual effect in soils. Because of its direct application onto soil, alachlor may leach into groundwater and thereby pose a potential health hazard to humans and animals (Monteiro 1997).

The oncogenic effects of a degradation by-product of alachlor, 2,6-diethylaniline (DEA) (Fig. 1, **3**) in rats and mice (Coleman et al. 1999, 2000; Hanioka et al. 2002) led the U.S. Environmental Protection Agency (EPA) to declare alachlor a potential human carcinogen. DEA is environmentally stable (Osano et al. 2002) and may undergo hydroxylation and oxidation reactions in the body to produce diethylbenzoquinonimine (DEBQI), a toxic molecule that interacts with DNA (Coleman et al. 1999, 2000).

Alachlor is degraded mainly by soil microorganisms, although no pure or mixed cultures able to extensively degrade or mineralize alachlor have been reported. Bacteria and fungi can degrade alachlor through co-metabolism (Smith and Phillips 1975; Novick and Alexander 1985; Sun et al. 1990; Ferrey et al. 1994; Shelton et al. 1996; de Schrijver and de Mot 1999). Natural biodegradation alone is insufficient to remove alachlor and alachlor derivatives have been detected in surface and groundwater (Aga and Thurman 2001; Osano 2002). The main alachlor biotransformation products reported include 2-chloro-2′,6′-diethylacetanilide (**2**), 2,6-diethylaniline (**3**), 2,6-diethyl-*N*-(methoxymethyl) aniline (**4**), 1-chloroacetyl-2,3-dihydro-7-ethylindole (**5**), 2,6-diethyl-*N*-(methoxymethyl) acetanilide (**6**) and *bis*-2-thio-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide (**7**) (Fig. 1) (Stamper and Tuovinen 1998).

Amongst bacteria, the actinomycetes exhibit a vast metabolic and physiological diversity, and play important roles in the degradation of a wide range of complex and polymeric compounds in nature (McCarthy and Williams 1992). Despite their interesting biotechnological properties, relatively few actinomycetes have been evaluated for their ability to degrade pesticides and other environmental pollutants.

In this study, six highly tolerant streptomycete strains, isolated from alachlor-enriched soil samples in Brazil,

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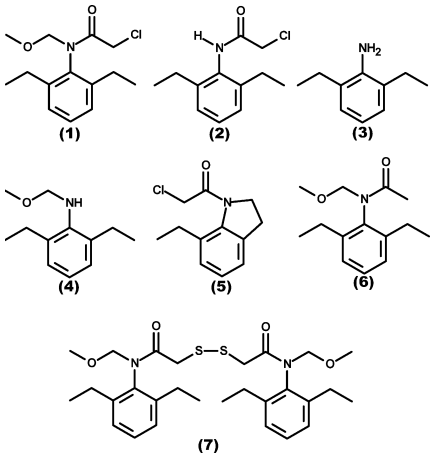

Fig. 1 Alachlor (**1**) and its main biotransformation derivatives reported by Stamper and Tuovinen (1998), including: 2-chloro-2′,6′-diethylacetanilide (**2**), 2,6-diethylaniline (**3**), 2,6-diethyl-*N*-(methoxymethyl) aniline (**4**), 1-chloroacetyl-2,3-dihydro-7-ethylindole (**5**), 2,6-diethyl-*N*(methoxymethyl)acetanilide (**6**), and *bis*-2-thio-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide (**7**)

were investigated for their ability to degrade alachlor based on HPLC analysis. The alachlor biotransformation products were identified using GC-MS. Two different alachlor samples (freshly diluted commercial alachlor and a long-term storage aqueous solution) were used in these experiments.

Materials and methods

Herbicide, microorganisms and culture conditions

Alachlor was obtained as an emulsifiable concentrated formulation (480 g l^{-1}) from Monsanto (Jacareí, SP, Brazil). Freshly prepared dilutions in sterile distilled water (sample 1) were used for degradation experiments and chromatographic analyses. To evaluate the formation of decomposition by-products upon dilution and storage, the herbicide was diluted in sterile distilled water and stored for up to 6 months at room temperature prior to analysis (sample 2).

Streptomyces sp. strains LS143 (DSM 41805, CBMAI 0001), LS151 (DSM 41806, CBMAI 0002), LS153 (DSM 41807, CBMAI 0003), LS166 (DSM 41808, CBMAI 0004), LS177 (DSM 41809, CBMAI 0005) and LS182 (DSM 41810, CBMAI 0006) used in this study were isolated from soil samples treated with alachlor (Sette

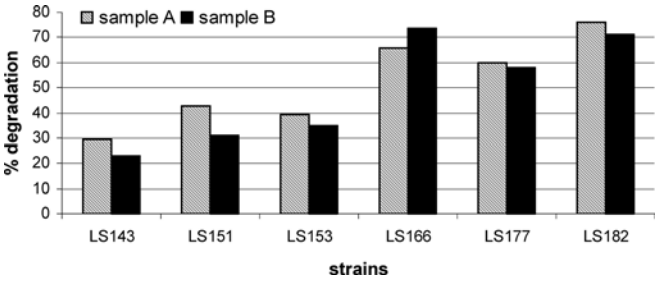

Fig. 2 Degradation of alachlor (144 mg l^{-1}) by pure cultures (replicate analyses) after incubation for 14 days (150 rpm, 30°C)

2001), and selected based on their tolerance to high concentrations of the herbicide (720 mg l^{-1}). The strains were deposited at the CBMAI (Coleção Brasileira de Microrganismos de Ambiente e Indústria, Campinas, Brazil) and DSMZ (http://www.dsmz.de) culture collections.

Starter cultures of spores and mycelia were grown in 150-ml Erlenmeyer flasks containing 10 ml liquid mineral salts medium (2 g Na_2SO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.65 g K_2HPO_4, 1 g NH_4Cl, 1 g KNO_3, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.1% yeast extract, in 1 l distilled water, pH 7.3), supplemented with 0.5% glucose. Flasks were incubated without shaking for 24 h at 30°C, followed by 48 h with shaking (150 rpm).

Biodegradation assays

Pure culture experiments were done in 150-ml Erlenmeyer flasks containing 30 ml liquid mineral salts medium, supplemented with alachlor (sample 2, as described previously) at a concentration of 144 mg l^{-1}. The flasks were inoculated with 10 ml of starter cultures and incubated on a rotary shaker (150 rpm) for 14 days at 30°C. Each experiment was carried out in duplicate and non-inoculated flasks were run as controls.

Analytical methods

Broths from pure cultures containing alachlor were centrifuged (15,000 *g* 10 min^{-1}), filtered (0.22-µm Millipore filter), and 20-µl aliquots were analyzed by HPLC using an LC-6A Shimadzu HPLC system equipped with a C18 column (25 cm×4.6 mm diameter, Varian, Brazil) and a UV-visible absorbance detector set at 220 nm. Acetonitrile/water (50:50, v/v) was used as the mobile phase at a flow rate of 1 ml min^{-1} at ambient temperature. The extent of degradation was expressed relative to the concentration (peak area) of control samples.

The biodegradation products were identified by GC-MS. Organic compounds were extracted from the centrifuged culture broth (40

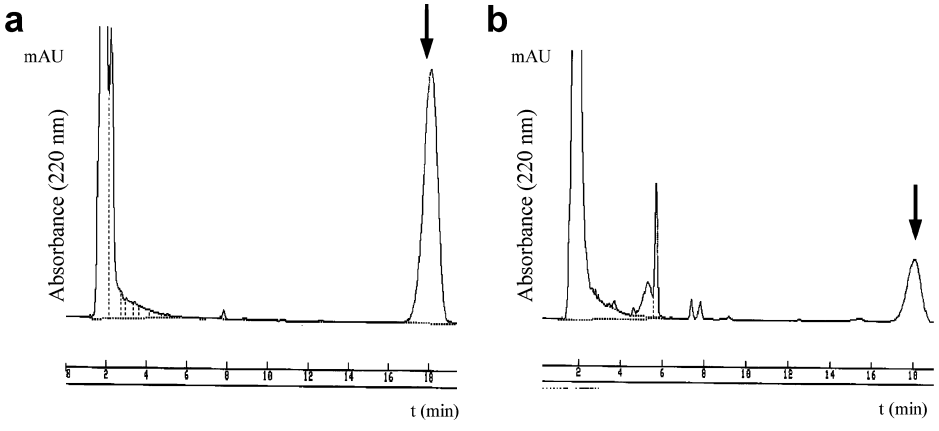

Fig. 3a, b HPLC chromatograms showing the degradation of alachlor (144 mg l^{-1}) by *Streptomyces* sp. LS182 after 14 days. **a** control, **b** after incubation (alachlor peak indicated by *arrows*)

Table 1 Alachlor decomposition products in aqueous solution and biodegradation compounds detected by GC-MS. *MW* Mass weight, *RT* retention time, *nd* not detected

Compound	Name	MW	RT	Kovats index[a]	Relative abundance (%)						
					Control	LS143	LS151	LS153	LS166	LS177	LS182
1	alachlor [2-chloro-*N*-(methoxymethyl)-*N*-(2′,6′-diethyl-phenyl)-acetamide]	269	10.84	1918	79.02	71.69	51.61	55.95	48.18	52.07	44.77
2	2-chloro-2′,6′-diethylacetanilide	225	9.39	1716	10.90	10.63	8.19	11.05	9.90	10.44	6.30
3	2,6-diethylaniline (DEA)	149	6.05	1117	2.29	2.32	3.88	<0.5	4.54	5.20	1.20
5	1-chloroacetyl-2,3-dihydro-7-ethylindole	223	8.83	1646	nd	nd	1.23	2.05	nd	nd	1.99
8	*N*-(2,6-diethylphenyl)-methyleneamine	161	5.61	1081	1.62	1.53	0.79	1.19	0.93	1.21	0.44
9	2,6-diethylacetanilide	191	8.57	1629	1.63	2.00	1.55	2.23	1.45	1.81	0.77
10	2-hydroxy-2′,6′-diethyl-*N*-acetanilide	251	10.46	1889	3.26	6.44	3.90	2.05	5.48	1.51	<0.5
11	7-ethylindole	147	6.18	1344	nd	nd	1,90	2.80	1.30	1,10	6.90
12	7-ethyl-3-methyl-2-methoxy-2,3-dihydroindole	191	6.34	1360	nd	nd	nd	0.90	nd	0.77	1.33
13	8-ethylquinoline	156	6.75	1395	nd	<0.5	2.71	8.36	4.92	10.01	12.11
14	7-ethyl-*N*-methylindole	159	8.07	1481	nd	nd	nd	0.63	nd	nd	1.18

[a]Calculated according to Buzan et al. (1969)

Table 2 Main fragments and relative abundance of some alachlor biodegradation products

Compound[a]	Main fragmentation ions (% relative abundance)
5	223 ($M^{\bullet+}$, 12), 208 (16), 174 (40), 146 (100)
11	147 ($M^{\bullet+}$, 60), 132 (100), 117 (60), 77 (16)
12	191 ($M^{\bullet+}$, 20), 160 (100), 144 (24), 132 (60)
13	157 ($M^{\bullet+}$, 60), 156 (100), 142 (10), 129 (32)
14	159 ($M^{\bullet+}$, 52), 158 (24), 144 (100), 130 (12)

[a]Names listed in Table 1

ml) using C18 micro columns (SEP-PAK cartridge, Waters, Milford. Mass., USA) followed by elution with water (3 ml) and diethyl ether (6 ml). The assembled organic fractions were evaporated under a nitrogen stream to 50–500 µl volume and 1-µl aliquots were analyzed by GC-MS (splitless mode) in a Hewlett-Packard 5890 gas chromatograph system, equipped with a DB-5 fused silica capillary column (30 m×0.25 mm×0.25 µm, J.W. Scientific, Folsom, Calif., USA) attached to an HP 5970 DMS mass spectrometer operated at 70 eV for ionization. The detector and injector temperatures were 260°C and 220°C, respectively. The pressure of the carrier gas (helium) was 10 psi; the oven temperature after sample injection was 80°C, but increased to 290°C at 15°C min^{-1}.

Results

The degradation of alachlor (144 mg l^{-1}) after 14 days of incubation is shown in Fig. 2. More than 55% degradation was observed with strain LS166, about 70% with strain LS177, and 75% with strain LS182. Some additional peaks corresponding to biodegradation products and other secreted compounds were also observed in the culture broth extracts (Fig. 3b).

GC-MS analysis of a sample of alachlor (sample 2) was used to identify the alachlor byproducts, which included 2-chloro-2′,6′-diethylacetanilide (**2**), 2,6-diethylaniline (**3**), *N*-(2,6-diethylphenyl)-methyleneamine (**8**), 2,6-diethylacetanilide (**9**) and 2-hydroxy-2′,6′-diethyl-*N*-acetanilide (**10**) (Fig. 4a, Fig. 5, and Table 1).

Comparison of the total ion chromatograms of samples from the biodegradation experiments revealed extensive similarity amongst generated products (Table 1). The main products were 1-chloroacetyl-2,3-dihydro-7-ethylindole (**5**), 7-ethylindole (**11**), 7-ethyl-3-methyl-2-methoxy-2,3-dihydroindole (**12**) and 7-ethyl-*N*-methylindole (**14**) (Fig. 4b, Fig. 6, Table 2), identified as indole derivatives, and a quinoline compound identified as 8-ethylquinoline (**13**), all identified based on their fragmentation patterns (Marx and Djerassi 1968; Draper and MacLean 1968). Additional compounds, including (**2**, **3**, **9**, **10**) were also detected (Fig. 4b, Fig. 5, and Table 1).

Alachlor degradation by LS182 using sample 1 was analyzed by GC-MS (Fig. 4d) and only two compounds, 2-chloro-2′,6′-diethylacetanilide (**2**) (Fig. 5) and 1-chloroacetyl-2,3-dihydro-7-ethylindole (**5**) (Fig. 6), were detected.

Discussion

The six alachlor-resistant *Streptomyces* strains used here were capable of degrading high concentrations (144 mg l^{-1}, or 144 ppm) of alachlor (sample 1) and other alachlor by-products (sample 2) in vitro. Most of the previous studies on alachlor degradation used up to 100 ppm of the herbicide (Novick and Alexander 1985; Capri and Walker 1993; Konopka 1994; Ferrey et al. 1994; Shelton et al. 1996), which was comparable to that used here (144 ppm). However, only one of these reports (Shelton et al. 1996) described the ability of *Streptomyces* sp. to transform alachlor (50 ppm). In this case, strain PS1/5 degraded 95% of the herbicide, although the culture medium included dextrin as an additional carbon source. This level of degradation was not directly comparable to that observed in our study, because of the different conditions used.

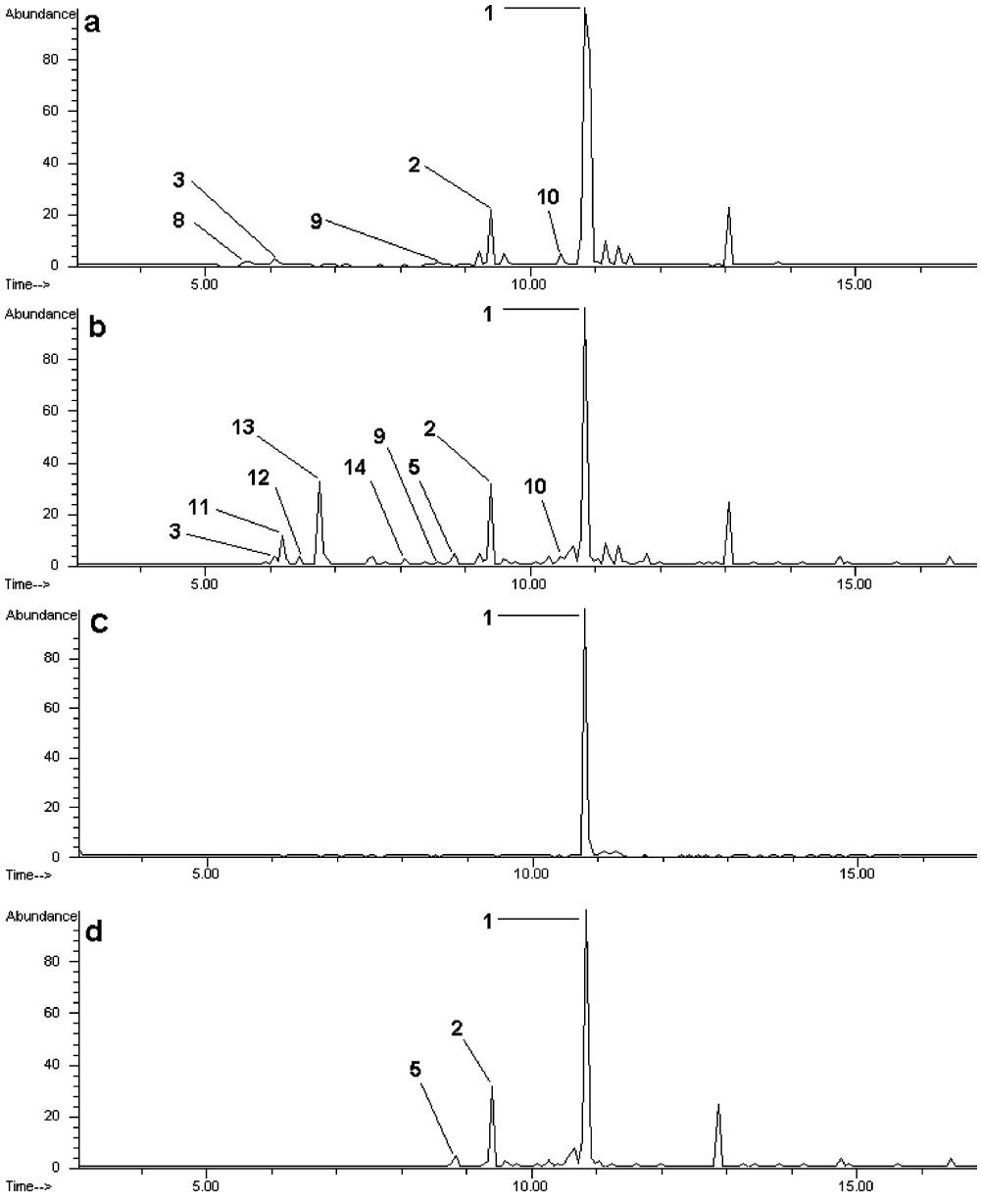

Fig. 4 GC-MS total ion chromatograms of **a** a control of sample 2, **b** the biotransformation of sample 2 by *Streptomyces* LS182, **c** a control of sample 1 and **d** the biotransformation of sample 1 by strain LS182 (see text for details). *Numbers* refer to the compounds shown in Fig. 5, Fig. 6, and Table 1)

GC-MS analysis showed that there was no aromatic-ring cleavage by the streptomycete strains tested. However, extremely slow mineralization of alachlor has been detected under conditions of co-metabolism (Novick et al. 1986; Ferrey et al. 1994; Yen et al. 1994). In agricultural soils, less than 4% of the alachlor aromatic carbon was mineralized in 30–120 days (Novick et al. 1986; Yen et al. 1994). Mineralization by white-rot fungi (18 ppm alachlor) was about 6–14% over 122 days of incubation (Ferrey et al. 1994).

The main biodegradation products in our experiments consisted of dechlorinated indole compounds (**11**, **12**, and **14**), a quinoline derivative compound (**13**) and a chlorinated indole derivative (**5**). Of these, compounds **13** and **14** have not been mentioned before as alachlor biodegradation products and their structures were suggested based on their mass spectra and fragmentation patterns. 8-ethylquinoline compound (**13**) was confirmed by comparison with the spectrum reported by Draper and MacLean (1968). The mass spectrum for 7-ethyl-*N*-methylindole (**14**) was not found in the literature, but its main fragments were analogous to those of methylindole, thus providing an additional argument for the proposed structure (Marx and Djerassi 1968). The remaining compounds (**5**, **11,** and **12**) were reported by Mangiapan (1997). Analysis of the biotransformation products indicated that 2-chloroacetyl was the nitrogen substituent most easily cleaved by the enzymes since among the biotransformation products detected only compound **5** had this group. Cyclization and the formation of *N*-heteroaromatic compounds was by far the most important biotransformation reaction as all of the detected compounds had an extra cycle in their structure when compared to alachlor. Tiedje and Hagedom (1975) proposed a pathway for the biotransformation of

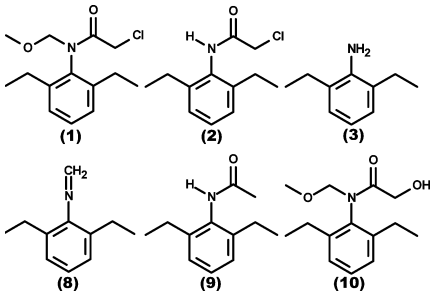

Fig. 5 Alachor (**1**) and derivatives arising from decomposition in water, as detected in control experiments without microbial inocula (names given in Table 1)

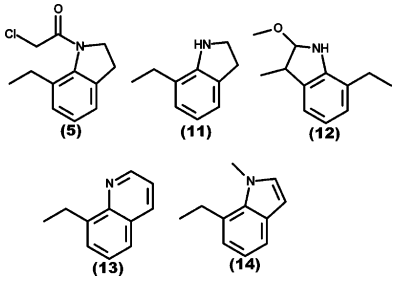

Fig. 6 Alachlor derivatives detected in the *Streptomyces* biodegradation assays (compound names given in Table 1)

alachlor into compound **5** (Fig. 7) that perfectly explains the formation of compound **11**. However, the biotransformation route had to be adapted in order to explain the remaining products (Fig. 7). Based on compound **8**, we were able to explain the origin of compounds **13** and **14**. The enzymatic cyclization of *N*-alkylaniline by horseradish peroxidase (Hanzlik et al. 2001) has been described and supports our biotransformation pathway (Fig. 7).

Compared to alachlor (water solubility of 242 mg l^{-1} at 25°C) (Zagorc-Koncan 1996), the biotransformation products detected in this study are relatively water-soluble, and have a weak adsorption to soil particles and aquifer organic material (Fetzner 1998). These compounds may be degraded further by microorganisms in nature (Shukla 1986; Aislabie et al. 1990; Pothuluri et al. 1993; Kaiser et al. 1996; Licht et al. 1997; Fetzner 1998).

The carcinogenic product 2,6-diethylaniline (DEA, **3**, Fig. 6) was detected in the alachlor (sample 2) degradation experiments (Table 1). This compound has been reported to be present in groundwater at twice the concentration of alachlor (Osano et al. 2002), indicating that it may be a potential environmental hazard. However, GC-MS analysis carried out using *Streptomyces* sp. strain LS182 indicated that DEA was a by-product of the herbicide in aqueous solution (Fig. 5), being detected only in control samples without microbial inoculum.

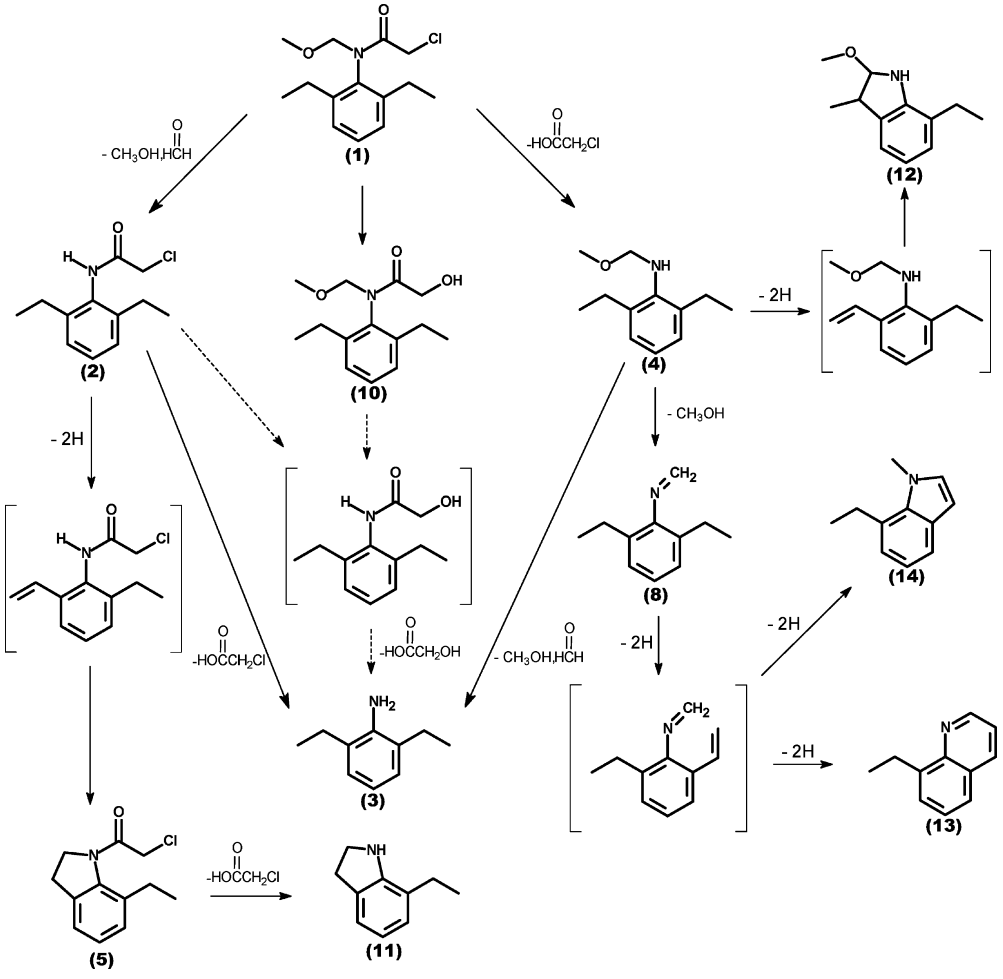

Fig. 7 Presumed pathway of alachlor biotransformation based on Tiedje and Hagedorn (1975) and on our results

Our results also indicated that strain LS182 may use alachlor by-products to generate compounds **11**, **12**, **13**, and **14** (Fig. 6, Fig. 7) since these are not detected when pure alachlor (sample 1) is added to the media. In the latter case, only compounds **2** and **5** were detected (Fig. 4d). Tiedje and Hagedorn (1975) reported that compounds **2** and **5** were preferentially formed by the fungus *Chaetomium globosum*, compared to production of DEA. Our data also suggest that *Streptomyces* strain LS182 transformed alachlor by bypassing the formation of DEA, an undesirable, toxic by-product.

In conclusion, we have demonstrated the dechlorination and biotransformation of alachlor by six *Streptomyces* strains, and have identified the main indole and quinoline derivatives in this process. Strains LS166, LS177, and LS182, which showed high degradation rates, may have potential applications in biotechnological and bioremediation processes, particularly in the initial steps of alachlor biotransformation.

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