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Enantiomeric Purity of Biodegradation Products of Juvenogens by Newly Isolated Soil Bacteria

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Two bacteria were isolated from sand RQ30, characterized as *Bacillus simplex* and *Bacillus* sp. strain 05 (GenBank EU399813), and were used as biocatalysts for a hydrolytic assay of stability of the cis or trans isomers of ethyl *N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]methyl}phenoxy}ethyl}carbamate, which are among insect hormonogen substances (juvenogens). The stability tests were performed using simple modeling under laboratory conditions. The structures of the products were assigned as ethyl (1*R*,2*R*)-*N*-{2-{4-[(2-hydroxycyclohexyl)methyl]phenoxy}ethyl}carbamate and ethyl (1*S*,2*R*)-*N*-{2-{4-[(2-hydroxycyclohexyl)methyl]phenoxy}ethyl}carbamate on the basis of ^{1}H and ^{13}C NMR, IR, and FAB-MS analyses.

KEYWORDS: ***Bacillus simplex***; ***Bacillus* sp.**; **hydrolysis**; **juvenogen**; **enantiomeric purity**

INTRODUCTION

At present, an environmentally safe means of insect pest management is represented by insect growth regulators (IGRs) acting on insect morphology and reproduction. A long-term investigation in this area resulted in designing several structurally different types of compounds (*1–3*). Among those IGR agents, juvenogens represent relatively convenient IGRs, which have already proven a high effectiveness against several nonrelated insect species, including termites (*1, 4*). Termites represent dangerous pests, typical in tropical and subtropical areas of the Earth, that already have started to migrate into mild climate areas due to global climate changes. Juvenogens usually display only a low biological activity in topical application against insects but may be activated through in vivo biochemical pathways. Selection of a suitable juvenogen for insect (termite) treatment should be based not only on its biological activity against the target species but also on its environmental impact on nontarget organisms, which is mainly given by metabolites of the applied IGR agent produced by soil microbial strains (*5*).

Bacteria produce and secrete lipases, which can catalyze both hydrolysis and synthesis of acylglycerols. These reactions usually proceed with a high regioselectivity and enantioselectivity, and therefore, lipases have become very important stereoselective biocatalysts used in organic chemistry (*6*). Some microbes in soil are generally able to metabolize insecticides to obtain energy and nutrients (*7*), while the insecticide may have a deleterious effect on other groups of organisms present in soil (*8, 9*). When pesticides are metabolized by microbes, their degradation may be affected by a population of microorganisms that is present on the site, the nutrient supply to the biodegradation agents, and other conditions that may affect their activity and the availability of the pesticide to the degrading population. One of the key microorganisms in soil belongs to the genus *Bacillus*, which is an extensive heterogeneous group encompassing 83 validly described species to date. Members of the group share a great deal of morphological and biochemical similarities (*10–12*). In contrast, the environmental and nonpathogenic species of this genus exhibit a wide range of physiologies, DNA base contents, and nutritional requirements (*13, 14*).

Soil microbial activities play an important role in organic matter turnover, element cycling, and plant growth. For these reasons, the assessment of both biological presence and activity should be taken into account following soil amendment (*15*). This finding can be helpful to evaluate the efficiency of a remediation treatment and/or its influence on soil functional recovery. For this purpose, standard microbiological methods can be combined with community approaches to detect, with a better level of resolution, any possible structural and/or functional changes of soil microbial population.

The objectives of this study were (a) the investigation of degradation of the racemic cis or trans isomers of ethyl *N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]methyl}phenoxy}ethyl}carbamate (insect hor-

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monogen substances (juvenogens)) by selected soil microorganisms under laboratory conditions and (b) isolation and structure assignment of the main degradation products. Two types of *Bacillus* species were newly isolated from the typical soil, characterized and used as the biodegradation agents in this study.

MATERIALS AND METHODS

Chemicals. Ethyl (*cis*)-*N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]methyl}-phenoxy}ethyl} carbamate (**1**) and ethyl (*trans*)-*N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]methyl}phenoxy}ethyl} carbamate (**2**) were synthesized as described previously (*16*).

Analytical Methods Used. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian UNITY 500 spectrometer (in FT mode) at the respective 499.8 and 125.7 MHz frequency values either in deuteriochloroform using tetramethylsilane ($\delta = 0$) as an internal reference or in hexadeuteroacetone using the central line of the solvent ($\delta = 2.13$) as an internal reference. The ^{19}F NMR spectra were recorded at 470.27 MHz frequency in deuteriochloroform using hexafluorobenzene as the external reference ($\delta = -162.9$). Column chromatography purifications were performed on silica gel 60 column (particle size 0.04–0.063 mm, Fluka) using light petroleum ether/diethyl ether as the mobile phase. TLC was performed on precoated silica gel TLC plates. A column (250 mm × 4 mm) filled with Biosphere Si-100 (5 μm, Watrex) was employed for HPLC analysis of the MTPA esters of **1a**, **2a**, **1c**, and **2c** using light petroleum/ether (9:1 v/v) as mobile phase at 1 mL/min.

Analyses of the chiral products were performed on a HPLC instrument (Waters: Delta 600E multisolvent delivery system, Waters 2996 PDA detector, and Empower 1 PDA software) under the following chromatographic conditions: column packed with a Nucleodex β-OH stationary phase (Macherey-Nagel, 200 mm × 4 mm, 5 μm particle size), using a mixture of mobile phase A (water) and mobile phase B (MeOH) at a flow rate of 0.3 mL/min. UV detection was monitored at 210, 220, 225, 254, and 275 nm. A gradient program (initial conditions of 30% A and 70% B, linearly increasing to 90% B over 30 min, holding for 10 min, returning to the initial conditions of 30% A and 70% B over 5 min, and holding for 15 min) was employed.

Microbial Strains Isolation and Selection. Standard quartz sand RQ30 (3 g; grain size 0.1–0.5 mm, Kaiser and Kraft) stored at 5 °C was shaken for 1 h with sterile distilled water (20 mL). The sand leach (pH 6.0) was quickly centrifuged (100*g*, 10 s), and the supernatant was collected. Because of a small amount of microorganisms in the sample, the supernatant was centrifuged (10 000*g*, 5 min), 15 mL of the upper layer of supernatant was removed, and the remaining portion (5 mL) was properly uniformly mixed. Then, 400 μL of this thickened leach was applied to Petri dishes with complete medium (yeast autolysate (5 g/L), peptone (3 g/L), casein hydrolysate (5 g/L), glucose (10 g/L), and agar (20 g/L)), malt agar (commercial, Sigma-Aldrich), and CASO agar (commercial, Fluka), and dishes were incubated for 2–3 days at 28 °C. Fifteen bacterial and three mold strains were isolated. Each isolate was inoculated on Petri dishes with minimal medium (bacteria: K_2HPO_4 (3.5 g/L), KH_2PO_4 (1.5 g/L), NH_4Cl (0.5 g/L), Na_2SO_4 (0.14 g/L), $MgCl_2 \cdot 6H_2O$ (0.15 g/L), and agar (12 g/L) and molds: yeast nitrogen base without amino acids (6.7 g/L) and agar (22 g/L)) and with 15 mg of **1** or **2**, respectively, as the only carbon source applied in 100 μL of ethanol to the surface of a medium in the Petri dishes. Dishes were incubated for 3–5 days at 28 °C. Eleven bacterial and all mold strains demonstrated the ability to grow on this medium. Two bacterial strains were selected for degradation experiments due to their most rapid growth on the medium with both compounds. Both strains were stored at −70 °C (each strain was cultivated for 24 h, then a volume of 700 μL of bacterial suspension was mixed with 300 μL of sterile glycerol and deep frozen; during revival, the suspension was applied on Petri dishes with complete medium). During a set of experiments, the strains were kept on slant agars at 4 °C. One of them was identified by biochemical tests: reduction of nitrate, hydrolysis of starch, hydrolysis of DNA, anaerobic growth, growth at 50 °C, catalase, growth at 10% NaCl, utilization of citrate, hydrolysis of aesculin, hydrolysis of casein, acid from glucose, acid from xylose, acid from celobiose, hydrolysis of casein, hydrolysis of gelatin, hydrolysis of Tween 80, and hydrolysis of tyrosine in the Czech Collection of Microorganisms, Faculty of Sciences, Masaryk University, Brno, as *Bacillus simplex*. The second strain was identified using 16S rDNA sequence analysis as *Bacillus* sp. strain 05.

Table 1. Phenotypic Character Strains *Bacillus* sp. Strain 05 and *B. psychrodurans*[a]

character	*Bacillus* sp. strain 05	*B. psychrodurans*
sporangium swollen	−	+
reduction of nitrate	−	+
hydrolysis of starch	−	+
hydrolysis of DNA	+	+
anaerobic growth	+	+
growth at 37 °C	+	−
catalase	+	+
growth at 10% NaCl	−	−
utilization of citrate	−	−
hydrolysis of aesculin	−	−
hydrolysis of casein	−	−
acid from xylose	−	−
hydrolysis of gelatin	−	+
hydrolysis of Tween 80	−	−
acid from glucose	+	+
growth at 4 °C	+	+
hydrolysis of tyrosine	−	−
cleavage of urea	−	−

[a] +: positive and −: negative.

16S rDNA Sequence Analysis. Nearly the entire 16S rRNA gene, between positions 27 and 1492 (*Escherichia coli* 16S rRNA gene sequence numbering), was amplified by PCR using universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR mixture contained reaction buffer, 100 μM concentrations of deoxynucleoside triphosphates, 0.2 μM concentrations of primers, ~80 ng of total DNA, and 2.5 U of Taq DNA polymerase (TopBio). PCR was performed in a Biometra T1 Thermocycler (Whatman Biometra) with the following conditions: initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. The resulting PCR product was extracted from 0.6% agarose gel with a Zymoclean Gel DNA Recovery Kit (ZymoResearch) and directly sequenced (Applied Biosystems 310 DNA sequencer, Big Dye Terminator cyclesequencing ready kit, PerkinElmer) using the following primers: F27, F357 (5′-ACTCCTACGGGAGGCAGCAG-3′), R922 (5′-CCGCTTGTGCGGGCCCCCGTC-3′), and R1492.

Sequence data were aligned manually with the known 16S rRNA sequence using conserved regions and secondary structure characteristics as references. A comparative analysis demonstrates that the 16S rRNA gene sequence from the tested strain has a significant identity (98–99%) to a number of strains of the Gram-positive bacterial genus of *Bacillus*. The greatest identity (10 bp differences in ca. 1430 bp) was found in the *Bacillus* sp. CK7 strain (GenBank AJ920000.1). Other close matches were found in the *Bacillus psychrodurans* strain DSM 11713 (15 bp difference, GenBank AJ277984). However, our strain shows some different biochemical characteristics (**Table 1**) than those for *B. psychrodurans* (*17*). On that basis, we characterized our strain as *Bacillus* sp. strain 05. The nucleotide sequence of the 16S rRNA gene from *Bacillus* sp. strain 05 is catalogued in the GenBank under accession number EU399813.

Biotransformation. *Bacillus simplex* and *Bacillus* sp. strain 05 (GenBank EU399813) were checked microscopically prior to each experiment. Overnight cultures (2 × 20 mL) grown in simple Erlenmeyer flasks (250 mL) sealed with aluminum foil at 28 °C in a minimal medium for bacteria with 3% (w/w) Casamino acids (Difco vitamin-free Casamino acids) and 1% (w/w) glucose were centrifuged (10 000*g*, 20 min) and collected. Cells were washed by 0.1 M phosphate buffer (pH 7.1; 3 × 10 mL) and resuspended in phosphate buffer (20 mL). These cell suspensions [20 mL; *B. simplex* or *Bacillus* sp. strain 05 or *B. simplex* (10 mL) and *Bacillus* sp. strain 05 (10 mL)] were used as sources of enzymes for biodegradation of **1** or **2** (0.047 mmol).

The biomass of both strains of *Bacillus* was determined by measuring the dry cell weight (DCW). The cells collected at stationary phase from

Scheme 1. Reaction Pathway

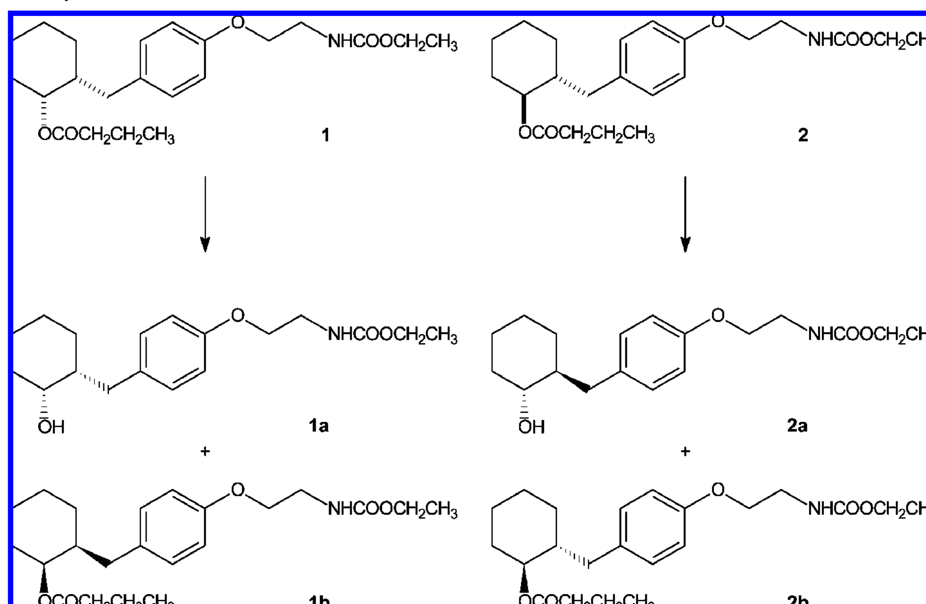


Table 2. Hydrolysis of **1** and **2** Catalyzed by *B. simplex* or *Bacillus* sp.[a]

substrate	ch. y. (%)	bioconversion activity (μmol/g DCW)	ee_p (%)	ee_s (%)	*c*	*E*
		B. simplex				
1	4.0	0.812	55.24	1.02	0.018	3.49
2	7.9	1.604	96.00	3.45	0.035	50.72
		Bacillus sp.				
1	0	0	0	0	0	0
2	3.5	1.814	67.52	1.34	0.019	5.22

[a] ch. y.: chemical yield; ee_p: enantiomeric purity of product **1a** or **2a**; ee_s: enantiomeric purity of product **1b** or **2b**; *c*: conversion, $c = ee_s/(ee_s + ee_p)$; *E*: enantiomeric ratio calculated according to the formula $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.

Table 3. Hydrolysis of **1** and **2** Catalyzed by *B. simplex* and *Bacillus* sp. in Concert[a]

	B. simplex and *Bacillus* sp.				
substrate	ch. y. (%)	ee_p (%)	ee_s (%)	*c*	*E*
1	4.2	57.20	1.05	0.018	3.71
2	9.7	>99	5.27	0.057	210.99

[a] Abbreviations as in **Table 2**.

fermentation broth were centrifuged for 20 min at 4500*g*; the pellet containing cells was washed repeatedly with distilled water. Then, the cells were filtered and dried at 65 °C until a constant weight was achieved to obtain the DCW measurement. Biomass concentration was 2.3 g DCW of *B. simplex* per liter and 0.9 g DCW of *Bacillus* sp. per liter. The solubility of **1** or **2** in phosphate buffer was improved by the addition of acetone (250 μL). The reaction progress was monitored by TLC. After 7 days of stirring at 28 °C, progress was not observed, and the reaction was ceased.

Final workup consisted of extracting the organic compounds into diethyl ether, drying the extract (Na_2SO_4), evaporating the solvent under reduced pressure, and chromatographic separation of the residue. The products were characterized as given by **1a** or **1b** and **2a** or **2b** (**Scheme 1**).

Chemical yields of the products are summarized in **Tables 2** and **3**. Three independent experiments were performed. In all experiments, the reaction conditions and analysis of the products were identical. The repeatability of the experiment was good. The repeatability of the standard deviation of obtained results (chemical yields of the reactions, enantiomeric purity of the products, and enantioselectivity of the process) was ~3−5% (based on absolute yield value).

Alkaline Hydrolysis of Chiral Esters 1b and 2b to Chiral Alcohols 1c and 2c. The respective cis or trans isomers of ethyl *N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]methyl}phenoxy}ethyl} carbamate **1b** or **2b** (5 mg, 0.012 mmol) were dissolved in a 1 M solution of sodium carbonate in absolute ethanol (2 mL). The solution was stirred for 4 h at 25 °C, and the reaction course was checked by TLC. When the reaction was quantitatively completed, the solvent was evaporated under reduced pressure. Purification of the product by column chromatography afforded the respective chiral alcohols **1c** or **2c** (**Scheme 2**).

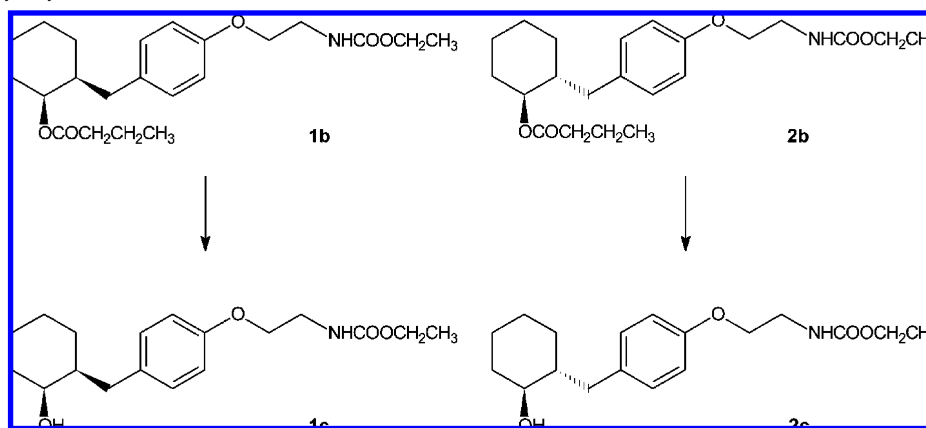
3,3,3-Trifluoro-2-methyl-2-phenylpropanoic Acid (MTPA) Ester Alcohols 1a, 1c, 2a, and 2c. A general procedure used for the preparation of MTPA esters in a milligram scale starting from the chloride of MTPA is described in detail in ref *18*. In a typical experiment, a solution of either enantiomers of 3,3,3-trifluoro-2-methyl-2-phenylpropanoyl chloride (0.07 mmol) in benzene (500 μL) or a solution of 4-(dimethylamino)pyridine (0.005 mmol) in pyridine (30 μL) was added to a solution of the respective alcohols **1a**, **1c**, **2a**, and **2c** (0.05 mmol) in benzene (200 μL). The mixture was stirred at laboratory temperature for 3−5 h. Thereafter, benzene was evaporated under reduced pressure, and the residue was dissolved in light petroleum ether and purified by column chromatography. The spectral data of the products are in agreement with earlier published data (*19, 20*).

RESULTS AND DISCUSSION

Juvenogens (*2*) are chemical compounds that usually display low or no biological activity toward insect species but are capable of liberating the biologically active ingredient under the effect of abiotic or biotic conditions. In this investigation, the earlier prepared fatty acid esters with the alcoholic portion derived from alcoholic juvenile hormone analogues **1** and **2** were studied. The effect of selected soil bacteria on the stability of juvenogens **1** and **2** was the subject of the present investigation, in which *B. simplex* and *Bacillus* sp. strain 05 were used for the degradation studies.

The strains of both tested species of *Bacillus* were cultivated in a minimal medium for bacteria with the addition of 3% (w/w) Casamino acids and 1% (w/w) glucose. All bacteria must utilize the energy sources in their environment to produce ATP, which is required for the biosynthetic processes that bacteria use for their maintenance and reproduction. Bacteria produce enzymes that allow them to transform environmental energy sources using mostly lipases (*21*) or oxidoreductases (*22, 23*). The energy sources that different bacteria use for biotransfor-

Scheme 2. Alkaline Hydrolysis of Chiral Esters **1b** and **2b** to Chiral Alcohols **1c** and **2c**



mations depend on specific enzymes that each bacteria produces (*21–23*). The effect of glucose concentration is most appreciable only when the extracellular concentration of glucose is below 2 g/L (*24*). At a higher value, the extracellular glucose concentration does not seem to have a significant effect, and the growth rate becomes the predominant factor of the glucose uptake rate. Studies of growth under energy sufficient conditions often use a very high input of glucose concentrations. This is probably a reason as to why the effect of glucose concentration is rarely observed and as to why only the effect of growth on the glucose uptake rate has been addressed even under energy sufficient conditions. Nevertheless, the results of Hueting and Tempest (*25*) suggested clearly that for some organisms and under certain growth conditions, the glucose uptake rate could be affected by the glucose concentration as well as the cell growth rate.

The carbon source requirement of the bacterium was tested by adding various organic acids, sugars, and alcohols to the minimal medium. Organic acid derivatives (citrate, succinate, fumarate, pyruvate, and acetate) supported its growth. Among the sugars (glucose, fructose, and sucrose that were tested), only glucose and fructose were utilized. The growth was highest at 1% concentration of succinate and glucose (*26*). If succinate and glucose were used at a lower concentration (0.5%) or a higher (5%) concentration, bacterial growth was low.

The degradation experiments were performed in phosphate buffer (pH 7.1). During the experiment, the samples of biotransformation products were checked by TLC. After 7 days, the progress of the reaction was not observed anymore using this analytical method, and the experiment was stopped. The enzymes present in the cells were intracellular or cell-bound enzymes. In the stationary phase of bacterial growth, the cells were removed from their broth, and the whole cells were used as sources of enzymes. Localization of the enzymes in the cells was not determined. The cells were transferred to the buffer containing the tested compound. We found that after 7 days, the enzymes become inactive, and biotransformation ceases.

The products of biodegradation were characterized as ethyl (1*R*, 2*R*)-*N*-{2-{4-[(2-hydroxycyclohexyl)methyl]phenoxy}ethyl} carbamate (**1a**) and ethyl (1*S*,2*R*)-*N*-{2-{4-[(2-hydroxycyclohexyl)-methyl]phenoxy}ethyl} carbamate (**2a**) (**Scheme 1**). There are numbers of examples given in the literature, in which the authors claim enantioselectivity of the processes catalyzed by bacterial lipases (for a review, see ref *6*). Therefore, obtaining products enriched by one of the enantiomers also was expected during our experiments. Both compounds (**1a** and **2a**) belong to a series of biologically active insect juvenile hormone bioanalogues (juvenoids). The remaining substrates, juvenoid esters (juvenogens, biochemically activated insect hormonogen compounds), were enriched by the opposite enantiomers and structurally assigned as ethyl (1*S*,2*S*)-*N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]-methyl}phenoxy}ethyl} carbamate and (1*R*,2*S*)-*N*-{2-{4-{[2-(butanoyl)oxycyclohexyl]methyl}phenoxy}ethyl} carbamate. For assignment of the absolute configuration and enantiomeric purity of products **1a**, **1b**, **2a**, and **2b** (**Scheme 1**), chiral HPLC analysis was combined with ^{1}H and ^{19}F NMR spectral analysis of diastereoisomeric esters of alcohols **1a**, **1c**, **2a**, and **2c** (**Schemes 1** and **2**) and the enantiomerically pure 3,3,3-trifluoromethyl-2-methoxy-2-phenylpropanoic acid (Mosher acid) (*20*). *B. simplex* yielded products with a low chemical yield; particularly, **1a** was obtained with only a poor 4.0% chemical yield. The stability of **1** is generally higher than that of **2**, and it may be documented by the chemical yields of the biodegradation of the compounds (**Tables 2** and **3**). Biodegradation of **1** by *Bacillus* sp. strain 05 was not observed. Mineralization of **1** and **2** was not observed either. After the reaction, a mixture of reaction products was obtained from the buffer and without enzyme in the expected quantity.

An identical biodegradation experiment was performed with both bacteria at the same time. At natural conditions, the bacteria operate in concert. The results of this experiment are summarized in **Table 3**. It is evident that the course of the reaction corresponds to that observed with single bacteria. On the basis of these results, it is evident that only hydrolytic enzymes (lipases) of the tested bacteria took part in biodegradation of the studied juvenogens **1** and **2**.

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