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Biodegradation of Trialkylacetic Acid Benzyl Esters

A. Millier[1], F. Prost[1], P. Chambon[2], and R. Longeray[3]

[1]Rhone-Poulenc, Laboratoire d'Ecotoxicologie, 69150 Decines, France; [2]Faculté de Pharmacie, Laboratoire de Toxicologie, 8 Avenue Rockefeller, 69008 Lyon, France; [3]Escil, Laboratoire de Synthèse et de Chimie Organique appliquée, 43 boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

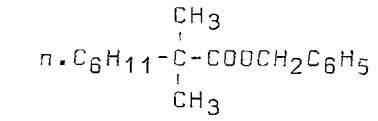
Benzyl esters of some particular trialkylacetic acids like versatic acids have shown interesting properties as dielectric media and could become valuable substitutes to PCB's provided it can be demonstrated that they are not objectionable from the environmental point of view (JAY & SCHWACHHOFER 1979). Since such chemicals may enter the environment through surface waters, it was thought of the utmost importance to check whether they could be degraded by aerobic river water bacteria.

In the present study, the biodegradation of two models has been evaluated under controlled laboratory conditions. A methodology had to be developed for testing these substances because of their very low solubilities in water.

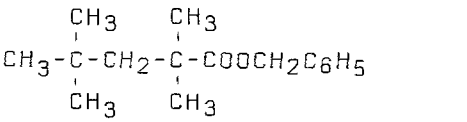
EXPERIMENTAL

Choice of models

Versatic 9-11 acid from which the most interesting of these compounds derived, is a complex mixture of branched-chain acids so it was found more convenient to carry out the tests with models. Since individual components of versatic acid are mainly trialkylacetic acids with at least one and often two methyl groups as substituents, two models were chosen with the following 2,2-dimethylalcanoic structure :



(I) Benzyl 2,2-dimethyl-octanoate



(II) Benzyl 2,2,4,4-tetramethyl-pentanoate

The first compound (I) has a straight chain third substituent expected to be easily attacked by bacteria while in substance (II) this alkyl substituent is branched.

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Synthesis of models

2,2 dimethyloctanoic acid and 2,2,4,4-tetramethylpentanoic acid were first synthesized following a general procedure for preparing 2,2-dimethylalcanoic acids (WHITMORE & BADERTSCHER 1933).

The corresponding benzyl esters were then obtained by adding 0.3 mole benzyl chloride within 2 h to a solution of 0.3 mole of either 2,2-dimethyloctanoic acid or 2,2,4,4-tetramethylpentanoic acid and 0.32 mole NaOH in 40 mL at 100° under vigorous stirring. The resulting mixture was then stirred under reflux for an additionnal 6 h. The crude ester was separated from the aqueous layer, washed twice with water and distilled under reduced pressure.

Both esters were obtained in 60 % yield as colorless liquids.

bp_{1mm} : (I) 125° (II) 114°

The structures were confirmed by NMR, IR and mass spectra since these compounds were not previously described in the literature.

Biodegradation experiments

The biodegradation assay was derived from the French norm procedure (AFNOR T 90-302, 1977) but an adaptation had to be made on account of the very low solubilities of both models in water (around 1 $mg.L^{-1}$).

A preliminary study showed that when these substances were simply dispersed by means of mechanical stirring into the aqueous test medium, coalescence soon occurred and losses caused by side-effects like adsorption and evaporation, were observed during the biodegradation experiments.

To minimize such undesirable phenomena, a procedure was developed where the tested substance was brought into the biological medium as a solution in ethanol with a non-toxic emulsifiant, Tween 80. Tween 80 is itself slowly degraded under the adopted conditions but its presence at the beginning of the test stabilizes the emulsion and blank tests showed that subsequent losses were insignificant.

The level of primary degradation was estimated from the rate of disappearance of the benzyl ester whereas metabolites were searched for within the culture medium.

All experiments were carried out in 2 L glass-stoppered conical flasks.

Biological medium

In ca. 800 ml of distilled water were dissolved 0,3 g $(NH_4)_2SO_4$; 0.15 g NH_4NO_3 ; 0.3 g KH_2PO_4 ; 2 g Na_2HPO_4, 12 H_2O ; 0.05 g $MgSO_4$, 7 H_2O ; 0.05 g $CaCl_2$, 2 H_2O. Then 1 ml of a solution of 0.1 g $FeSO_4$, 7 H_2O ; 0.1 g $MnSO_4$, H_2O ; 0.025 g $Na_2B_4O_7$, 10 H_2O ; 0.025 g $Co(NO_3)_2$, 6 H_2O ; 0.025 g $CuCl_2$, 2 H_2O ; 0.025 g $ZnCl_2$ and 0.01 g NH_4VO_3 in 100 mL distilled water was added and the final volume made up to 1 L with water. The pH was adjusted to 7.5.

Inoculum

Bacteria were isolated from urban sewage waters by filtration on membrane with average pore size 0,2 µm then resuspended in a physiological medium (Ringer solution) so that the concentration was 5 x 10^7 microorganisms per mL.

Procedure

200 mL of the biological medium were introduced in each flask. 100 µl of a solution of the benzyl ester (50 g.L-1) and Tween 80 (100 g.L-1) in ethanol were added under vigorous stirring to ease the dispersion. The flasks were then stoppered and sterilized by autoclaving for 30 mn at 120°. The inoculum (2 mL) was added except in the flasks designed for blank tests. All the flasks were placed in a dark room on a shaking agitator with a temperature of 25 $\pm$ 1°.

The tests were performed in a batch system. At chosen time intervals (0, 3, 7, 14, 21 and 28 days), three flasks (two designed for biodegradation assay and one blank) were taken for analysis. Simultaneously a test with a standard compound (glucose) was run to check the activity of the inoculum.

Analytical methods

Determination of the residuel benzyl ester

The growth medium was made alkaline (pH 11) with NaOH.N. The whole content of each flask was then extracted three times with 50 mL hexane in a 500 mL separating funnel. The separation of the two layers was sometimes difficult (especially at the beginning of the test when Tween 80 was still present in large quantities) and required a

combination of centrifugation, addition of NaCl and filtration steps. The three hexane extracts were combined and the finalvolume made up to 200 mL with hexane. This fraction was then analysed by gas chromatography using the following conditions : Instrument Carlo Erba Fracto-Vap 2350 fitted with a flame ionisation detector ; glass solumn (2 m by 2 mm I.D) packed with 4 % SE 30 on Chromosorb W (HP) 80-100 mesh ; carrier gas : nitrogen 30 mL. min^{-1} ; temperatures : injector 250°, column 180° isothermal ; sample size : 1 µL. The sample concentrations were determined by external calibration with standardsof benzyl ester in hexane.

Metabolites study

The aqueous fraction from hexane extraction was acidified to pH 2 with HCl (2 N) and extracted three times with 50 mL portions of ethyl ether in order to recover the supposed acidic metabolites. The combined extracts were filtered through a small cotton-wool plug, dehydrated on anhydrous Na_2SO_4 and concentrated to a final volume of 20 mL under a flow of dry nitrogen. The resulting concentrate was then trated with diazomethane and analysed by gas chromatography mass spectrometry on a Finnigan 3300 GC-MS instrument fitted with a glass column (1.2 m by 2 mm I.D.) packed with 4 % SE 30 on Chromosorb W (HP) 80-100 mesh ; carrier gas : helium ; temperatures : injector 250°, column : programmed from 80 to 200° at 4°/mn, sample size : 0,5 µL.

The concentrations of 2,2-dimethyloctanoic and 2,2,4,4-tetramethylpentanoic acids were determined by gas chromatography using external calibration with standards of the corresponding methyl esters in ethyl ether.

RESULTS

The results of the biodegradation experiments are shown in Figs 1. 2.

Benzyl 2,2-dimethyloctanoate

This compound was degraded completely within 23 days. The blank tests showed that the initial concentration remained unchanged after that period so no degradation phenomena other than bacterial attack could have contributed to the disappearence of the substance during the experiment. 2,2-dimethyloctanoic acid was found to be the main metabolite at the beginning (3 and 7 days) but soon disappeared

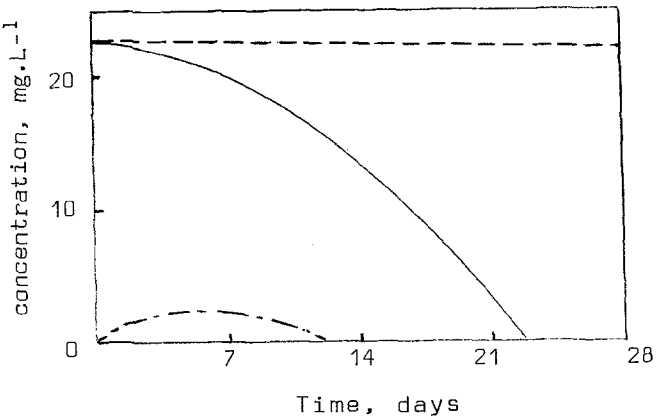

Fig 1. Biodegradation of benzyl 2,2-dimethyl octonoate

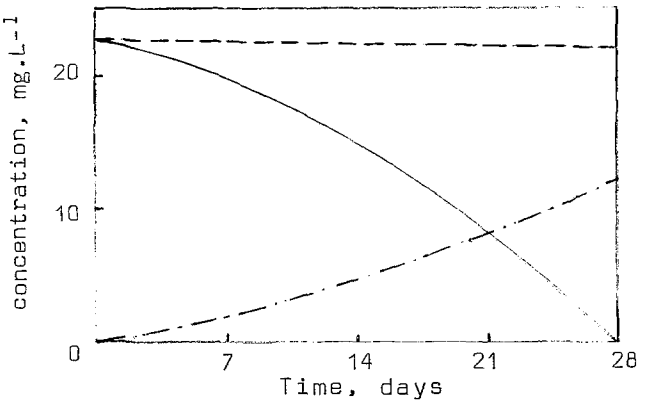

Fig 2. Biodegradation of benzyl2,2,4,4-tetra-methylpentanoate

——— Biodegradation test (concentration of benzyl ester)

- - - - - - Blank (concentration of benzyl ester)

—·—·— Biodegradation test (concentration of corresponding acid)

from the culture medium (absent after 21 and 28 days) indicating that it was probably degraded as soon as it was formed from the initial breakdown of its benzyl ester. From 14 days onwards a new peak was observed on the chromatogramm of the ether extract. The corresponding compound was identified by mass spectrometry as 2,2-dimethylsuccinic acid. The con-

centration of this substance increased regularly as the test proceeded showing that 2,2-dimethylsuccinic acid could be the terminal product of the bacterial metabolism of benzyl 2,2-dimethyloctanoate under the adopted test conditions.

A metabolic pathway can be proposed to account for the formation of 2,2-dimethylsuccinic acid from the initial molecule. This would involve the initial hydrolysis of the ester giving 2,2-dimethyloctanoic acid while the benzyl moiety would have been rapidly degraded. 2,2-dimethyloctanoic acid in turn, could have been metabolized through the ω-oxydation mechanism (DEN, 1965) to 2,2-dimethylsuberic acid and then through the well-known β-oxydation sequence to 2,2-dimethyladipic acid and in the end, 2,2-dimethylsuccinic acid. This last substance was considered quite resistant to soil microorganisms degradation (HAMMOND & ALEXANDER 1972).

Benzyl 2,2,4,4-tetramethylpentanoate

The complete degradation of this compound took a little longer than that of benzyl 2,2-dimethyloctanoate. 2,2,4,4-tetramethylpentanoic acid was found in the culture medium at the beginning of the test indicating that hydrolysis of the ester was the first step of the bacterial metabolism but this metabolite turned out to be quite resistant to bacterial degradation as its concentration increased regularly to reach around 10 $mg.L^{-1}$ at the end of the test (28 days). This suggests that 2,2,4,4 tetramethylpentanoic acid could persist at least to some extent in natural environment.

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