Mechanisms of Biodegradation of Dibenzoate Plasticizers

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Abstract

Biodegradation mechanisms were elucidated for three dibenzoate plasticizers: diethylene glycol dibenzoate (D(EG)DB), dipropylene glycol dibenzoate (D(PG)DB), both of which are commercially available, and 1,6-hexanediol dibenzoate, a potential green plasticizer. Degradation studies were done using *Rhodococcus rhodochrous* in the presence of pure alkanes as a co-substrate. As expected, the first degradation step for all of these systems was the hydrolysis of one ester bond with the release of benzoic acid and a monoester. Subsequent biodegradation of the monobenzoates of diethylene glycol (D(EG)MB) and dipropylene glycol (D(PG)MB) was very slow, leading to significant accumulation of these monoesters. In contrast, 1,6-hexanediol monobenzoate was quickly degraded and characterization of the metabolites indicated that the biodegradation proceeded by way of the oxidation of the alcohol group to generate 6-(benzoyloxy) hexanoic acid followed by β-oxidation steps. This pathway was blocked for D(EG)MB and D(PG)MB by the presence of an ether function.

The use of a pure hydrocarbon as a co-substrate resulted in the formation of another class of metabolites; namely the esters of the alcohols formed by the oxidation of the alkanes and the benzoic acid released by hydrolysis of the original diesters. These metabolites were biodegraded without the accumulation of any intermediates.

Key words: Biodegradation, plasticizers, 1,6-hexanediol dibenzoate, metabolites

INTRODUCTION

To improve the flexibility and workability of plastic resins, it is often necessary to incorporate plasticizers into polymeric matrices (Sears and Darby, 1982). This has resulted in the broad application of plasticizers in industries for the production of electrical cables, paints, wall papers and other construction materials and, to a lesser extent, food packaging films and medical products (Staples et al., 1997). The global demand for plasticizers was approximately 5×10^9 kg in 1999 and has been estimated to be growing by approximately 2.8% annually (Lerner, 2003).

Due to their widespread use in such large quantities, extensive research has been conducted to investigate the impacts associated with the release of plasticizers into the environment during manufacturing, while they are being used, and following their disposal (Cadogan et al., 1993; Staples et al., 1997; Bauer and Herrmann, 1997). The most commonly used class of plasticizers is the phthalates (Rahman and Brazel, 2004), for which significant concerns have been raised about the health and environmental consequences associated with their use (Wams, 1987; Staples et al., 1987; Scholz et al., 1997; Tickner et al., 2001). For example, phthalates and their intermediary metabolites have been detected in aquatic and terrestrial environmental samples (Roslev et al., 1998; Cartwright et al., 2000, Horn et al. 2004, Otton et al., 2004), as well as in human plasma and urine (Wahl et al., 2001; Wahl et al., 2004; Sathyanarayana et al., 2008). Moreover, the findings of numerous toxicological studies have resulted in several phthalates and their metabolites being placed in the list of priority pollutants of the United States Environmental Protection Agency (Keith and Telliard, 1979). Our earlier work identified toxic metabolites from the degradation of phthalates and adipates and elucidated the

breakdown mechanism of their biodegradation by a common soil microorganism, *Rhodococcus rhodochrous* (Nalli et al., 2002; Nalli et al., 2006a, 2006b).

The health and environmental implications of phthalates and increasingly strict environmental legislation has led to their partial replacement in a number of plastics applications with dibenzoate plasticizers (Wypych, 2004; Rahman and Brazel, 2004). This is due to the higher biodegradation rates and lower toxicity of the dibenzoates (Arendt and Lang, 1998; Lang and Stanhope, 2001). The European Chemical Agency has recently approved dibenzoates as alternatives to phthalates (Deligio, 2009). However, earlier studies have shown that the interaction of *Rhodotorula rubra* with dibenzoate-based plasticizers resulted in incomplete microbial degradation leading to the accumulation of monobenzoates, which had significantly higher toxicity than the original plasticizers (Gartshore et al., 2003).

Consequently, it is of considerable importance to identify the functional groups that influence the biodegradation pathways of these dibenzoate plasticizers. This will provide insight that can be used to design alternative plasticizers that do not result in the accumulation of toxic intermediates when interacting with common microorganisms.

The two most important commercial dibenzoate plasticizers, di-ethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB),, both contain ether functions. The objective of this study was to investigate the effect of the ether function on the biodegradation mechanisms of dibenzoate plasticizers. This was done by comparing the biodegradation mechanisms of the two commercial dibenzoate plasticizers with that of 1,6-hexanediol dibenzoate, a potential green plasticizer.

MATERIALS AND METHODS

Chemicals and reagents

1,6-Hexanediol (99%), n-hexadecane (99%), benzoyl chloride (99%), D(EG)DB (96%) and D(PG)DB (98%) were purchased from Sigma-Aldrich (Oakville, ON). BactoTM Brain/Heart infusion was obtained from Difco Microbiology (Montréal, QC). [$^2H_{30}$]Tetradecane (98 atom % D) was purchased from CDN isotopes (Montréal, QC). Pentadecane (99%) was purchased from A&C American Chemicals (Montréal, QC), bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Chromatographic Specialties (Brockville, ON), and all other chemicals were purchased from Fisher Scientific (Montréal, QC). 1,6-Hexanediol dibenzoate was synthesized as described previously (Kermanshahi pour et al., 2009).

Microorganism and growth conditions

R. rhodochrous ATCC 13808 (ATCC, Manassas, VA, USA) was maintained at -70 ºC in plastic vials containing 20% glycerol and the optimal growth medium of Bacto Brain/Heart infusion broth, as recommended by the ATCC. The contents of the vials were used to grow inocula in sterile Brain/Heart infusion broth. One mL of this inoculum was transferred to shake flasks containing 100 mL of the sterilized minimum mineral salt medium (MMSM), 0.1 g L^{-1} yeast extract and 2.5 g L^{-1} of one of n-hexadecane, n-tetradecane or [$^2H_{30}$]tetradecane. The concentration of 2.5 g L^{-1} of the hydrocarbon was chosen to ensure that growth of bacteria was not carbon-limited.

The MMSM consisted of 4 NH_4NO_3, 4 KH_2PO_4, 6 Na_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2 \cdot 2H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, and 0.014 disodium ethylenediaminetetraacetic acid (in g L^{-1}). When the stationary phase was reached, 2 mL of this microbial culture was

used to inoculate shake flasks containing 200 mL of sterile MMSM, 2.5 g L^{-1} of the appropriate hydrocarbon, and either 1.6 g L^{-1} D(EG)DB or 1.3 g L^{-1} D(PG)DB or 1.5 g L^{-1} 1,6-hexanediol dibenzoate. These shake flasks were incubated on a rotary incubator shaker (Series 25, New Brunswick Scientific, Edison, NJ, USA) set at 250 rpm and 30 ^{o}C.

Sample preparation for GC analyses

Triplicate samples of 3 mL were taken from the shake flasks, usually once per day. The pH was reduced to approximately 2 by the addition of sulfuric acid and the sample was then extracted with 3 mL of chloroform containing 1.5 g L^{-1} pentadecane as an internal standard. The mixture was stirred vigorously for 1 min and then the organic phase was transferred to a glass vial using a glass syringe. The samples were stored at 4 ^{o}C until analysis by GC.

GC analyses

The concentrations of the plasticizers and the metabolites were determined by GC/FID. Aliquots (1 μL) of the chloroform extracts were analyzed using a Varian CP-3800 GC equipped with a FID detector and a fused silica 8CB column (Varian, Montreal, QC) with a length of 30 m and an inner diameter of 0.32 mm. The chromatographic conditions were as follows: injection port temperature of 250 °C; initial column temperature of 40 °C; initial time of 2 min; heating rate of 10 °C min^{-1}; final temperature of 300 °C; and detector temperature of 300 °C. Helium was used as the carrier gas at a flowrate of 1.5 mL min^{-1}.

Sample preparation for GC/MS Analyses

Samples for GC/MS were treated in the same manner as for GC/FID analysis except that the chloroform used for extraction did not contain pentadecane. The solvent was removed with a dry nitrogen stream and the residues were dissolved in 50 µL of anhydrous pyridine. Trimethylsilyl (TMS) derivatives were made by the addition of 50 µL of BSTFA to the pyridine solutions in capped auto injector vials and these were heated in an aluminum block at 60 °C for 15 min.

GC/MS analyses

Aliquots (1 µL) of the TMS derivatized extracts were analyzed in low resolution GC/MS mode with a GC (time-of-flight) mass spectrometer (Micromass, Manchester UK) fitted with a 30 m DB-1 capillary column having a 0.25-mm inner diameter and 0.25-µm film thickness. The temperature varied from 80 ºC after a 1 min holding time to 300 ºC at 10 °C min^{-1} under controlled conditions followed by a bake-out period of 6 min at 300 °C. The injector was operated in a 1:100 split mode at 250 ºC with a constant helium pressure of 70 kPa. The GC re-entrant temperature was 250 °C. The ion source was operated in election ionization mode at 70 eV and 200 °C.

Un-derivatized extracts were also analyzed in GC/MS mode on a 30 m long, 0.32-mm inner diameter HP-5 column programmed at 10 ºC min^{-1}, with all other parameters remaining the same as those above.

RESULTS

The metabolites generated from the biodegradation experiments of 1,6-hexanediol dibenzoate or D(EG)DB by *R. rhodochrous* growing on various hydrocarbons as a co-

160 substrate are presented in Table 1. Many of these compounds have been identified
161 previously in experiments using glucose or hexadecane as the co-substrate (Gartshore et
162 al., 2003; Kermanshahi pour et al., 2009). The new compounds identified here were 6-
163 (benzoyloxy)-3-hydroxy butanoic acid, 4-(benzoyloxy)-3-hydroxy butanoic acid, 1-
164 [$^{2}H_{29}$]tetradecyl benzoate and 2-[2-(benzoyloxy)ethoxy] acetic acid. These were all
165 identified by GC/MS using the molecular weight of the parent ions (Table 1) and by
166 comparison with the fragmentation patterns of similar compounds reported previously
167 (Kermanshahi pour et al., 2009).

168 Figure 1a demonstrates a typical degradation of D(EG)DB by *R. rhodochrous* in
169 a medium containing hexadecane. There was a significant accumulation of di-ethylene
170 glycol monobenzoate accounting for 70% of the initial D(EG)DB. During the same time
171 period there was considerable accumulation of benzoic acid. Both of these metabolites
172 were eventually metabolized further.

173 Figure 1b shows data for the degradation of D(PG)DB. This compound has three
174 different possible isomers depending on the positions of the methyl branches on the
175 central propylene glycol dimer and all of these seem to degrade at the same rate. The
176 monoester metabolite D(PG)MB also has isomers but only two peaks were seen in
177 significant concentrations. These both had similar patterns of appearance and
178 degradation. The concentrations increased to maximum values at about the time that the
179 D(PG)DB disappeared and then two monobenzoates slowly degraded. A small amount of
180 benzoic acid was also observed early in the experiment, but this quickly disappeared.

181 The results of biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous* are
182 presented in Figs. 2a and 2b with either hexadecane or tetradecane, respectively, as the

co-substrate carbon source. In both cases, the dibenzoate was degraded and benzoic acid was produced and then degraded. The other metabolites are more interesting. 4-Benzoyloxy-butanoic acid was observed in both sets of experiments but, while it was the major metabolite when tetradecane was a co-substrate (Fig. 2b), it was only observed in trace amounts when hexadecane was used (Table 1). The metabolite 1-hexadecyl benzoate was only observed when hexadecane was used. In all of these experiments, the other metabolites listed in Table 1 were either not observed or only seen in small amounts and had degraded by the end of the experiments.

The same type of experiment was repeated in the presence of [$^{2}H_{30}$]tetradecane as a co-substrate and the same major metabolites seen in Fig. 2b, 4-benzoyloxybutanoic acid and benzoic acid, were observed to behave in a similar fashion. The only deuterium-containing metabolite observed was 1-[$^{2}H_{29}$]tetradecyl benzoate and this was only detected in trace amounts (Table 1).

DISCUSSION

The biodegradation of the commercial plasticizers D(EG)DB and D(PG)DB by the yeast, *R. rubra,* resulted in the formation of substantial amounts of the metabolites, diethylene glycol monobenzoate (D(EG)MB) or dipropylene glycol monobenzoate (D(PG)MB) (Gartshore et al., 2002). These monoesters were shown to be toxic and, especially in the case of D(PG)MB, resistant to further degradation. In the current study, using a common soil bacterium, *R. rhodochrous*, it was shown that while the monoesters were again generated, this bacterium was more effective at degrading these metabolites.

The compounds containing dipropylene glycol (both the diester and the monoester) exist as isomers because there are several possible variations depending on

206 the position of the methyl groups. Each of the two moieties of propylene glycol has a
207 single methyl substituent. Both methyl groups can be on the carbon atoms adjacent to the
208 central ether function; both can be on the carbon atoms adjacent to the alcohol functions;
209 or one moiety can have a methyl group adjacent to the ether and the other a methyl group
210 adjacent to the alcohol. It was possible to differentiate among some of these with the GC
211 column being used and it is clear that the presence of these methyl branches can slow
212 down the rate of hydrolysis of the monoesters. In the results presented here, both the
213 diester and monoester of diethylene glycol were biodegraded more quickly than those of
214 the dipropylene glycol. However, there was no evidence of a difference in stability of the
215 isomers of either the D(PG)DB or D(PG)MB compounds. Therefore, the actual
216 placement of the methyl groups on the propylene glycol fragment is not a significant
217 factor in the biodegradability of these compounds. In the cases of commercial plasticizers,
218 biodegradation led to significant accumulation of D(EG)MB and D(PG)MB accounting
219 for up to 70% and 80% of the initial molar concentration of D(EG)DB and D(PG)DB,
220 respectively (Figs. 1a and b). These metabolites were stable over the course of the
221 experiment, even though they did eventually biodegrade.
222 To develop a green plasticizer analogue of this class of compounds, it would be
223 essential to ensure that there was not a build-up of a toxic monoester, or any other toxic
224 metabolite, during interaction with microorganisms. While the removal of the methyl
225 groups did not eliminate the production of a monoester, replacing the ether function of
226 D(EG)DB with an ethylenic group achieved this goal. Biodegradation of the new
227 compound, 1,6-hexanediol dibenzoate, by *R. rhodochrous* resulted in only trace amounts

228 of the corresponding monoester, 1,6-hexanediol monobenzoate, and this was quickly
229 degraded.

230 Several other metabolites were produced, but most of these were detected in only
231 trace amounts (Table 1). The most noticeable exception was benzoic acid, which was
232 observed as a metabolite for the biodegradation all of the dibenzoates, using any of the
233 co-substrates (Table 1). While benzoic acid was observed in the studies with the
234 potential green plasticizer, it was not observed in concentrations as high as those
235 observed with the two commercial plasticizers and benzoic acid was not resistant to
236 further degradation. Even if small amounts of benzoic acid are released by biodegradation
237 of this green plasticizer, benzoic acid is not a source of significant environmental concern
238 and, in fact, is approved for use as a food preservative (US FDA, 1973).

239 The presence of significant amounts of benzoic acid early on in all of the
240 biodegradation experiments is consistent with the hydrolysis of one ester bond being the
241 first step in the biodegradation of all of the dibenzoate plasticizers. The hydrolysis of the
242 second ester bond seems to be much slower, at least in the cases of D(EG)MB and
243 D(PG)MB, and it is possible that the free hydroxyl function on the monoester inhibits the
244 enzyme activity.

245 The pattern for the biodegradation of 1,6-hexanediol dibenzoate (Fig. 2a) is
246 significantly different to that of the two commercial plasticizers. In particular, there were
247 high concentrations of 1-hexadecyl benzoate. It was previously suggested that this was
248 formed by the esterification reaction between benzoic acid, released by hydrolysis of the
249 plasticizer, and hexadecanol, a metabolite of hexadecane (see Fig. 3) (Kermanshahi pour
250 et al., 2009). This hypothesis is now confirmed. The data in Table 1 show that the nature

of the benzoate ester formed was dependent on the co-substrate. Thus, substitution of hexadecane by deuterated tetradecane resulted in the side product being 1-[$^{2}H_{29}$]tetradecyl benzoate, which could only come from the deuterated co- substrate. This indicates that the creation of this type of intermediate should not be considered an environmental risk. Its presence is an artifact of working with a pure culture growing on a sufficient amount of an easily oxidized alkane to generate excess amounts of the alcohols faster than the microorganism is metabolizing these alcohols. It is unlikely that there would be appreciable amounts of these alkanes in most environmental sites so the formation of this type of intermediate is not an issue.

However, the presence of an alkyl benzoate does create a problem in the interpretation of the data and the elucidation of the mechanism of biodegradation of dibenzoates. As mentioned above, the biodegradation of 1,6-hexanediol dibenzoate does not generate a stable monoester, but the data in Table 1 show the presence of three metabolites, (benzoyloxy)hexanoic acid, 6-(benzoyloxy)-3-hydroxy hexanoic acid and 4-(benzoyloxy) butanoic acid, which all could have originated from the progressive β-oxidation of 1-hexadecyl benzoate (Fig. 3). However, when the alkane used as a co-substrate was [$^{2}H_{30}$]tetradecane, the same, non-deuterated, oxidation products were observed. Thus, these must have originated from the monoester, not 1-hexadecyl benzoate. The elimination of the pathway in Fig. 3 leads to the pathway shown in Fig. 4. This mechanism was developed after identifying the various metabolites shown in Table 1.

There are actually two possible pathways for the monoester derived from 1,6-hexanediol dibenzoate shown in Fig. 4 and both are probably operating. The monoester

could undergo a second hydrolysis to release the diol and a second molecule of benzoic acid. It is important to note that this pathway is the only possibility available for the monoesters D(EG)MB and D(PG)MB because of the presence of the ether bond in the diol fragment. If the ether bond is removed as in 1,6-hexanediol benzoate, the other pathway becomes an option and the monoester can be oxidized to 6-(benzoyl)hexanoic acid followed by β-oxidation to generate 6-(benzoyl)-3-hydroxy hexanoic acid and then 4-(benzoyloxy)butanoic acid.

The biodegradation of D(EG)DB leads to small amounts of a compound [2-(benzoyloxy)ethoxy]acetic acid, which must be the oxidation product of the monoester. However, there is no evidence of the products from β-oxidation of this compound, thereby confirming that this pathway is not possible if the ether bond is present. Overall, it seems likely that the only pathway for the biodegradation of the monoesters from D(EG)MB or D(PG)MB is the relatively slow hydrolysis of the second ester bond.

All of these considerations lead to the conclusion that a stable monoester will be generated if the β-oxidation pathway was limited by the presence of an internal ether bond. This implies that the monoester is resistant to hydrolysis of the second ester bond but that this mechanism does slowly break down this toxic intermediate. In the case of the monoester from 1,6-hexanediol dibenzoate, the β-oxidation pathway is very efficient but this implies that, again, the monoester is resistant to hydrolysis of the second ester bond. It seems reasonable to conclude that the free hydroxyl function is somehow interfering with the action of the esterase enzymes on the monoesters.

CONCLUSIONS

In this study, it has been demonstrated that biotransformation of two commercial dibenzoate plasticizers, D(PG)DB and D(EG)DB, by *R. rhodochrous* results in the accumulation of monoester metabolites. In contrast, the biodegradation of 1,6-hexanediol dibenzoate, a potential green plasticizer analogue to D(EG)DB, did not result in the accumulation of the corresponding monoester, 1,6-hexanediol monobenzoate. The biodegradation pathway established for 1,6-hexanediol dibenzoate shows that 1,6-hexanediol monobenzoate degraded via oxidation and β-oxidation.

Biodegradation mechanisms established for the dibenzoate plasticizers demonstrate that the presence of the ether function leads to significant quantities of toxic metabolites. This, in turn, can be seen to be an important consideration in the design of green plasticizers.

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387 **FIGURE CAPTIONS**

388 **Figure 1a.** Biodegradation of D(EG)DB (●) by *Rhodococcus rhodochrous* ATCC 13808
389 and the corresponding accumulation of D(EG)MB (Δ) and benzoic acid (□). The initial
390 concentration of D(EG)DB was 5.2 mM and the growth medium contained 2.5 g L^{-1} of
391 hexadecane. **b.** Biodegradation of D(PG)DB by *Rhodococcus rhodochrous* ATCC 13808.
392 The three isomers of D(PG)DB (●, ▲ and ■), the two major isomers of D(PG)MB (○
393 and Δ) and benzoic acid (□) are all indicated. The initial concentration of D(PG)DB was
394 3.8 mM and the growth medium contained 2.5 g L^{-1} of hexadecane.

395 **Figure 2a.** Biodegradation of 1,6-hexanediol dibenzoate (●) by *Rhodococcus*
396 *rhodochrous* ATCC 13808 in the presence of hexadecane and the corresponding
397 accumulation of 1-hexadecyl benzoate (Δ) and benzoic acid (□). The initial concentration
398 of 1,6-hexanediol dibenzoate was 4.6 mM and the growth medium contained 2.5 g L^{-1} of
399 hexadecane. **b.** Biodegradation of 1,6-hexanediol dibenzoate (●) by *Rhodococcus*
400 *rhodochrous* ATCC 13808 in the presence of tetradecane and the corresponding
401 accumulation of 4-benzoyloxybutanoic acid (Δ) and benzoic acid (□). The initial
402 concentration of 1,6-hexanediol dibenzoate was 4.6 mM and the growth medium
403 contained 2.5 g L^{-1} of tetradecane.

404 **Figure 3.** Proposed mechanism for the formation of 1-hexadecyl benzoate arising from
405 the biodegradation of 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous* in the
406 presence of hexadecane as a co- substrate, including a possible mechanism for the
407 formation of 4-(benzoyloxy)butanoic acid.

408 **Figure 4.** Proposed biodegradation pathway of 1,6-hexanediol dibenzoate by
409 *Rhodococcus rhodochrous*.