Metabolites from the Biodegradation of 1,6-Hexanediol Dibenzoate, a Potential Green Plasticizer, by *Rhodococcus rhodochrous*

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ABSTRACT

Metabolites from the biodegradation of a potential plasticizer, 1,6-hexanediol dibenzoate by the common soil organism *Rhodococcus rhodochrous* and in the presence of n-hexadecane as a co-substrate, were identified using GC/MS and FTMS techniques. Trimethylsilylation of compounds from the biodegradation broth permitted detection of the following metabolites: 1-hexadecyl benzoate, 6-benzoyloxyhexanoic acid, 4-benzoyloxybutanoic acid, 6-benzoyloxyhexan-1-ol and benzoic acid. The presence of these metabolites was confirmed by repeating the biodegradation with 1,6-hexanediol di[$^{2}H_5$]benzoate, by measurement of their exact masses in FTMS and by comparison with available authentic materials. The results show that biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous* does not lead to the accumulation of persistent metabolites as has been reported for commercial dibenzoate plasticizers.

Key words: 1,6 hexanediol dibenzoate, plasticizer, biodegradation, metabolites GC/MS, FTMS

INTRODUCTION

The interaction of microorganisms with xenobiotic chemicals in the environment is a critical issue that must be studied when assessing their toxic impacts on ecological systems and human health [1,2,3,4]. Incomplete biodegradation of the parent compounds in the environment may lead to their accumulation and the production of metabolites of increased mobility, toxicity and persistence [5,6,7,8]. Therefore, in order to fully assess the impacts of these xenobiotics in the environment, it is important to identify the full range of compounds that are produced through biodegradation and to assess their toxicity and biodegradability.

In addition, the development of alternative compounds to replace conventional plasticizers requires monitoring of the consequences of their biodegradation to ensure that their metabolites have minimal toxicological impacts in the environment. Plasticizers, which are the most widely used additives in polymer manufacturing [9], have raised serious health and environmental concerns in recent years [10,11]. In addition, the concerns raised above about the potential impacts of metabolites have been shown to be the case for a number of widely used commercial plasticizers including phthalates and adipates [4,6,7,8]. Studies of the biodegradation of phthalate and adipate plasticizers have demonstrated the production of several different metabolites with greater toxicity than the parent compounds [4,6,7,8].

For example, 2-ethylhexanoic acid, a potent peroxisome proliferator [12,13], was identified from the biodegradation of di-2-ethylhexyl adipate, di-2-ethylhexyl phthalate and di-2-ethylhexyl terephthalate [6]. Mono-2-ethylhexyl phthalate, a metabolite expected in the biodegradation of di-2-ethylhexyl phthalate [14], is classified as an endocrine disruptor [15]. Detection of these and related metabolites of phthalates and adipates in mice, rats and human plasma and urine has led to greater concerns and stricter environmental regulations [16, 17, 18] .

In recent years, dibenzoate plasticizers such as diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB) have been proposed as alternatives to the more commonly used compounds because they tend to degrade more rapidly under the action of common microorganisms [19,20]. While this tendency

appears to make them attractive as alternatives to phthalates and adipates, it has been shown that the incomplete microbial hydrolysis of D(EG)DB and D(PG)DB when microorganisms are growing on glucose as a primary co-substrate leads to the accumulation of diethylene glycol monobenzoate and dipropylene glycol monobenzoate, respectively, which exhibit significant toxicity [21].

However, the rapid degradation of the dibenzoates indicate that a potential route to the development of a "green" plasticizer may be to start with the basic structure of the more easily degraded dibenzoate plasticizer and modify it to reduce the accumulation of metabolites when undergoing biodegradation. Therefore, the aim of this study was to monitor the biotransformation of 1,6-hexanediol dibenzoate, a potential plasticizer, by *Rhodococcus rhodochrous*, a common soil organism in the presence of hexadecane as a primary carbon source and to identify all of the metabolites created during biodegradation. Low resolution GC/MS with electron ionization was used for the identification of the metabolites as their trimethylsilyl (TMS) derivatives. FTMS was also used to obtain their underivatized accurate masses with electrospray ionization (ESI).

EXPERIMENTAL

Chemicals and reagents

1,6-Hexanediol 99%, 1-hexadecanol 99%, n-hexadecane 99% and benzoyl chloride 99% were purchased from Sigma-Aldrich (Oakville, ON). [$^2H_5$]Benzoyl chloride 99.1 atom% D was purchased from CDN isotopes (Montréal, QC). Bacto^{TM} Brain/Heart infusion and yeast extract were obtained from Difco Microbiology (Montréal, QC) and Fisher Scientific (Montréal, QC), respectively. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Chromatographic Specialties (Brockville, ON). Pentadecane was purchased from A&C American Chemicals (Montréal, QC). All other chemicals were obtained from Fisher Scientific (Montréal, QC).

Synthesis of 1,6-hexanediol dibenzoate and 1,6-hexanediol di[2H5]benzoate

1,6-Hexanediol dibenzoate was synthesized by refluxing 5 grams of 1,6-hexanediol with 20 mL of benzoyl chloride (4 equivalents) under nitrogen in 120 mL of acetone in a

round bottom flask for 7 hours. The reaction mixture was cooled to room temperature and then diluted with 100 mL of chloroform. The mixture was washed three times with 100 mL of a saturated sodium bicarbonate solution and concentrated to a yellow oil, which, on standing, yielded an off-white powder. This was re-crystallized from heptane.

The synthesis of 1,6-hexanediol di[$^2H_5$]benzoate was achieved in a similar manner by reacting 1,6-hexanediol (0.39 g) with 1.2 mL of benzoyl-[$^2H_5$]-chloride (3 equivalents) in 11 mL of acetone. The procedure used for work-up and re-crystallization is described above.

The proton NMR spectra of the synthesized 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate were consistent with the spectra expected for these compounds (Figure 1). The EI spectrum of the unlabelled diester was virtually identical to the spectrum published in the NIST/EPA/NIH 1998 Mass Spectral Library of the United States Department of Commerce.

Synthesis of 1-hexadecyl benzoate

1-Hexadecyl benzoate was synthesized by reacting 8 grams of 1-hexadecanol with 5 mL of benzoyl chloride (1.3 equivalents) in 120 mL of refluxing acetone in a round bottom flask for 7 hours. The reaction was carried out under nitrogen. The reaction mixture was cooled to room temperature and then diluted with 100 mL of chloroform. The mixture was washed three times with 100 mL of a saturated sodium bicarbonate solution. The solvent was then evaporated and 1-hexadecyl benzoate was crystallized from the residue. The proton NMR spectrum obtained from the synthesized 1-hexadecyl benzoate was in agreement with the spectrum expected for this compound.

Biodegradation Studies

Biodegradation of 1,6-hexanediol dibenzoate or 1,6-hexanediol di[$^2H_5$]benzoate was conducted in 500 mL Erlenmeyer flasks with a sponge cap. The medium for the experiments consisted of 100 mL of the sterilized minimum mineral salt medium (MMSM) and 0.1 g/L of yeast extract and 2.5 g/L n-hexadecane. Either 1,6-hexanediol dibenzoate or 1,6-hexanediol di[$^2H_5$]benzoate (3 mmol/L) were added to the flasks individually prior to autoclaving. The MMSM contained 4 g/L NH_4NO_3, 4 g/L KH_2PO_4,

6 g/L Na_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, and 0.014 g/L disodium ethylenediamine-tetraacetic acid.

Rhodococcus rhodochrous ATCC 13808 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored at -70 ºC in plastic vials containing 20% glycerol and a sterile growth medium of Bacto™ Brain/Heart infusion broth.

To prepare the initial inoculum, the contents of a vial were thawed and transferred to a 500 mL shaker flask containing sterile growth medium composed of Brain/Heart infusion (30 g/L Brain/Heart infusion broth in 100 mL of distilled water) and then incubated on a rotary shaker (Series 25, New Brunswick Scientific, Edison, NJ, USA) set at 250 RPM and 30 ºC. After one day, a new shaker flask containing 100 mL of 30 g/L sterile growth medium of Brain/Heart infusion in distilled water was inoculated with 1 mL of the initial inoculum.

When exponential growth was reached, this microbial culture was used to inoculate 100 mL of the sterilized MMSM containing 0.1 g/L yeast extract and 2.5 g/L n-hexadecane. This was used to inoculate the shaker flasks containing either 1,6-hexanediol dibenzoate or 1,6-hexanediol di[$^2H_5$]benzoate for the biodegradation study. The shaker flasks were incubated for a period of 7 days on a rotary incubator shaker set at 250 RPM and 30 ºC.

Sample preparation for GC/MS and GC/FID analyses

Over the course of biodegradation of 1,6-hexanediol dibenzoate, triplicate samples of 3 mL each were taken from the biodegradation broth every day. The samples were adjusted to pH 2 through the addition of sulfuric acid and extracted with 3 mL of chloroform. For GC/MS analysis, the extracts were evaporated to dryness under a dry nitrogen stream and the residues were taken up 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, which were heated in an aluminum block at 60°C for 15 minutes. For GC/FID analysis, chloroform extracts of the samples were used without derivatization.

GC/MS Analyses

Aliquots (1 μL) of the underivatized extracts were analyzed in low resolution GC/MS mode with a GCT (Micromass, Manchester UK) fitted with a 30 m HP-5 capillary column having a 0.32 mm i.d. and 0.25 μm film thickness. The temperature was programmed from 80 ºC after 1 min hold to 300 ºC at 10 °C /min followed by a bake-out period of 6 min at 300 °C. The injector was operated in 1:100 split mode at 250 ºC with a constant helium pressure of 70 kPa. The GC re-entrant temperature was 250 °C. The EI ion source was operated at 70 eV and 200 °C. The scan range was m/z 80 to 600 to avoid m/z 73 common to TMS derivatives.

TMS derivatized extracts and the synthesized 1-hexadecyl benzoate were analyzed in GC/MS mode on a 30 m, 0.25 mm i.d. DB-1 column operated as described above.

FTMS Analyses

High-resolution measurements of the un-derivatized extracts were made in positive ion electrospray mode with an IonSpec 7.0 tesla FTMS (Lake Forest, CA) calibrated with polyethylene glycol 300. The instrument was equipped with a "Z"-spray source from Waters Corporation (Milford, MA), an accumulation hexapole, a collision cell, a hexapole ion guide, a standard cylindrical ion cyclotron resonance (ICR) cell and Omega 9 software. The analyses employed a direct infusion flow rate of 2 to 3 μL/min in solution with 90:10 v/v methanol:water. Formic acid (1%) and sodium iodide were added to enhance cationization and to provide a secondary mass scale calibrating ion (Na_2I^+, m/z 172.8835). The "Z" spray source employed capillary and cone voltages of 3899 and 30 V, respectively. Ions were accumulated in the hexapole for 300 to 1500 msec with a rod voltage of 70 V. For the transfer of ions to the ICR cell through the hexapole ion guide, the low mass range coil with a frequency of 3020 kHz was used along with a voltage of 80 V. For detection, ions were excited through an arbitrary waveform in a range of m/z 100 to 1000 with an amplitude of 135 V(b-p); the ADC rate for the MS was 2 MHz for a scan range of m/z 75-500. Transients were 1M data points long. A waiting time of 5 sec before the detection step was used to allow the pressure in the ICR cell to return to its nominal value of 2×10^{-9} torr.

184 **GC/FID analyses**

185 Aliquots (1 μL) of the chloroform extracts were analyzed in a Varian CP-3800 gas
186 chromatograph equipped with a 30 m x 0.32 mm i.d. fused silica 8CB column (Varian,
187 Montreal, QC) programmed to 300 ºC at 10 ºC after a 2 min hold initially at 40 ºC . The
188 injection port and FID were 250 ºC and 300 ºC respectively. Helium was used as a carrier
189 gas at a flowrate of 1.5 mL/min. The concentration of the metabolites were estimated by
190 GC/FID

191 **RESULTS AND DISCUSSION**

192 1,6-Hexanediol dibenzoate was synthesized in an attempt to develop a more
193 environmentally benign version of the standard dibenzoate plasticizers. It has been shown
194 that analogous compounds can be converted to stable toxic metabolites by
195 microorganisms while growing on an easily metabolized substrate [21]. The purpose of
196 this work was to determine whether co-metabolism of 1,6-hexanediol dibenzoate by a
197 typical soil microorganism, *R. rhodochrous* resulted in the production of stable
198 metabolites.

199 Figure 2 shows the total ion current GC of an extract of an experiment using 1,6-
200 hexanediol dibenzoate without derivatization (panel A) and after trimethylsilylation
201 (panel B). Retention times and elution order are different in panel A and panel B due to
202 the use of a HP-5 column and 10 ºC program (panel A) *vs* a DB-1 column and a 5 ºC
203 temperature program for the derivatized sample (panel B).

204 Figures 3A and 3B show the EI mass spectra of 1,6-hexanediol dibenzoate and 1,6-
205 hexanediol di[$^{2}H_5$]benzoate, respectively. Weak molecular cations at *m/z* 326 and 336
206 were observed in their mass spectra, respectively. Fragmentation pathways are proposed
207 in Scheme 1. Fragment ions at m/z 204/209 are formed by loss of benzoic acid via a
208 McLafferty mechanism and with a possible subsequent elimination of the elements of
209 formaldehyde to yield ions at m/z 174 and 179. Formaldehyde elimination, as proposed
210 in Scheme 1, is not observed in the dibenzoates of 1,5-pentanediol or 1,4-butanediol (data
211 not shown, available in the NIST/EPA/NIH 1998 Mass Spectral Library), and this may be
212 related to the alkyl chain length. There are three possible precursor ions to m/z 174/179;
213 these are m/z 221/226, 204/209, and the $M^{+\bullet}$. The first would violate the even electron

'rule', and it is not possible to decide between the last two on the basis of the present data. Fragments at m/z 123/128, nominally protonated benzoic acid, may be formed in a 4-centred elimination of a radical olefin. Alternatively, m/z 123/128 may have the Ph-$C(OH)_2^+$ structure. Subsequent loss of water by *m/z* 123/128 results in ions at *m/z* 105/110. Formation of this last ion by a direct C(O)-O fission in the molecular ion cannot be discounted.

Figures 4A and 4C are the mass spectra of 1-hexadecyl benzoate and 1-hexadecyl [$^2H_5$]benzoate, respectively, isolated from the broth. The mass spectrum of the synthesized 1-hexadecyl benzoate is shown in Figure 3B. The proposed fragmentation scheme for 1-hexadecyl benzoate and 1-hexadecyl [$^2H_5$]benzoate in Scheme 2 accounts for the formation of fragments at *m/z* 123/128 and 105/110 in a manner parallel to that suggested in Scheme 1 for 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate. In Scheme 1, while m/z 123/128 is drawn as protonated benzoic acid, the ion structure may be that of a α,α-dihydroxybenzyl cation. M/z 105/110 may be formed by H_2O loss or directly from the $M^{+\bullet}$. A McLafferty rearrangement in the molecular cations generates *m/z* 224/224, which undergo the sequential loss of 2 ethylene groups.

Figures 5A and 5B show the mass spectra of the trimethylsilylated metabolites identified as 6-benzoyloxyhexanoic acid and 6-[$^2H_5$]benzoyloxyhexanoic acid, respectively. In support of this assignment, Scheme 3 accounts for the major ion fragments and deuterium labelling found in Figure 5A and 5B. Loss of methyl radical by the molecular radical cations yields m/z 293 and 298. Subsequent loss of CO_2 by m/z 293/298 yields m/z 249/254. M/z 179/184 is formed by the loss of the elements of ε-caprolactone by m/z 293/298, and goes on to lose CO_2 to form m/z 135/140 and $Si(CH_3)_2O$ resulting in m/z 105/110. M/z 105/110 formation directly from the molecular cation cannot be excluded here, nor in the case of Scheme 4. M/z 117 is an ion characteristic of TMS derivatives of compounds with carboxy groups, which corresponds to $^+$COOTMS [22]. Weak m/z 117 ions were observed in both Figures 5A and 5B.

Figures 6A and 6B represent the spectra of the TMS derivatives of 6-benzoyloxyhexan-1-ol and 6-[$^2H_5$]benzoyloxyhexan-1-ol, which were expected metabolites in the biodegradation of 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate, respectively, by analogy to the metabolites reported for related

commercial plasticizers [21]. Scheme 4 proposes fragmentations that account for the major ions in the mass spectra. A novel Me_2Si migration is proposed to occur in the loss of CO_2 for the transition m/z 179/184 - 135/140. The latter ion is drawn as a silicon analogue of an α,α-dimethylbenzyl cation, but the actual structure is not known. M/z 135 is an intense fragment in the spectrum of the TMS ester of benzoic acid (NIST/EPA/NIH 1998 Mass Spectral Library) and is formed by losses of methyl radical and subsequently CO_2.

The spectra of the TMS derivatives of 4-benzoyloxybutanoic acid and 4-[$^2H_5$] benzoyloxybutanoic acids are illustrated in Figures 7A and 7B, and show homology with the spectra of 6-benzoyloxyhexanoic and 6-[$^2H_5$]benzoyloxyhexanoic acids (Figure 5A and 5B). M/z 265 and 270 are formed by the loss of methyl radical by the molecular radical cations (m/z 280 and 285, not detected), and go on to lose CO_2 forming m/z 221 and 226. Ions at m/z 179/184, 135/140 and 105/110 likely have the same structures as those proposed in Scheme 3. M/z 117 was also observed in both Figures 7A and 7B indicating the presence of a carboxyl group.

Figure 8A and 8B are respectively the mass spectra of the TMS derivatives of benzoic and [$^2H_5$]benzoic acids found in the derivatized extracts. The molecular ions at m/z 194 and 199 show fragmentations similar to those found in Figures 5, 6 and 7.

The experimental and calculated exact masses obtained for 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate and their metabolites are presented in Table 1. The difference between the calculated and experimental masses was 2.3 ppm or better,

None of the above metabolites were observed in abiotic control experiments, eliminating the possibility of the formation of any of these metabolites by chemical hydrolysis or oxidation.

Table 2 contains data for the highest observed concentrations and maximum lifetime for each of the metabolites. All of these metabolites eventually disappeared. The most important of these is the monoester, 6-benzoyloxyhexan-1-ol. This compound is analogous to the monoesters produced by co-metabolism of the commercial plasticizers diethylene glycol dibenzoate and dipropylene glycol dibenzoate [21]. However, the monoesters (diethylene glycol monobenzoate and dipropylene glycol monobenzoate) from biodegradation of the commercial plasticizers were not only resistant to further

biodegradation but were also shown to exhibit significant toxicity in screening assays [21]. Therefore, the fact that the monoester of 1,6-hexanediol was only observed in small quantities and also degraded rapidly supports the hypothesis that this compound may represent a more environmentally benign dibenzoate plasticizer.

The most long lived of the metabolites in Table 2 and one that was observed at an order of magnitude greater concentration than the monoester was 1-hexadecyl benzoate. This is the only metabolite that can not originate directly from the degradation of 1,6-hexanediol dibenzoate. It is hypothesized that the formation of 1-hexadecyl benzoate is the result of an enzymatic conjugation of benzoate hydrolyzed from 1,6-hexanediol dibenzoate or its metabolites to hexadecanol, a metabolite of hexadecane degradation. This is consistent with the fact that biodegradation of 1,6-hexanediol di[$^2H_5$]benzoate resulted in formation of 1-hexadecyl [$^2H_5$]benzoate.

This ester of 1-hexadecanol is not a potential problem. It is an artifact arising from the use of hexadecane as the primary carbon and energy source. Hexadecane was convenient for these experiments because *R. rhodochrous* grows well on hydrocarbons [4] and a hydrophobic substrate helps to disperse the water insoluble plasticizer. However in an environmental situation, this benzoate is unlikely to be formed because there will not be appreciable amounts of alkanes or alcohols present.

CONCLUSIONS

GC/MS and FTMS were used to identify metabolites arising from the biodegradation of 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous.* All of these metabolites were confirmed by repeating the experiments with deuterated analogues.

In contrast to commercially available dibenzoate plasticizers, metabolism of 1,6-hexanediol dibenzoate did not result in accumulation of persistent metabolites. Furthermore the most stable of the metabolites would not be expected to be observed in the environment. These results support the potential to use 1,6-hexanediol dibenozate as a green plasticizer.

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Table 1. Accurate masses of labelled and unlabelled 1,6-hexanediol dibenzoate and metabolites measured in positive ion electrospray

Compound	Ion Composition	Found	Required	Error (ppm)
1,6-Hexanediol dibenzoate	$C_{20}H_{22}O_4Na$	349.1410	349.1410	0.0
	$C_{20}H_{23}O_4$	327.1591	327.1591	0.0
1,6-Hexanediol di[$^2H_{10}$]benzoate	$C_{20}H_{12}{}^2H_{10}O_4Na$	359.2037	359.2038	0.3
	$C_{20}H_{13}{}^2H_{10}O_4$	337.2222	337.2218	1.1
1-Hexadecyl benzoate	$C_{23}H_{39}O_2$	347.2953	347.2945	2.3
1-Hexadecyl [$^2H_5$]benzoate	$C_{23}H_{34}{}^2H_5O_2$	352.3252	352.3258	1.7
6-Benzoyloxyhexanoic acid	$C_{13}H_{16}O_4Na$	259.0942	259.0941	0.4
6-[$^2H_5$]Benzoyloxyhexanoic acid	$C_{13}H_{11}{}^2H_5O_4Na$	264.1254	264.1255	0.4
6-Benzoyloxyhexan-1-ol	$C_{13}H_{18}O_3Na$	245.1148	245.1148	0.0
6-[$^2H_5$]Benzoyloxyhexan-1-ol	$C_{13}H_{13}{}^2H_5O_3Na$	250.1457	250.1462	2.0
4-Benzoyloxybutanoic acid	$C_{11}H_{12}O_4Na$	231.0628	231.0628	0.0
4-[$^2H_5$]Benzoyloxybutanoic acid	$C_{11}H_7{}^2H_5O_4Na$	236.0942	236.0942	0.0
Benzoic acid	$C_7H_7O_2$	123.0441	123.0441	0.0
[$^2H_5$]Benzoic acid	$C_7H_2{}^2H_5O_2$	128.0757	128.0754	2.3

Table 2. Metabolites from the biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous**

Metabolites	Highest concentration observed, mmol/L **	Time of observation, hr	Time of confirmed disappearance, hr
6-benzoyloxyhexan-1-ol	0.03 ± 0.01	40	87
6-benzoyloxyhexanoic acid	0.30 ± 0.04	40	87
4-benzoyloxybutanoic acid	0.07 ± 0.01	64	87
Benzoic acid	0.20 ± 0.02	40	87
1-hexadecyl benzoate	0.31 ± 0.01	40	185

*Initial concentration of 1,6-hexanediol dibenzoate was 3 mmol/L.
** Values are the average of triplicate samples. Samples were taken once per day.

Legends

Figure 1. NMR spectrum of synthesized 1,6- hexanediol dibenzoate. Relative integration of protons on indicated carbon atom (A:B:C:D:E:F=2:2:1:2:2:2). A,B,C exhibit the expected patterns for a phenyl group. D and F are triplets and E is a multipet (assumed to be a triplet of triplet) as expected for this part of the structure.

Figure 2. Gas chromatograms obtained for the un-derivatized broth extract (panel A) and for the extract after trimethylsilylation (panel B). In the former, peaks are identified as: 1: hexadecane, 2: 1-hexadecyl benzoate, 3: 1,6-hexanediol dibenzoate. In the latter, peaks are identified as: 1: benzoic acid TMS derivative, 2: 1,6-hexanediol TMS derivative, 3: pentadecane added to the extract as a retention time marker, 4, hexadecane added to the broth as a co-metabolite, 5: 4-benzoyloxybutyric acid TMS derivative, 6: 6-benzoyloxyhexan-1-ol TMS derivative, 7: 6-benzoyloxyhexanoic acid TMS derivative, 8: 1,6-hexanediol dibenzoate, and 9: 1-hexadecyl benzoate. Other eluting peaks in B originate in the solvent and derivatizing reagent. The elution order for 1-hexadecylbenzoate and 16-hexanediol dibenzoate is reversed by switching between DB-1 and HP-5 columns.

Figure 3. Electron ionization mass spectra of **A**: 1,6-hexanediol dibenzoate and **B**: 1,6-hexanediol di[$^2H_5$]benzoate isolated from the incubation mixtures. Their mass spectra and GC retention times are identical to those for the diesters synthesized for this study. The intensities of ions above m/z 220 are multiplied by 20.

Figure 4. Electron ionization mass spectra of **A:** 1-hexadecyl benzoate, **B:** synthesized 1-hexadecyl benozate and **C:** 1-hexadecyl [$^2H_5$]benzoate. The intensities of ions above m/z 240 are multiplied by 50.

Figure 5. Electron ionization mass spectra of the TMS derivatives of **A:** 6-benzoyloxy-hexanoic acid and **B:** 6-[$^2H_5$]benzoyloxyhexanoic acid isolated from the incubation mixtures. The intensities of ions above m/z 210 are multiplied by 20.

Figure 6. Electron ionization mass spectra of the TMS derivatives of **A:** 6-benzoyloxyhexan-1-ol and **B:** 6-[$^2H_5$]benzoyloxyhexan-1-ol isolated from the incubation mixtures.

Figure 7. Electron ionization mass spectra of the TMS derivatives of **A:** 4-benzoyloxybutanoic acid and 4-[$^2H_5$] benzoyloxybutanoic acid isolated from the incubation mixtures.

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Figure 8. Electron ionization mass spectra of the TMS derivatives of benzoic acid and [$^2H_5$] benzoic acid isolated from the incubation mixtures.

Schemes

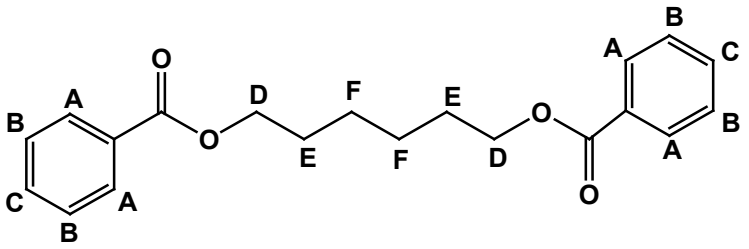
Scheme 1. Proposed fragmentation scheme for 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate. The second m/z value refers to the labelled diester.

Scheme 2. Proposed mass spectrometric fragmentation scheme for 1-hexadecyl benzoate and 1-hexadecyl [$^2H_5$]benzoate. The second m/z value refers to the labelled ester.

Scheme 3. Proposed mass spectrometric fragmentation scheme for the TMS derivatives of 6-benzoyloxyhexanoic and 6-[$^2H_5$]benzoyloxyhexanoic acids. The second m/z value refers to the labelled TMS ester.

Scheme 4. Proposed mass spectrometric fragmentation scheme for the TMS derivatives of 6-benzoyloxyhexan-1-ol. The second m/z value refers to the labelled TMS ester.

547



548

D

B

E F

A

C

8 7 6 5 4 3 2 1 0

PPM

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551
552
553 **Figure 1**
554

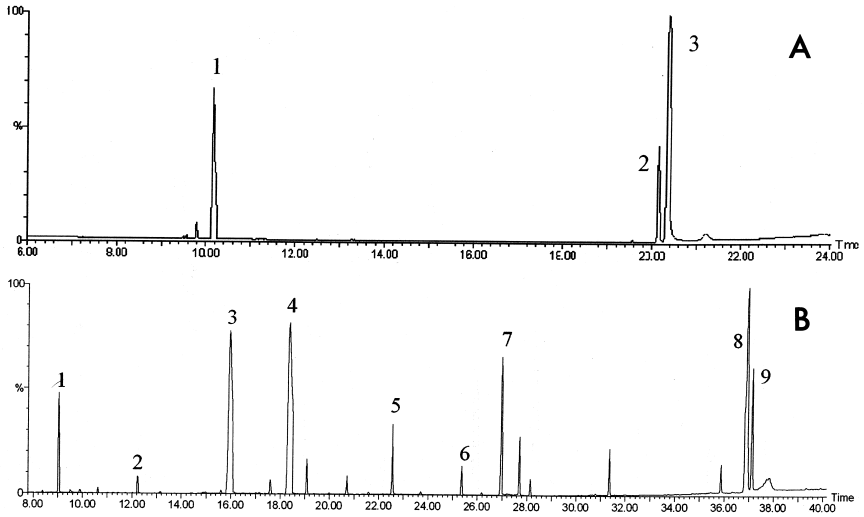
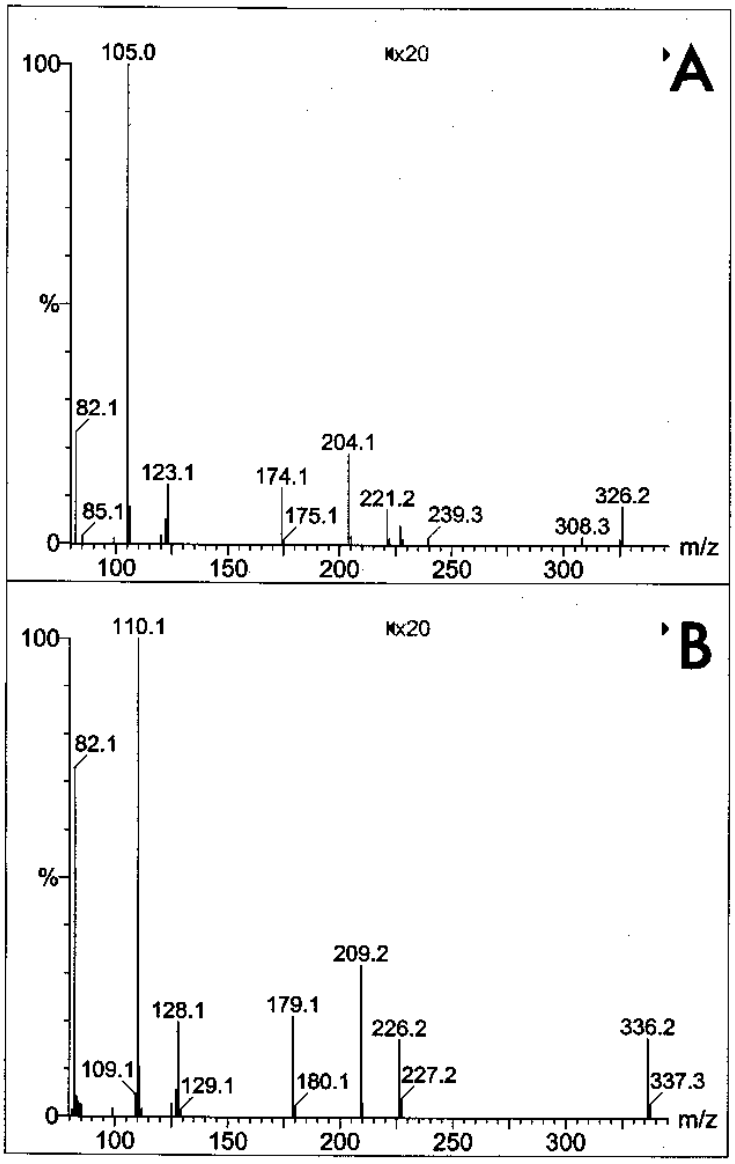


Figure 2

565



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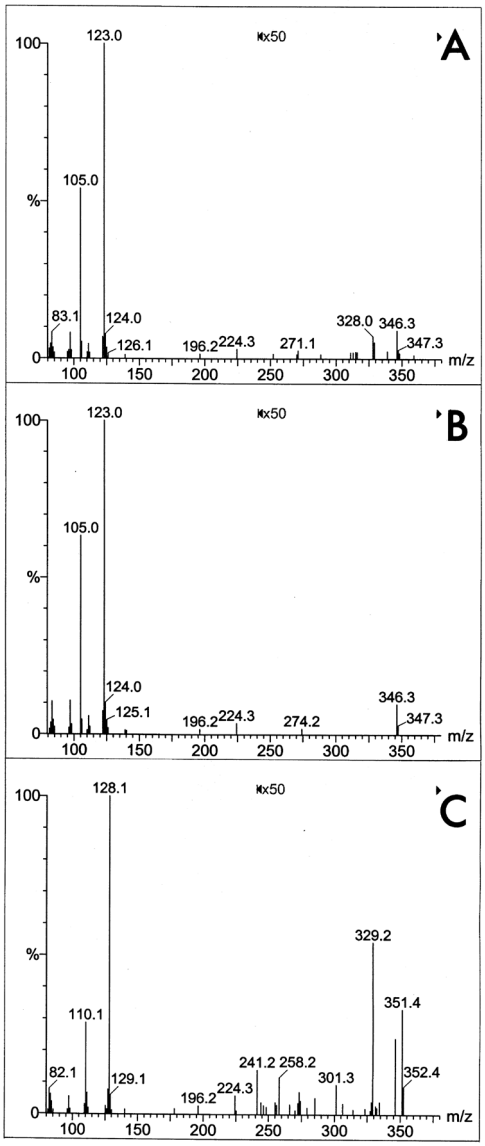

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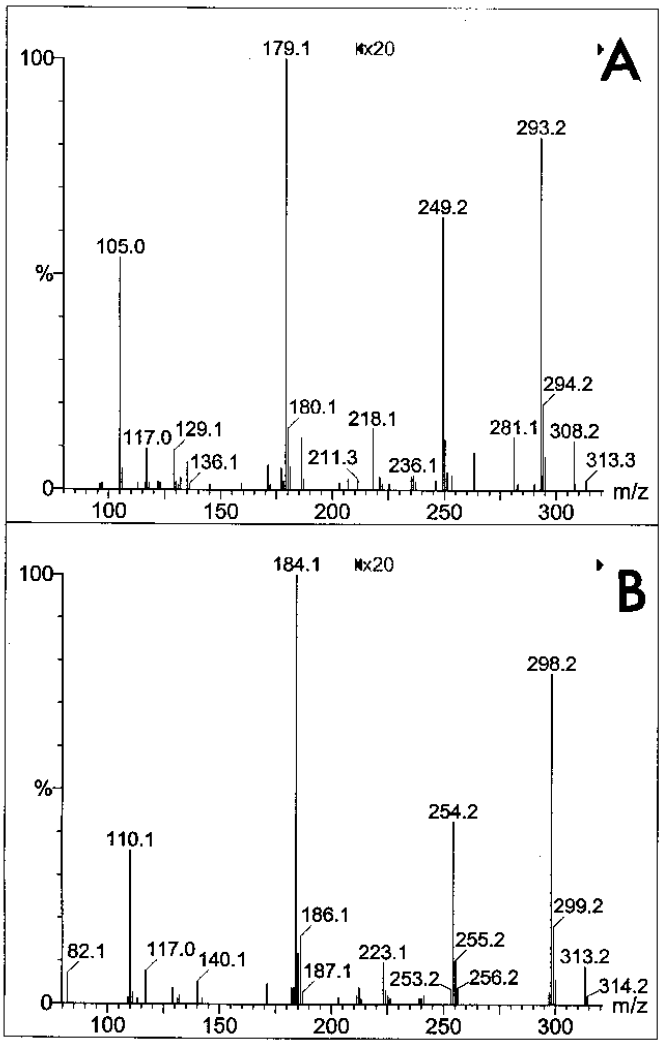
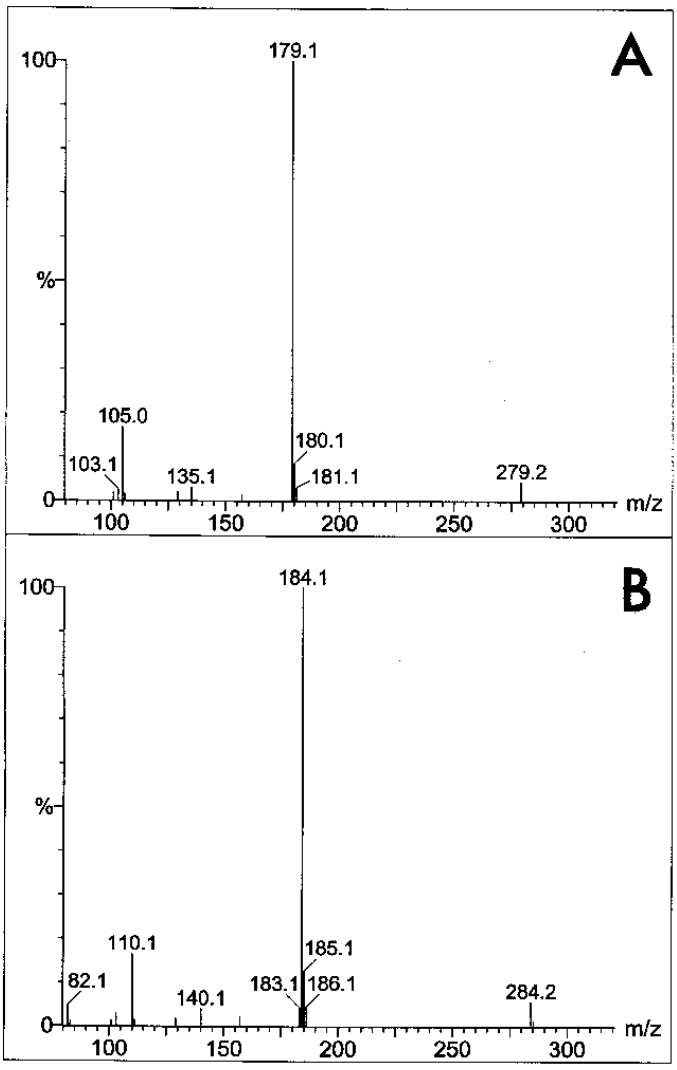


570

571 **Figure 5**

572



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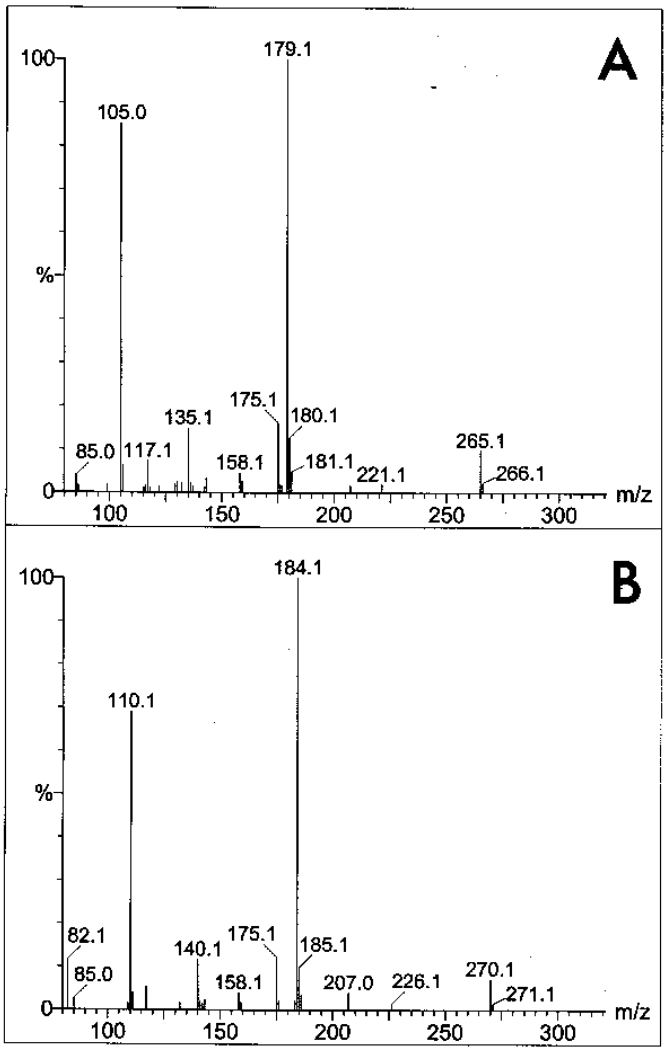
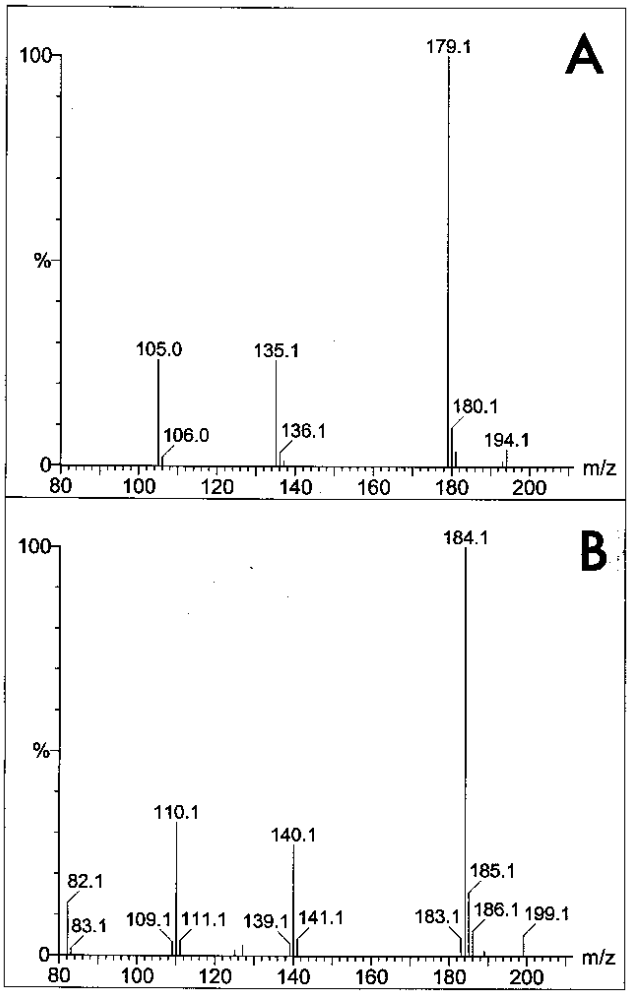
574 **Figure 6**



575

576 **Figure 7**

577



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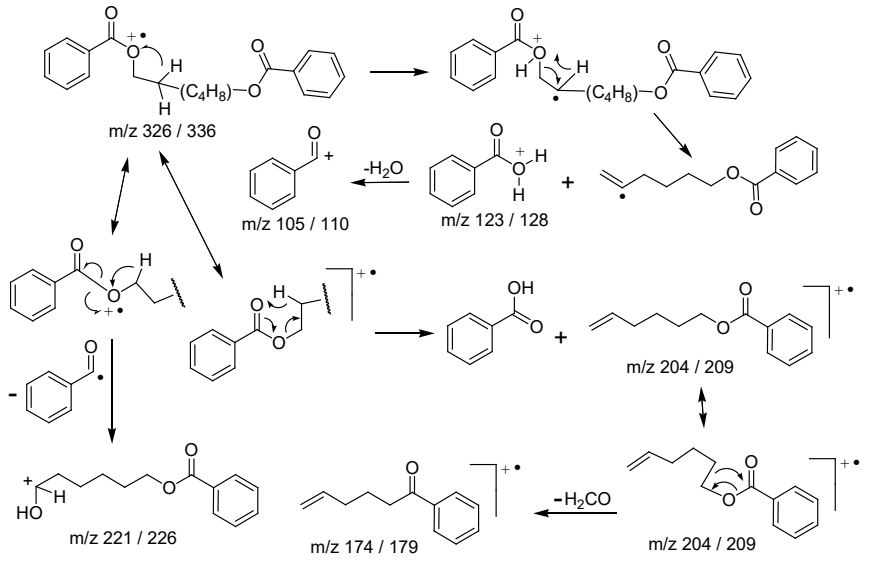
579 **Figure 8**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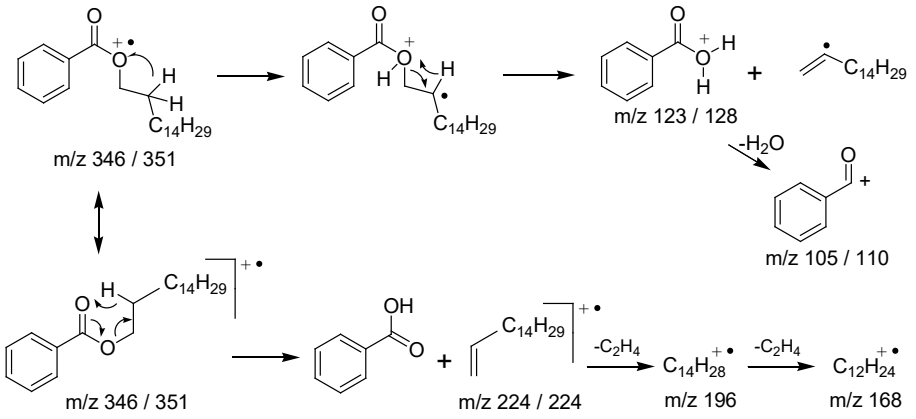
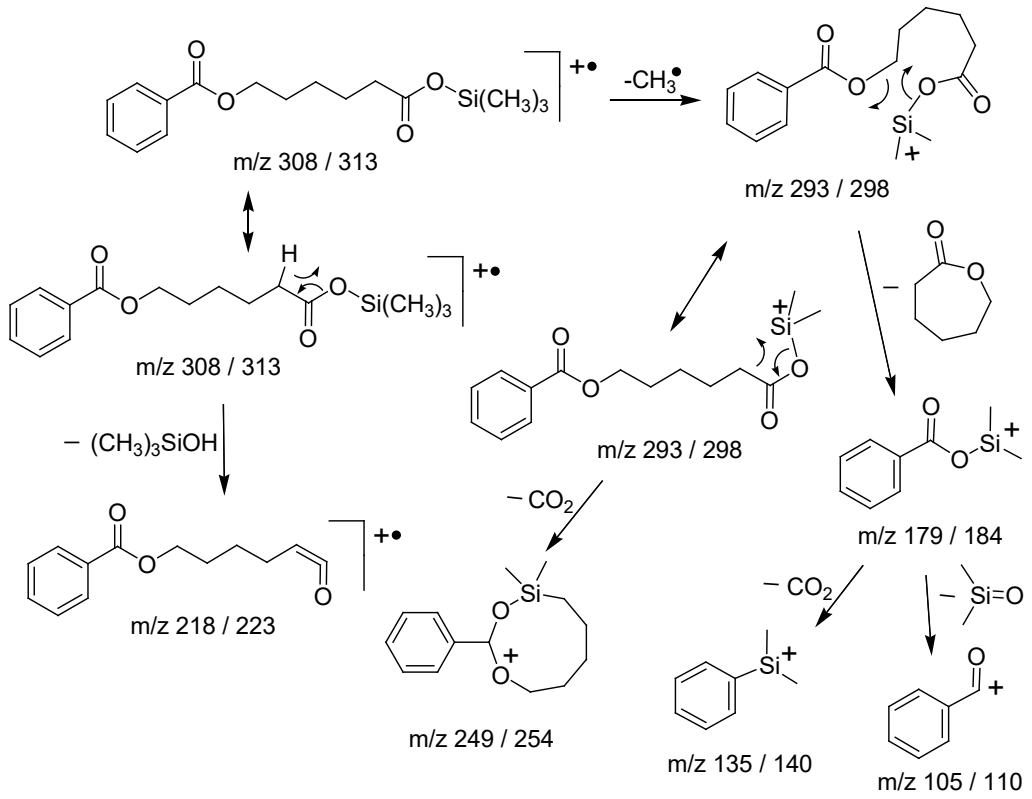
-H2O
m/z 105 / 110
m/z 123 / 128
m/z 204 / 209
m/z 221 / 226
m/z 174 / 179
-H2CO
m/z 204 / 209

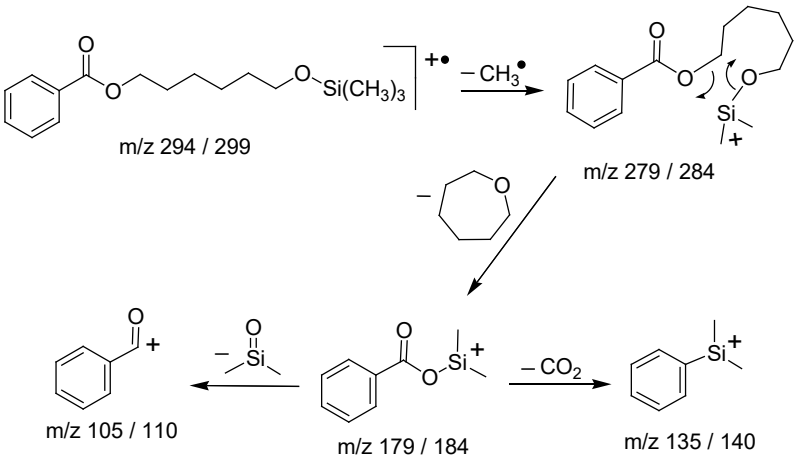
Scheme 1


m/z 346 / 351
m/z 123 / 128
m/z 105 / 110
m/z 346 / 351
m/z 224 / 224
m/z 196
m/z 168

Scheme 2


m/z 308 / 313
-CH3•
m/z 293 / 298
m/z 308 / 313
– (CH3)3SiOH
m/z 293 / 298
– CO2
m/z 179 / 184
m/z 218 / 223
m/z 249 / 254
– CO2
– Si=O
m/z 135 / 140
m/z 105 / 110

Scheme 3



Scheme 4