Biodegradation **9:** 487–499, 1998.

Biological, thermal and photochemical transformation of 2-trifluoromethylphenol

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Key words: Bacillus, biotransformation, NMR, thermophilic, trifluoromethylphenol

Abstract

This is the first report on the metabolism of trifluoromethyl aromatics in a thermophilic bacterium, *Bacillus thermoleovorans* A2. Enzymes of the phenol degradation pathway are induced when cultivating *Bacillus thermoleovorans* A2 on complex medium. Direct measurements of fluorinated xenobiotics in cell suspensions using ^{19}F-NMR made it possible to follow quantitatively the biotransformation routes. During the biotransformation of 2-CF_3-phenol by *B. thermoleovorans* A2, a fluorinated metabolite, 2-hydroxy-6-oxo-7,7,7,-trifluorohepta-2,4-dienoate (7-TFHOD), accumulated. This metabolite is transformed non-enzymatically when exposed to sunlight. The accumulation of 7-TFHOD as an intermediate in the 2-CF_3-phenol pathway was rationalized by calculating molecular properties of a series of *meta*-cleavage products.

Abbreviations: CF_3 – trifluoromethyl; CHF_2 – difluoromethyl; CH_2F – monofluoromethyl; 7-TFHOD – 2-hydroxy-6-oxo-7,7,7,-trifluorohepta-2,4-dienoic acid; HOD – 2-hydroxy-6-oxo-hepta-2,4-dienoic acid; HMS – 2-hydroxymuconic acid semialdehyde; LUMO – lowest unoccupied molecular orbital; LB – Luria Bertani, NMR - nuclear magnetic resonance; MTBSTFA – N(*tert*-butyldimethylsilyl)-N-methyl-trifluoroacetamide

Introduction

The use of NMR spectroscopy to characterise and quantify xenobiotic metabolites in biological fluids is well established. ^{19}F-NMR is of particular value in studies on fluorinated xenobiotics since concentrations as low as 0.1 μM have been detected and quantified in biological samples with typically no interference from endogenous compounds (Parisot et al. 1991; Serre et al. 1997). Minor structural changes occurring far away from the fluorine atom in a molecule can be detected because of the large chemical shift range, thus providing a subtle probe of metabolic transformation reactions (Mabury & Crosby 1995). ^{1}H-NMR spectroscopy is generally much less useful for the direct analysis of drug metabolites in biofluids because of extensive overlap of the xenobiotic signals with those from endogenous metabolites (Bollard et al. 1996).

Fluorinated hydrocarbons are widely used as herbicides, fungicides and human medicines. Fluorine remains frequently an outsider among the halogens, as its apparent relatively simple electronic structure nevertheless leads to a wide variety of reactivity patterns. These patterns – both the influence of C-F moieties on other parts of a molecule as well as the reactivity of the C-F bond itself – are often not well understood. Yet, this chemistry becomes of increasing importance as more than 10% of all pesticides contain fluorine, in most cases as a trifluoromethyl (CF_3) group. CF_3 groups have been the subject of significant interest by both synthetic (Banks & Tatlow 1994), mechanistic (Zuilhof et al. 1997; Koch et al. 1998) and medicinal chemists (Edwards 1994). Despite the fact that most of

the fluorinated agrochemicals contain CF_3 groups (e.g. trifluralin, a large-scale herbicide used to control a variety of grasses and broadleaf weeds) there are only a few reports on their microbial degradation pathways (Golab et al. 1979; Zeyer & Kearney 1983; Gennari et al. 1991; Kulowski et al. 1997; Zayed et al. 1983; Abernethy & Walker 1993) .

Generally, tests with agrochemicals in laboratory and ecosystems revealed incomplete degradation with still intact CF_3 groups (Key et al 1997). On the other hand, this metabolic deactivating effect is a useful property for some pharmaceuticals e.g. mefloquine, an antimalaria agent with half-lives in the range of weeks in the human body. Eventually, upon release into the environment an accumulation of CF_3 parent molecules and/or metabolites may occur.

It was postulated that many of the CF_3-containing aromatics are biologically metabolised to CF_3-catechols by bacterial enzymes (Engesser et al. 1988a; Engesser et al. 1988b). This raised the question about the occurrence and fate of these CF_3-catechols. Polymerisation of catechols at elevated temperatures is a possible non-biological reaction. Under thermophilic conditions the rate of polymerisation of chlorocatechols has been studied (Reinscheid et al. 1997). With an initial concentration of 0.1 mM the concentration of 3-chlorocatechol had decreased by 3% after 20 min at 60 °C representing a promising method to treat solid waste.

Our decision to study the different aspects of the transformation reactions of CF_3 organics in the environment was mainly based on two facts: the mass production of CF_3 aromatics as agrochemicals and the strong resistance of the CF_3 group to biological processes involving enzymes (Engesser et al. 1990). This combination directly leads to their world-wide abundance in high concentrations in the environment (Franklin 1993). A recent review article about fluorinated organics in the biosphere summarises that in a number of key areas major uncertainties exist concerning the distribution, fate, and biological behaviour of these compounds in the environment (Key et al. 1997). This situation deserves a thorough study of the environmentally relevant biological, chemical and photochemical reaction types.

Many environmental modifications of agrochemicals may be ascribed to photochemical reactions (Lodder & Cornelisse 1995). The photo-degradation of these toxic, halogenated compounds is in aqueous solutions one of the factors determining their persistence, and is as such a topic of environmental relevance. 2,3,7,8-Tetrachlorodibenzo-p-dioxin, for example, has a very low water solubility, volatility and is thermally stable. However, in the presence of surfactants it undergoes photo reduction with UV light. Many photo-products are formed, with salicylic acid reported as the final product. Taylor et al. (1993) measured a partial release of fluorine anions using an ion-sensitive electrode when irradiating an aqueous solution of 7-TFHOD, an intermediate in the degradation pathway of fluorinated benzoates and fluorinated catechols (Engesser et al. 1988a; Engesser et al. 1988b; Engesser et al. 1990). In the study of Taylor et al. (1993) it remained however unclear to what extent defluorination of 7-TFHOD occurred. In the present study purified 7-TFHOD obtained by anion-exchange chromatography was irradiated.

As model compounds the series of CF_3-phenol isomers was selected. The model organism is a thermophilic and metabolically versatile *Bacillus* species that has been isolated by Mutzel et al. (1996). The degradation pathway for phenol and substituted phenols has been investigated (Reinscheid et al. 1997; Duffner & Müller 1998). Data on the taxonomical position revealed that the organism of this study, *Bacillus thermoleovorans* A2, is a typical representative of the phenol-degrading group of thermophilic Bacilli (Duffner et al. 1997). The widespread occurrence of these spore-forming bacteria, especially dominating self-heated compost (Strom 1985), leads directly to questions about their metabolic potential against CF_3 aromatics. As a valuable tool for investigating the metabolism of fluorinated compounds ^{19}F-NMR permits a direct monitoring without sample clean-up. In this study we focus on the biological, thermal and photochemical transformation characteristics of 2-CF_3-phenol.

Materials and methods

Bacteria, media and culture conditions

The phenol-degrading *Bacillus thermoleovorans* A2 (Mutzel et al. 1996) was maintained at 60 °C on solidified, chloride-free medium containing phenol as sole carbon and energy source (Reinscheid et al. 1997). Growth was measured photometrically at 540 nm. For the induction experiments and the production of induced cells a Luria-Bertani (LB) medium (Sambrook et al. 1989) was used. All incubations were carried out at 60 °C. Screw-capped vials equipped with

a Teflon-lined rubber septum were used to prevent evaporation.

Test for growth-substrates and cometabolic transformation by resting cells

The fluorinated substrates 2-trifluoromethylphenol (2-CF_3-phenol), 3-CF_3-phenol and 4-CF_3-phenol were tested as sole carbon and energy source by incubating the organism in chloride-free medium at 60 °C. Sterile controls permitted the determination of abiotic substrate loss due to elevated temperatures. After 48 hours of incubation, the optical densities were determined. Substrate concentrations were measured using ^{19}F-NMR.

For the resting cells experiments LB-grown cells in the late-exponential growth phase were centrifuged, washed with sodium phosphate buffer (0.1 M; pH = 7.2) and resuspended to a final cell concentration of 1×10^9/ml. The above mentioned fluorinated compounds and salicylic acid were added to a final concentration of 1 mM each. The vials were incubated and samples were withdrawn for direct ^{19}F-NMR measurements. For HPLC and GC-MS analysis the supernatant obtained after centrifugation of the samples was used. After 20 min of incubation an aliquot of the 2-CF_3-phenol containing cell suspension was withdrawn for the anion-exchange chromatography.

Whole-cell enzyme activity measurements

For the induction experiments the cells were centrifuged and resuspended in 0.1 M sodium phosphate buffer (pH = 7.2). Catechol 2,3-dioxygenase activity was assayed by measuring the increase in absorbance due to the formation of the yellow *meta*-cleavage product HMS formed with catechol as substrate using $\epsilon_{375\,nm}$ = 33,400 cm^{-1} M^{-1}. Phenol hydroxylase activity was detected by observing the formation of HMS with phenol as substrate in the presence of catechol 2,3-dioxygenase activity in the intact cells. Both substrates were added to a final concentration of 1 mm. Specific enzyme activities in whole cells are expressed as activity per unit OD_{540nm} ($\Delta OD_{375nm} \times min^{-1} \times OD^{-1}_{540nm}$).

Temperature stability of 2-trifluoromethylphenol

A 1 mm solution of 2-CF_3-phenol in sodium phosphate buffer (0.1 M; pH = 7.2) was incubated at 60 °C. After appropriate time intervals samples were withdrawn and analysed using HPLC, ^{19}F-NMR and ^{1}H-NMR.

Purification of 7-TFHOD by anion-exchange chromatography

A sample of the cometabolic transformation of 2-CF_3-phenol was centrifuged and the supernatant brought to 0.1 M KCl. This solution was applied to a MonoQ column (bed volume 1 ml). After washing the column with phosphate buffer plus 0.1 M KCl, the *meta*-cleavage product was eluted with a linear gradient of 0.1 M to 2 M KCl and appeared at 0.8 M KCl. Detection wavelengths were set at 380 nm and 270 nm. The salt gradient was followed by conductivity measurements. Fractions containing the *meta*-cleavage product were collected.

Photolysis and temperature stability of the meta-cleavage product

Photolysis was examined by exposure of solutions of HMS or 7-TFHOD in sodium phosphate buffer (0.1 M; pH = 7.2) to full sunlight (between 10:00 a.m. and noon) in covered quartz cuvettes. To test for the influence of oxygen, solutions were flushed with air or argon for 20 min. During the light exposure temperatures never exceeded 20 °C. Control cuvettes were kept in the dark. Temperature stability was tested at 60 °C in sodium phosphate buffer (0.1 M; pH = 7.2). After appropriate time intervals the solutions were analysed photometrically and, in case of 7-TFHOD solutions, by ^{19}F-NMR.

Analytical methods

2-CF_3-phenol, 3-CF_3-phenol, 4-CF_3-phenol, salicylic acid and 2,3-dihydroxybenzoic acid were analysed by HPLC using a photo diode-array-detector (Waters 996). Identification was done by comparing UV spectra and retention times with those of reference substances. Reversed-phase HPLC was carried out on a RP18 column (Nucleosil 5μ, 4.6 mm × 150 mm) using methanol/water/acetic acid (600/400/10, v/v/v) as eluent with a flow rate of 1 ml/min.

7-TFHOD and HMS concentrations were calculated from their molar extinction coefficients (Engesser et al. 1988b; Bayly et al. 1966).

For the identification with the GC-MS system the same sample clean-up procedure was used as already described (Reinscheid et al. 1997). The residues were

redissolved in 50 μl ethyl acetate and 10 μl of derivatizing reagent (MTBSTFA; Aldrich) that introduces *tert*-butyldimethylsilyl groups were added. After 5 minutes of heating the cooled mixture was concentrated by evaporation under a stream of argon. The analyses were performed on a HP 5890 GC System connected to a mass selective detector (5970 series) equipped with a capillary column (HP5) of 30 m $\times$ 0.25 mm dimensions at a helium flow rate of 20 ml/min. Injector and detector temperatures were 150 °C and 280 °C, respectively, and the column temperature was raised from 80 °C to 250 °C at a rate of 5 °C/min with an initial holding time of 4 min. The mass spectra were obtained at 70 eV.

^{19}F-NMR spectra were obtained on a Bruker DPX 400 spectrometer equipped with a 10 mm fluorine probehead operating at 376.498 MHz. To samples of cell suspensions (1.9 ml) 100 μl ^2H_2O was added as a field-frequency lock. For quantification *p*-fluorobenzoate was used as internal standard. Chemical shifts are reported relative to $CFCl_3$. Generally, spectra were acquired at 25 °C with 5 μs pulses (30 degree flip angles), sweep width of 100 ppm, 32 K data points, line broadening of 1 Hz, and between 3000 and 8000 scans for each experiment depending on the concentration of the compounds. Selectivity tests were performed with cell suspensions of metabolically inactive *B. thermoleovorans* A2 cells (cell density = 1.0 $\times$ 10^{10}/ml) in sodium phosphate buffer (0.1 M; pH = 7.2). For routine ^{19}F NMR measurements ^{1}H couplings were eliminated using broad-band proton decoupling using a Waltz-16 pulse sequence. To test for the formation of CF_2H or CFH_2 groups no ^{1}H decoupling was used. ^{1}H-NMR spectra were measured on the same spectrometer as described above, now using a 5 mm dual ^{13}C/^{1}H probe operating at 400.13 MHz. Samples were prepared in phosphate-buffer with ^2H_2O as solvent. Generally, spectra were acquired at 7 °C, sweep width of 12 ppm, 32 K data points and line broadening of 0.5 Hz. The water signal was presaturated during the relaxation delay.

Computational chemistry

Molecular orbital calculations were carried out using Spartan 4.1.1 (Wavefunction Inc., Irvine, CA) with the default convergence criteria implemented in there. Restricted Hartree-Fock computations were performed within the framework of semi-empirical computations using the AM1 Hamiltonian (Dewar et al. 1985).

From the thus calculated wavefunction the three dimensional density of electrons can be computed, but it is impossible to uniquely partition this among the atomic centers in a totally unarbitrary manner. We therefore calculated the sum of two electrostatic potential charges as relevant charge indicator. This sum is indicative of the charge felt by approaching nucleophiles and suffers relatively little from arbitrary demarcations of the electron clouds, and is thus used throughout as an charge indicator.

Chemicals

Phenol, salicylic acid (Fluka, Buchs, CH) and 2,3-dihydroxybenzoic acid (Acros Chimica's-Hertogenbosch, The Netherlands) were used. All trifluoromethylphenol isomers were obtained from Fluorochem Limited (Derbyshire, UK). Stock solutions of the trifluoromethylphenols were kept at 4 °C in the dark.

Results

Direct measurement of CF_3-phenol in cell suspensions using ^{19}F-NMR

^{19}F-NMR was used to investigate the biological transformation of CF_3-phenols. Direct measurements of cell suspensions without any extraction procedure could be done because of the high selectivity of ^{19}F-NMR.

Under our experimental conditions we could easily measure fluorinated metabolites in the μM range within 30–60 min. We determined the chemical shift values for all three CF_3-phenols to test for peak resolution capacity. We observed clearly distinct chemical shift values for all three isomers with $\Delta\delta$ values of at least 0.4 ppm.

Cell growth

Expression of the phenol hydroxylase and the catechol 2,3-dioxygenase does not require the addition of phenol in the growth medium. During growth on LB medium the catechol 2,3-dioxygenase activity could be observed after 13 h in the exponential phase (Figure 1). Three hours later phenol hydroxylase activity was detected. Both activities decreased immediately when the cells had reached the stationary phase. It is therefore of crucial importance to harvest the cells at a

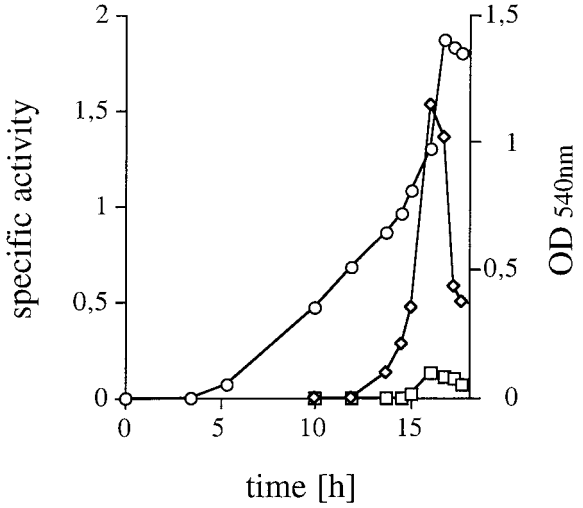

Figure 1. Growth and enzyme activities in whole-cells of *B. thermoleovorans* A2 with LB medium as substrate: ○, OD_{540nm}; □, phenol hydroxylase activity; ◇, catechol 2,3-dioxygenase activity.

well defined time point. For the resting cells transformation of 2-CF_3-phenol cells were harvested after 16 h of incubation.

Tests for CF_3-phenols as growth substrates – temperature stability of 2-CF_3-phenol

None of the tested three CF_3-phenols could serve as sole carbon and energy source under the conditions used as judged from growth tests in the chloride-free medium. However, a temperature-dependent chemical process that led to the release of fluorine anions with a concomitant decrease in the substrate concentration was observed in ^{19}F-NMR measurements in case of 2-CF_3-phenol and 4-CF_3-phenol.

The ^{1}H-NMR spectrum of 2-CF_3-phenol is shown in Figure 2A. Figure 2B shows the ^{1}H NMR spectrum of 2-CF_3-phenol after incubation for 20 min at 60°C without bacterial cells. The new signals correspond to salicylic acid by comparison with the reference compound.

^{19}F-NMR measurements of these samples clearly demonstrated the temperature-induced release of fluorine anions. At every time interval the molar amount of fluorine anions was three times the molar amount of 2-CF_3-phenol being transformed indicating the release of all three fluorine atoms of the CF_3 group. No fluorinated intermediates could be detected.

Upon GC-MS analysis after extraction and derivatization the following data were determined retention time = 29.1 min, masses at 309 u and 251 u representing $[M - \text{t-butyl}]^+$ and $[M - \text{t-butyl-Si}(CH_3)_2]^+$, respectively.

These data matched up with the masses obtained by analysis of derivatized salicylic acid as a reference compound. Quantitative measurements by HPLC revealed that 34% of the initital 2-CF_3-phenol was hydrolysed to salicylic acid within 20 min at 60 °C. The same type of experiment at 25 °C showed no detectable decomposition of substrate within 2 hours. However ^{19}F NMR measurements at 37 °C showed that decomposition of the substrate already can be detected within this timeframe.

Biotransformation of 2-CF_3-phenol by resting cells of B. thermoleovorans A2

^{19}F NMR experiments showed that 2-CF_3-phenol was transformed biologically by resting cells. However, neither a decrease in concentration nor the occurrence of additional peaks in the ^{19}F spectra due to biological processes could be observed for the other two isomers. Therefore, the biological transformation of 2-CF_3-phenol was further examined.

Biotransformation of 2-CF_3-phenol resulted in the accumulation of a yellow metabolite. The UV/VIS analysis of this product revealed an absorption maximum at 387 nm at neutral pH which shifted to 325 nm after acidification to pH 2. This spectral transition has an apparent pk_a value of 5.75. These data are in accordance with a *meta*-cleavage product of the corresponding 3-trifluoromethylcatechol (Engesser et al. 1988a; Engesser et al. 1988b; Engesser et al. 1990). The resting cells suspension was measured by ^{19}F-NMR without any extraction procedure (Figure 3). The peak at −66.14 ppm is due to the fluorinated substrate and the major metabolite signal is observed at −78.73 ppm.

The signals at −114.17 ppm and −123.0 ppm are ascribed to the internal standard *p*-fluorobenzoate and fluorine anions. NMR experiments without decoupling revealed the absence of CHF_2 and CH_2F groups because no splitting of the signal at −78.73 ppm was observed which would arise from geminal proton-fluorine interactions. The chemical shift value of −78.73 agrees with literature data of a CF_3 group at an α-position to a carbonyl function (Wray 1983). Additionally, after purification by anion-exchange chromatography no aldehydic proton was detectable in ^{1}H-NMR indicating that the catechol derivative is cleaved between the trifluoromethyl substituent and the adjacent hydroxyl function. GC-MS analysis allowed the

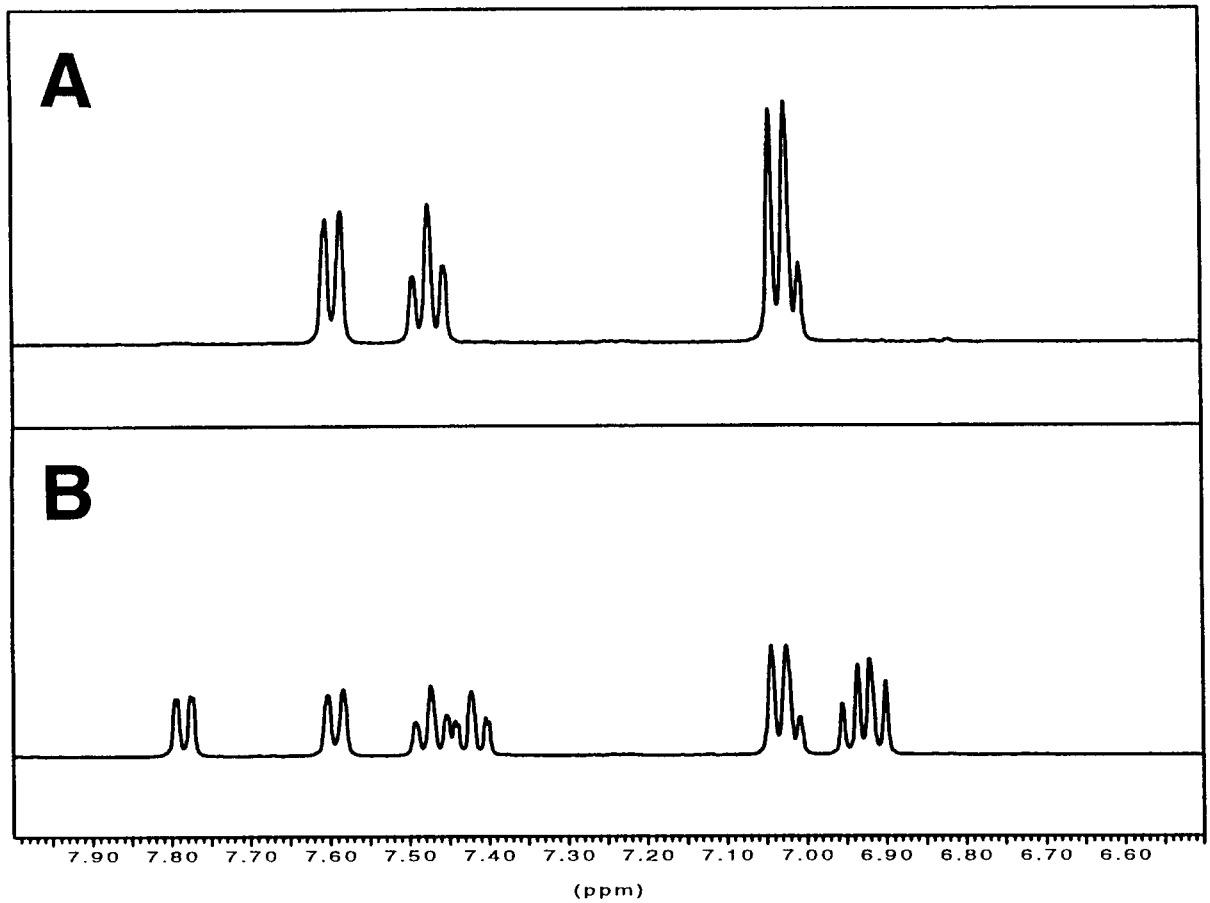

Figure 2. (A) ^{1}H-NMR spectrum of 2-trifluoromethylphenol in phosphate buffer; (B) ^{1}H-NMR spectrum of 2-trifluoromethylphenol after partial hydrolysis to salicylic acid.

identification of this metabolite as 7-TFHOD. At a retention time of 21.0 min a peak was detected with the following masses: 381 u, 353 u and 324 u, indicating $[M - \textit{tert}\text{-butyl}]^+$, $[M - \textit{tert}\text{-butyl} - CO]^+$ and $[M - \textit{tert}\text{-butyl} - \textit{tert}\text{-butyl}]^+$ ions, respectively (Figure 4).

The derivatisation produced the *tert*-butyldimethylsilyl derivative of 7-TFHOD. Due to the occurrence of two functional groups the mass of the product (210 u) will be raised by 2∗114u = 228 u. The spectra of TBDMS derivatives are normally dominated by $[M - 57]^+$ ions, in this case leading to the fragment ion of 381 u. By HPLC and GC-MS analysis of the resting cells suspension no catecholic intermediates, 3-CF_3-catechol and/or after hydrolysis of the CF_3 group 2,3-dihydroxybenzoic acid, could be found. Moreover, no conversion of salicylic acid was observed.

Purification of 7-TFHOD

Anion-exchange chromatography allowed the direct application of a centrifuged sample of the resting cells mixture. Stability problems did not occur as judged by ^{19}F-NMR analysis of the sample before and after the chromatographic separation. Simultaneous detection at 270 nm and 380 nm revealed the clear separation of a product with an absorbance at 380 nm and no absorption at 270 nm (7-TFHOD) from the contaminating compounds, 2-CF_3-phenol and salicylic acid, only absorbing at 270 nm.

Temperature- and photochemical stability of 7-TFHOD

Upon incubation at 60 °C without exposure to light 7-TFHOD remained stable for at least 4 hours. The absorption value at 387 nm remained constant and no additional absorption band occurred. No fluorine release was measured by ^{19}F-NMR, indicating that

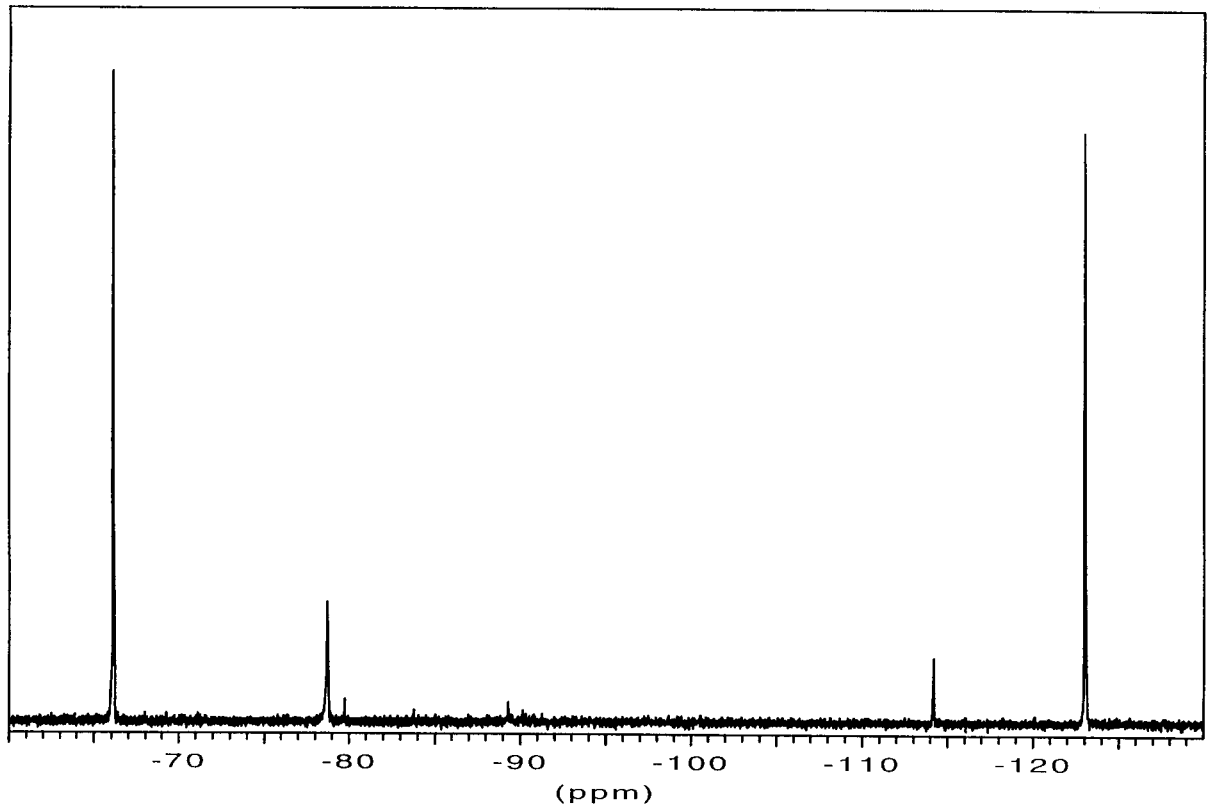

Figure 3. ^{19}F-NMR spectrum of a resting cells incubation of *Bacillus thermoleovorans* A2 with 2-trifluoromethylphenol as substrate.

not only the chromophore remained intact, but that also the trifluoromethyl group was not hydrolysed. However, exposure to sunlight of oxic solutions of 7-TFHOD and HMS-used as a reference-led to a rapid decrease in absorption (Figure 5). Within the first 20 min 28% of the 7-TFHOD was decomposed. First-order decay kinetics were followed at low substrate concentrations. Both compounds behaved similar in this way. The decomposition rates were equal for the experiments with anoxic solutions.

^{19}F-NMR measurements revealed a decreasing concentration of 7-TFHOD (−78.73 ppm) while no increase of the fluorine anion concentrations was detected. The photolytical breakdown of the chromophore is therefore not accompanied by a defluorination reaction leading to fluoride anions. No other signals appeared in the ^{19}F NMR spectrum resulting in a negative fluorine balance.

Molecular properties of a series of meta-cleavage products

Semiemperical AM1 molecular orbital properties computations were performed to obtain information on the molecular properties of a number of semialdehydes in their dianionic, monoanionic and neutral form (Table 1). These calculations revealed that within this series the lowest LUMO energy was found for 7-TFHOD (neutral form: −1.74 [eV]). This LUMO orbital is mainly located on the carbonyl carbon. In comparison to the derivative 2-hydroxy-6-oxo-hepta-2,4-dienoic acid (HOD) this property could lead to a facilitated nucleophilic attack of water or an OH-group of an enzyme. Generally, the alkyl derivatives form a clear distinct group of semialdehydes in comparison to 7-TFHOD. As expected from the strong electron-withdrawing effect of fluorine atoms, the sum of electrostatic potential charges on C6 and C7 – see experimental section for details – increases monotonically with an increasing number of fluorine atoms in going from HOD (+0.33) via 2-hydroxy-6-oxo-7,7,-difluoromethyl-hepta-2,4-dienoic acid and 2-

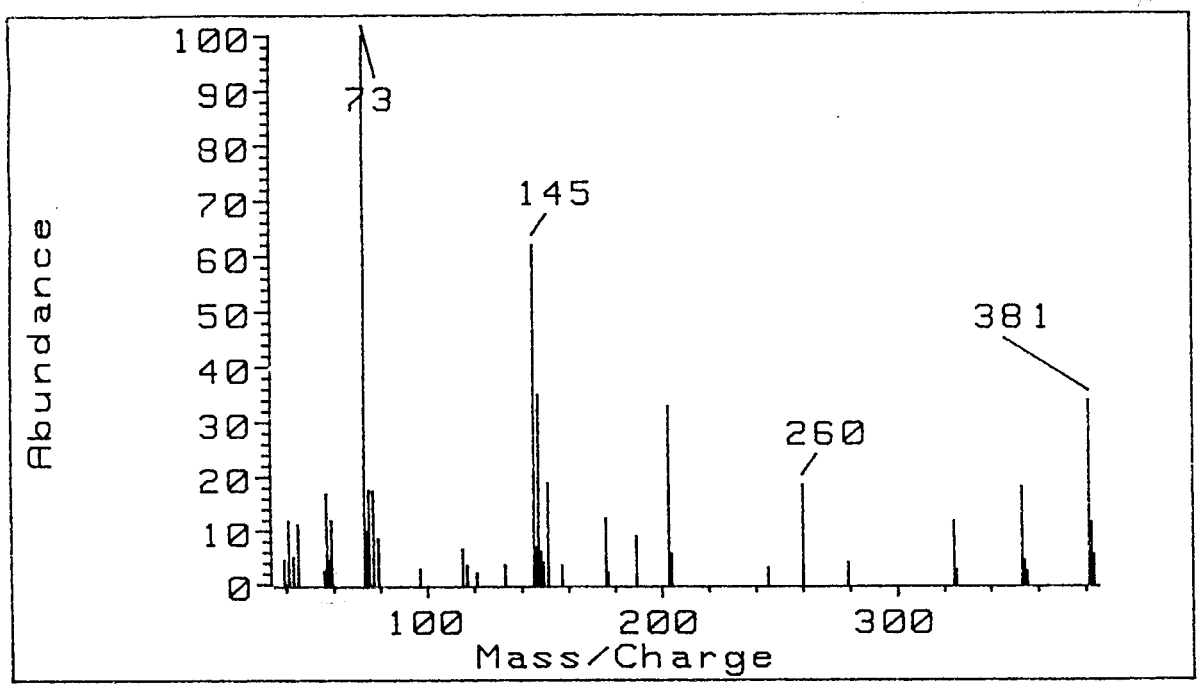

Figure 4. Mass spectrum of the t-butyldimethylsilyl derivative of 7-TFHOD. The spectra of t-butyldimethylsilyl derivatives are normally dominated by large $[M\text{-}57]^+$ ions, in this case leading to the fragment ion of 381 u.

hydroxy-6-oxo-7-fluoromethyl-hepta-2,4-dienoic acid to 7-TFHOD (+1.00). Substituent effects on the LUMO energy differences remained almost constant for the dianionic, monoanionic and neutral form.

Table 2 shows the calculated heat of formation for the monoanionic form and the corresponding hydrate. The differences in energy of the two aldehydes HMS and 5-methyl-HMS are similar to the calculated value for 7-TFHOD (−73.9 kcal/mol). The alkyl-substituted ketones show energy differences above −70 kcal/mol. The introduction of a fluorine atom in the substituent on C6 is lowering the energy difference below −70 kcal/mol.

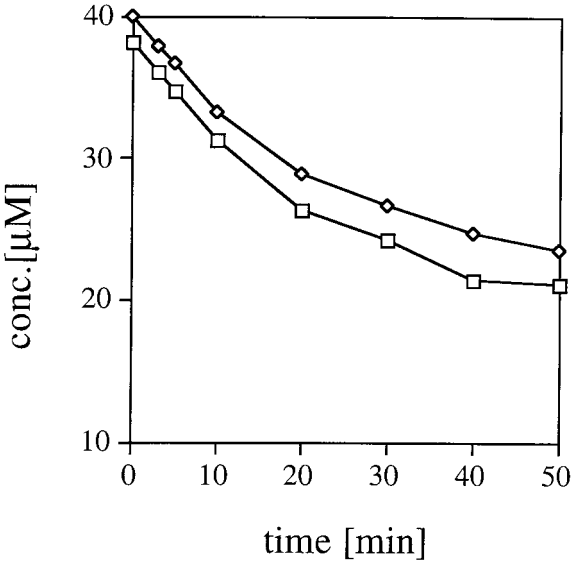

Figure 5. Decomposition of 7-TFHOD and HMS in phosphate-buffer solution (pH 7.2) during exposure to sunlight: ◇ 7-TFHOD; □ HMS.

Discussion

Biotransformation of 2-CF_3-phenol

The regulatory system of the *o*-cresol catabolism of *Pseudomonas* CF600 has been investigated. It has been reported by Chau Sze et al. (1996) that a growth phase-dependent response, mediated by the σ^{54}-dependent DmpR activator, was only a property of fast-growing cultures cultivated on carbon-rich (LB) medium. Under these conditions, the response to *o*-cresol is suppressed until the exponential-to-stationary

Table 1. Calculations of LUMO energies and electrostatic potential charges

Compound	LUMO energy (eV)		Sum of electrostatic potential charges on C6 and C7 for the dianionic form
	Dianion	Neutral	
HMS	+7.84	−1.32	–
5-methyl-HMS	+7.79	−1.29	–
HOD	+7.75	−1.28	+0.33
7-ethyl-HMS	+7.69	−1.30	+0.41
7-isopropyl-HMS	+7.66	−1.08	+0.37
7-propyl-HMS	+7.66	−1.07	+0.39
7-fluoromethyl-HOD	+7.52	−1.26	+0.58
7-difluoromethyl-HOD	+7.40	−1.48	+0.79
7-trifluoromethyl-HOD	+7.14	−1.74	+1.00

Table 2. Calculated heat of formation of *meta*-cleavage products

Compound	Heat of formation (kcal/mol)		
	Monoanionic form	Hydrate	Difference
HMS	−167.9	−240.9	−73.0
5-methyl-HMS	−174.1	−247.2	−73.1
HOD	−171.1	−241.2	−70.1
7-ethyl-HMS	−176.8	−246.4	−69.6
7−isopropyl-HMS	−182.4	−249.3	−66.9
7-propyl-HMS	−184.9	−252.3	−67.4
7-fluoromethyl-HOD	−220.5	−291.6	−71.1
7-difluoromethyl-HOD	−266.9	−342.6	−75.7
7-trifluoromethyl-HOD	−321.5	−359.4	−73.9

phase transition. Obviously, the expression of the first two enzymes in the phenol catabolism of *B. thermoleovorans* A2 when growing on LB medium, is also suppressed until the cells enter the late-exponential phase. However, at this moment it is still unclear if only a growth phase-dependent regulation controls the expression of the aromatic degradation pathway or if this is a fortuitous induction, i.e., a component of the LB ingredients is utilized in the late-exponential phase controlled by a regulatory system also operating on the phenol pathway.

Analogously to *ortho*-substituted monohalophenols (Reinscheid et al. 1997) we propose a pathway for 2-CF_3-phenol metabolism in *B. thermoleovorans* A2 (Figure 6). After hydroxylation to 3-CF_3-catechol, a *meta*-cleavage produces 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoic acid (7-TFHOD).

Since catechol 2,3-dioxygenase is converting 3-CF_3-catechol to 7-TFHOD and also since the hydrolysis of the initial substrate 2-CF_3-phenol is lowering the total amount of 3-CF_3-catechol formed, it seems polymerisation and/or hydrolysis of 3-CF_3-catechol is of importance in our system. Consequently, we could not detect neither 3-CF_3-catechol nor 2,3-dihydroxybenzoic acid. Nevertheless, under real environmental conditions it is possible that polymerisation comes into play due to thermal enzyme inactivation. The thermal stability of the catechol 2,3-dioxygenase is currently under investigation in our laboratory.

During our work it became clear that the analysis of semialdehydes by GC-MS poses a problem. Higson & Focht (1992) could not detect the methyl derivative of 5-chloro-HMS with an EI detector the ion energy set at the typical value of 70 eV. In this case the detection was achieved by lowering the ion energy to 20 eV or using a chemical ionization method. Even with a 20 eV setting Koh et al. (1997) detected a methyl derivative of 3,5-dichloro-HMS only in the selective ion

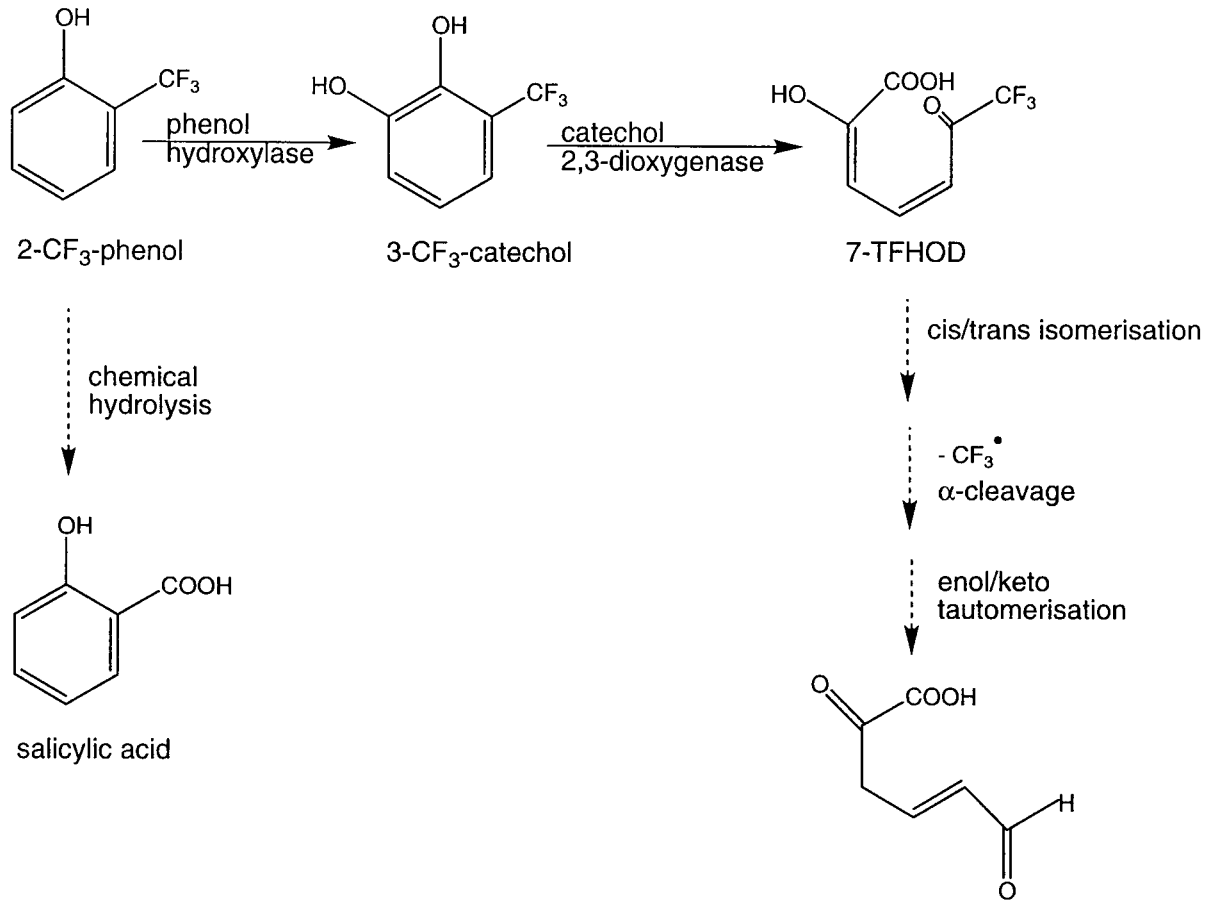

Figure 6. Overview of biological and non-biological transformations of 2-trifluoromethylphenol.

monitoring mode with an estimated 500fold increased sensitivity. With a possible initial concentration in the range of 0.1 M this difficulty cannot be due to initially small amounts of analyte. More likely, in the course of extraction, derivatisation, separation and detection, the instability of at least the methyl derivatives of the semialdehydes is leading to these analytical problems.

In our study the GC-MS detection of 7-TFHOD was achieved with the *tert*-butyldimethylsilyl derivative. Two factors might have been important:

(a) These derivatives are expelling a *tert*-butyl moiety very easily, thereby reducing the excess energy of the molecular ion and preventing further fragmentation. Further support of the idea that the kind of derivative is important for successful analysis by GC-MS is given by Sung Bae et al. (1996). They were able to analyse the trimethylsilyl derivative of 5-chloro-HMS at 70 eV ion energy.

(b) The derivatisation solution was again evaporated to dryness and the analyte redissolved in only 5 μl of solvent. This last step increased the concentration by a factor of 200 compared to the ethyl acetate extract. A clear GC signal for the 7-TFHOD derivative was obtained at 70 eV and without a selective ion monitoring mode.

Thermal transformation of 2-CF_3-phenol

The detection of salicylic acid as a major chemical degradation product of 2-CF_3-phenol was very surprising since it is known that the hydrolysis of a CF_3 group attached to an aromatic nucleus frequently requires rather energetic conditions, e.g., heating in concentrated sulphuric acid or strongly basic conditions (12N NaOH; Jones 1947) (Figure 6). In our study the critical step, the fission of the halogen-carbon bond, occurred under mild conditions. This process is a topic of further investigation. At 60 °C the hy-

drolysis is competing with the enzymatic formation of 7-TFHOD via 3-CF_3-catechol.

In a recent study the effect of incubation temperature on the reductive dechlorination of 2,3,4,6-tetrachlorobiphenyl was investigated (Wu et al. 1997). Temperature affected the lag time and the preferred route of dechlorination. The studied temperature range was between 4 °C and 66 °C, therefore real thermophilic conditions have been applied.

Photochemical decompositon of 7-TFHOD

Using ^{19}F-NMR and photometrical analysis it was established that decomposition of 7-TFHOD involves both the disappearance of the chromophore and the loss of the ^{19}F NMR signal. The latter process is, however, not accompanied with the formation of fluoride anion, and a negative fluorine mass balance is observed. This can be explained via an α-cleavage reaction of the photo-excited molecule, yielding a conjugated acyl radical and a trifluoromethyl radical. It has been shown that α-cleavage can occur in conjugated systems, e.g., CH_2 = CH-C(= O)H (Klessinger & Mich 1989). We expect α-cleavage to occur more easily in our conjugated system than in, e.g., CH_2 = CH-C(= O)H (where α-cleavage was shown to occur) because the loss of a CF3 radical is much less endothermic than the loss of a H radical in CH_2 = CH-C(= O)H, the difference being more than 25 kcal/mol according to PM3 calculations.

When the CF3 radical is liberated, this species can abstract a hydrogen atom from water to form trifluoromethane (fluoroform). This component has a low solubility in water, which in combination with its low boiling point (−84 °C) will lead to loss of fluorine atoms from the aqueous solution. A second independent photochemical process that can occur is cis-trans isomerisation around the C(4)–C(5) bond, to relieve some of the steric strain that exists in the depicted isomer. From the computed heats of formation (AM1, using COSMO with ϵ = 78 to simulate solvation in water) this is estimated to yield about 1–5 kcal/mol. But neither of these processes explains the disappearance of the chromophore. However, loss of absorption at 387 nm could be due to enol-keto tautomerisation which yields about 2 kcal/mol. The driving force of this process is the intrinsic higher stability of the keto-form in comparison to the enol-tautomer, in combination with the observation of a non-planar π-system, which diminished the stabilisation offered by through-conjugation from C1 to C6. Interestingly, this enol-keto tautomer [(E)-2,6-dioxo-hex-4-enoic acid] depicted in Figure 6 would be analogous to the first step in the enzymatic hydrolysis mechanism proposed by Engesser et al. (1988b).

Molecular orbital calculations

Growth tests with all three isomers of CF_3-phenol turned out to be unsuccessful indicating no productive pathway in the organism. A possible explanation is the observation of Engesser et al. (1990) that semialdehyde-hydrolases are not converting 7-TFHOD as a substrate.

Experimental data about the enzymatic hydrolysis of semialdehydes in *meta*-pathways of catechol degradation are scarce (Nordlund & Shingler 1990; Duggleby & Williams 1986; Bayly et al. 1987; Horn et al. 1991). Horn et al. (1991) suggested that the hydrolase of *P. putida* mt-2 may possess a serine hydrolytic enzymatic mechanism. Under these conditions molecular properties calculations can be helpful for testing the above mentioned hypotheses made on common grounds of organic chemistry.

Molecular orbital calculations have already been introduced in the field of phenol degradation (Cozza & Woods 1992; Peelen et al. 1995). It becomes clear that on the enzyme level turnover rates of a series of compounds can be correlated to computated molecular properties. The LUMO energy of the series of semialdehydes fit the proposed enzyme mechanism of Engesser et al. (1988) for the semialdehyde-hydrolase. The LUMO energy is of importance for the nucleophilic attack by the enzyme which results in the formation of a bounded semiketal (Fleming 1985).

Kinetic measurements of the hydrolase encoded by the TOL plasmid pWW0 from *P. putida* mt-2 showed that the higher catalytic efficiency with ketone substrates over aldehydes was the result of higher V_{max} values rather than lower K_m values (Duggleby & Williams 1986). Bayly & Di Berardino (1978) reported on the hydrolase activity of a *Pseudomonas putida* strain against different *meta*-cleavage products. Methyl substitution in the 7 position of 7-TFHOD resulted in reduced activity in comparison with the native compound. These results are in accordance to the calculated similar LUMO energy values for the alkyl derivatives which would result in similar K_m values but would not automatically affect V_{max}.

According to the Curtin-Hammett principle (Carey & Sundberg 1990) the energies for the different ionic forms had to be calculated. The rationale for this is that

under conditions where these forms have low interconversion energy barriers compared to the following reaction i.e. hydration, it is not possible to state which form is the starting compound for the reaction. Since proton transfer reactions belong to the fastest reactions in chemistry the corresponding activation energies are in fact very low. Supporting the idea that the trifluoromethyl substituent is facilitating a nucleophilic attack irrespective of the degree of ionisation (neutral species, mono-anion or dianion) 7-TFHOD displays the lowest LUMO energy in all three species under study.

In line with these calculated data about LUMO energies, the CF_3-substituted compound also possesses the highest positive charge at the center of attack, also facilitating nucleophilic attack (Fleming 1985). Additionally, differences in the reactivity of 7-TFHOD in comparison with alkylated analogous compounds (e.g., HOD) can also be related to the larger driving force for semi-ketal formation for the CF_3 compound (Table 2). The larger (>4 kcal/mol) driving force for the hydration of the CF_3-substituted compounds itself, however, directly suggests a reason for the observation that 7-TFHOD is not a substrate for the hydrolase (Duggleby & Williams 1986). The occurrence of a stabilised hydrate as intermediate might in fact hamper subsequent enzymatic steps in a thermodynamic fashion.

Acknowledgement

This work was supported by an EU Training Mobility and Research grant ERBFMGE-CT95-0066 (access to large scale facility Wageningen NRM Centre). U.M. Reinscheid wishes to acknowledge the LSF for provision of research facilities.

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