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Spontaneous release of fluoride during the dioxygenolytic cleavage of 5-fluorosalicylate by the salicylate 1,2-dioxygenase from Pseudaminobacter salicylatoxidans BN12

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One sentence summary: A novel reaction catalysed by a bacterial enzyme is described that splits with the help of oxygen the carbon-fluorine bond in non-natural compounds.

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Abstract

The alpha-Proteobacterium Pseudaminobacter salicylatoxidans BN12 forms a peculiar gentisate 1,2-dioxygenase (SDO) that oxidatively cleaves gentisate (2,5-dihydroxybenzoate) and additionally 1-hydroxy-2-naphthoate, salicylate and various amino-, chloro-, fluoro-, hydroxy- and methylsalicylates. In the present study, the conversion of 5-fluorosalicylate by this enzyme was analysed using various analytical techniques. Spectrophotometric assays showed that the conversion of 5-fluorosalicylate by the purified enzyme resulted in the formation of a new unstable intermediate showing an absorbance maximum at $\lambda_{max} = 292$ nm. The analysis of the enzymatic reaction by HPLC showed that two main products with absorbance maxima at $\lambda_{max} = 292–296$ nm were formed from 5-fluorosalicylate. The same two products (although in different relative proportions) were also formed when the SDO transformed 5-chlorosalicylate or when a purified 5-nitrosalicylate 1,2-dioxygenase from Bradyrhizobium sp. JS329 oxidized 5-nitrosalicylate. A whole cell system with recombinant Escherichia coli cells overexpressing the SDO activity was established in order to produce larger amounts of the reaction products. The reaction products were subsequently identified by $^1$H-NMR and mass spectrometry as stereoisomers of 2-oxo-3-(5-oxofuran-2-ylidine)propanoic acid. The release of fluoride in the course of the dioxygenolytic cleavage reaction was confirmed by ion-chromatography and $^{19}$F-NMR.

Keywords: Pseudaminobacter salicylatoxidans; salicylate 1,2-dioxygenase; gentisate 1,2-dioxygenase; ring-fission dioxygenases; fluorinated aromatics; biodegradation

Introduction

The carbon–fluorine bond is one of the strongest chemical bonds and organofluorine compounds are only rarely formed in nature. In contrast, fluorinated organic compounds are synthesized in large quantities by the chemical industry and it has been stated that about 20%–30% of all sold pharmaceuticals and agrochemicals are fluorinated compounds (e.g. top-selling compounds such as Prozac, Lipidor and Ciprobay). The large majority of the fluorinated compounds produced by industry are xenobiotics, which are not known from natural sources. It is therefore not surprising that fluorinated compounds are often...
recalcitrant to biodegradation and accumulate in the environment (Key, Howell and Criddle 1997; Müller, Faeh and Diederich 2007; Kiel and Engesser 2015).

There are only very few enzymatic reactions known which result in defluorination of aromatic compounds and it has been demonstrated for isomeric fluorobenzoates that different enzymatic reactions can result in the release of fluoride (Natara-jan et al. 2005; Murphy, Clark and Amadio 2009; Murphy 2010). Thus, for a 2-fluorobenzoate degrading pseudomonad, an initial dioxygenolytic simultaneous decarboxylation/defluorination to catechol has been found (Engesser and Schulte 1989). In the case of 3-fluorobenzoate (and fluorobenzene) the intermediate formation of 3-fluorocatechol and 2-fluoromuconate was reported (Boersma et al. 2004; Strunk and Engesser 2013). 4-Fluorobenzoate is initially converted by various bacteria via 4-fluorocatechol and 3-fluoromuconate and it was found that the fluoro originating from 4-fluorobenzoate can be finally released from 4-fluoromuconolactone by a hydrolytic dehalogenation resulting in the formation of maleylacetic acid (Schlömann et al. 1990). For 4-fluorobenzoate, there also exists a different degradative pathway which involves a 4-fluorobenzoate dehalogenase forming 4-hydroxybenzoate (Oltmanns et al. 1989; Amorim et al. 2014).

The a-proteobacterium Pseudaminobacter salicylatoxidans BN12 codes for a peculiar ring-fission dioxygenase (SDO) with the ability to cleave salicylate in the 1,2-position to 2-oxohepta-3,5-dienoic acid (Fig. 1). The enzyme has been intensively studied during the last years by using different biochemical, genetical and crystallographic techniques. These investigations demonstrated that the SDO belongs to the cupin superfamily of enzymes. The holoenzyme consists of four identical subunits and each subunit binds one ferrous iron ion by three histidine ligands (Hintner et al. 2001; Hintner, Reemtsma and Stolz 2004; Matera et al. 2008; Ferraroni et al. 2012a,b, 2013; Eppinger et al. 2015).

The SDO converts in addition to salicylate a wide range of substituted salicylates and it was observed that the enzyme converted 5-fluorosalicylic acid rather efficiently. In addition, evidence was obtained that the direct ring-fission product of 5-fluorosalicylic acid underwent some further reactions which could result in the release of fluoride (Fig. 1) (Hintner, Reemtsma and Stolz 2004).

A similar anionic elimination reaction was recently described for the reaction catalysed by the 5-nitrosalicylate 1,2-dioxygenase (NSDO) from Bradyrhizobium sp. strain JS329. This dioxygenase converted 5-nitrosalicylate into two products (which were tentatively identified as lactones) and nitrite was released in the course of this reaction (Qu and Spain 2011).

The conversion of 5-fluorosalicylic acid by the SDO appeared to be an interesting reaction with respect to the increasing importance of fluorinated compounds as environmental contaminants and the scarcity of known defluorinating enzymes. Therefore, the reaction was studied in detail in the present communication.

MATERIALS AND METHODS

Plasmids

Plasmid pJPH100exN was used for the expression of the wildtype SDO. This plasmid is a derivative of plasmid pET28a and encodes an aminoterminallyHis-tagged variant of the SDO from P. salicylatoxidans (Hintner, Reemtsma and Stolz 2004). Plasmid pJS804 was used as source for the gene coding for the NSDO from Bradyrhizobium sp. JS329 (Qu and Spain 2011).

Construction of an expression plasmid for the synthesis of a His-tagged variant of the NSDO from Bradyrhizobium sp. strain JS329

The gene (naaB) coding for the NSDO was amplified from plasmid pJS804 (kindly provided by E. Barton and J. Spain, Georgia Tech, GA) by using the primers naaB-F (AAAAACATATG-AAATGGAGCAACAAAGA) and naaB-R (AAAAACTCGAGATTTCGCTCTTGGTACGAA) the amplified DNA fragment was cut with the restriction enzymes Ndel and Xhol (restriction sites underlined). Subsequently, plasmid pET28a (Novagen, Madison, WI) was cut with the same restriction enzymes and both DNA fragments were ligated. This resulted in the formation of plasmid pEE001 which coded for an N-terminally His-tagged variant of the NSDO.

Enzyme expression

Cells of Escherichia coli BL21 (DE3)(pJPH100exN) were grown at 37°C overnight in dYT medium with kanamycin (50 μg ml−1) and then transferred at 30°C into fresh dYT medium with kanamycin to an initial optical density (OD600 nm) of about 0.4. The cells were grown to an OD600 nm of 0.8 and the expression of the enzyme induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1 mM). The cells were harvested about 5 h after IPTG addition by centrifugation (8000 rpm, 15 min, 4°C) and washed twice with 20 mM Tris/HCl buffer (pH 8).

The NSDO was produced by using E. coli Rosseta(DE3)-pLySSpE001). The cells were grown at 37°C overnight in LB medium with kanamycin (50 μg ml−1) and chloramphenicol (25 μg ml−1), and then transferred into fresh LB medium with kanamycin and chloramphenicol to an optical density (OD600 nm) of about 0.1. The cells were incubated at 37°C until an OD600 nm = 0.6–0.7 was reached. Subsequently, the cultures were shaken at room temperature and the expression of the NSDO was induced by adding IPTG (0.1 mM). After 5 h, the cells were harvested by centrifugation (8000 rpm, 15 min, 4°C) and washed twice with ice-cold K-phosphate buffer (pH 7, 20 mM).

Enzyme purification

The E. coli cells were suspended to an OD600 nm of about 200 in Tris/HCl (20 mM, pH 8) or K-phosphate-buffer (20 mM, pH 7) and disrupted by using a French press (Aminco, Silver Spring, USA) at 4°C and 80 MPa. Intact cells and cell fragments were removed by ultracentrifugation (35 000 rpm, 60 min, 4°C). The His-tagged enzyme variants were purified from the crude extracts by using a nickel nitritoltriacetic acid (NiNTA) agarose matrix (Qiagen, Hilden, Germany).
The SDO protein was eluted by using buffers containing 20 mM Tris/HCl (pH 8) plus 300 mM NaCl and increasing concentrations of imidazole. The active fractions eluted at an imidazole concentration of about 150 mM. Imidazole was removed from the active fractions by using HiTrap desalting columns (Amersham Bioscience, Freiburg, Germany).

The NSDO was eluted by using buffers containing 20 mM K-phosphate (pH 7) and increasing concentrations of imidazole. The active fractions eluted at an imidazole concentration of about 200 mM and the imidazole was removed as described above.

Spectrophotometric assays
The overlay spectra were recorded using a Cary 100 spectrophotometer (Agilent, Santa Clara, CA).

HPLC
The reaction products were separated by HPLC (Agilent 1100) by ion-pair chromatography on a Pro C18 AQ column (5 μm particles, 12.5 cm × 4 mm inner diameter; Trentec, Germany) at room temperature. Eluent A was H₂O/MeOH (80:20 v/v) and eluent B was H₂O/MeOH (5:95 v/v) with 1 mM tributylamine and 1 mM acetic acid each. Gradient elution started with 10% (v/v) eluent B at t = 0 min. At t = 5 min, a linear gradient was started which reached 10% (v/v) eluent A, 90% (v/v) eluent B at t = 16 min. The flow rate was 1 ml min⁻¹. Substrates and products were analysed by using a diode array detector at 210, 280, 295 and 305 nm.

LC/MS/MS
Product identification was performed by liquid chromatography-mass spectrometry (Agilent 1100) coupled to a triple quadrupole mass spectrometer API2000 (Perkin-Elmer Sciex Instruments, Waltham, MA) using electrospray ionization in the negative ion mode. The reaction mixtures were injected (5 μl) into the HPLC system without any pretreatment. Analytes were separated by ion-pair chromatography basically as described above, but using a flow rate of 0.5 ml min⁻¹.

Ion chromatography
The release of fluoride was quantified using ion chromatography (Metrohm 761 Compact IC, Metrohm AG, Filderstadt, Germany).

NMR
The nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AV250 spectrometer.

RESULTS
Spectrophotometric analysis of the conversion of 5-fluorosalicylate by the purified SDO
It was previously shown that the pH optimum of the SDO for the oxidation of salicylate lies at pH 8–9 (Hintner et al. 2001). Therefore, the purified His-tagged variant of the SDO was incubated with 5-fluorosalicylate at pH 8 in Na-phosphate buffer and the reaction analysed by UV/Vis overlay spectroscopy. The spectra demonstrated that 5-fluorosalicylate (λmax = 304 nm) was converted and that a new absorbance maximum at λmax = 292 nm was formed (Fig. 2A). The reaction product(s) was/were not stable under the reaction conditions and a bathochromic (new λmax at 328 nm) and hypochromic shift was observed (Fig. 2B).

The initial reaction of 5-fluorosalicylate resembled the previously studied conversion of salicylate to the 1,2-cleavage product 2-oxohepta-3,5-dienedioic acid that showed an absorption maximum at λmax = 283 nm (Hintner et al. 2001). In control experiments with salicylate no indications for significant decomposition reactions were detected even after several hours of incubation. This indicated that the instability of the products formed from 5-fluorosalicylate was due to the presence of the fluorine substituent.

Spontaneous decomposition of the formed products
In the following, it was examined if the observed decrease of the absorbance maximum at λmax = 292 nm was due to an enzymatic activity or to a spontaneous decomposition reaction. Therefore, 5-fluorosalicylate (0.2 mM) was converted by the purified SDO and the reaction monitored spectrophotometrically till it reached the maximal absorbance at 292 nm. Then, the enzyme was removed by ultrafiltration and the further reactions analysed. Thus, it was found that the changes in the UV/Vis overlay spectra were basically identical to those recorded for the decay reactions in the presence of the enzyme (see Fig. 2B). This indicated that the product showing a λmax at about 330 nm was spontaneously formed from the direct ring-fission product(s). This UV-Vis spectrum strongly resembled the well-known absorption spectrum of malemypyrurate, which already had been described as product formed from gentisate by gentisate 1,2-dioxygenases. The identity of this reaction end product with malemypyrurate was also suggested by the observation that the absorbance maximum at 330 nm disappeared immediately after acidification followed by a much slower increase in absorbance in the same wavelength region. These reactions have been previously described for the conversion of malemypyrurate to fumarlypyruvate (Lack 1959).

Reaction analysis by HPLC
The conversion of 5-fluorosalicylate by the purified SDO was subsequently analysed by HPLC using a solvent gradient with increasing methanol concentrations having a pH of about 5 (see materials and methods). Thus, it was found that two main products with retention times (Rt) of about Rt = 4.2 min (‘product X’) and Rt = 6.5 min (‘product Y’) were formed from 5-fluorosalicylate (Rt = 12.0 min). The in situ recorded absorbance maxima (λmax) of both products were at 292–296 nm. After prolonged incubation, both products slowly disappeared (Fig. 3).

Conversion of 5-fluorosalicylate by whole cells
The initial experiments with the purified SDO described above were performed in cuvettes or HPLC vials and therefore limited by the low solubility of oxygen in water. Therefore, in these experiments only about 0.25 mM of 5-fluorosalicylate could be converted. In order to properly characterize the products, significantly higher product concentrations were required. Therefore, it was attempted to convert higher concentrations of 5-fluorosalicylate by the purified enzyme in heavily stirred reaction vessels. Unfortunately, it was found that the reactions stopped rather rapidly and it was assumed that the catalytically active ferrous iron ions were either removed or oxidized under these conditions. Therefore, it was tested if the intended biotransformation was also possible with whole cells. Induced cells
Figure 2. Conversion of 5-fluorosalicylate by the purified SDO. The cuvette contained in a total volume of 1 ml 20 mM Na-phosphate (pH 8.0) and 0.2 mM 5-fluorosalicylate. The reactions were started by the addition of 5 µg of the purified SDO and one spectrum was recorded per minute. In (A) the initial increase in the absorbance is shown. In (B) the subsequent decomposition reaction was analysed for 4 h (spectra taken in 10 min intervals).

Figure 3. Conversion of 5-fluorosalicylate by the purified SDO in Na-phosphate buffer. The reaction mixture contained in 1 ml 20 mM Na-phosphate buffer (pH 8.0) plus 0.2 mM 5-fluorosalicylate. The reaction was started by the addition of 3 µg of the purified enzyme. At the indicated time intervals samples were taken and aliquots (5 µl each) instantaneously directly injected into the HPLC apparatus. The separated compounds were detected at 295 nm: □ 5-fluorosalicylate, Δ ‘product X’, and ▲ ‘product Y’.

Detection of fluoride release by ion-chromatography and \(^{19}\text{F}-\text{NMR}\)

The experiments described above demonstrated that it was possible to produce the same metabolites from 5-fluorosalicylate with whole cells as with the purified enzyme. Therefore, it was tested by ion-chromatography and \(^{19}\text{F}-\text{NMR}\) if in the course of the formation of these metabolites fluoride was released.

5-Fluorosalicylate was converted by resting cells of E. coli BL21 (DE3)(pPH100exN) and the reaction supernatants simultaneously analysed by HPLC and ion-chromatography. This demonstrated that the turn-over of 5-fluorosalicylate and the formation of the two products coincided with the release of fluoride.

Subsequently, samples from the same experiment were examined by \(^{19}\text{F}-\text{NMR}\). Initially, the spectral shifts of fluoride and the fluorine atom of 5-fluorosalicylate were determined as 119.5 and 125.1 ppm, respectively (Fig. 4A–C). Then, samples taken at different time points of the biotransformation experiment were analysed by \(^{19}\text{F}-\text{NMR}\). No signals for other fluorine-containing metabolites than 5-fluorosalicylate and fluoride were obtained. This demonstrated that no (sufficiently stable) fluorinated organic intermediates were formed during the conversion of 5-fluorosalicylate to fluoride by the SDO.

Reaction analysis by LC-MS/MS and \(^{1}\text{H}-\text{NMR}\)

In order to further characterize the reaction products, the reaction mixtures were reanalysed by LC-MS/MS. Thus, for both main products formed from 5-fluorosalicylate parent anions at m/z 167.2 were recorded. Furthermore, fragments of m/z 95.0 (−CO₂-CO) and m/z 122.9 (−CO₂) were observed. These data demonstrated together with the observed fluoride release and the analysis of the reaction by \(^{1}\text{H}-\text{NMR}\) (see Supporting Information) that ‘products X and Y’ were cis- and trans-2-oxo-3-(5-oxofuran-2-ylidene)propanoic acid. Therefore, the reaction scheme shown in Fig. 5 can be proposed for the conversion of 5-fluorosalicylate to fluoride by the SDO.

Comparison of the products formed by the SDO from 5-fluorosalicylate and 5-chlorosalicylate and by the 5-nitrosalicylate 1,2-dioxygenase from 5-nitrosalicylate

It was recently suggested that Bradyrhizobium sp. strain JS329 converted 5-nitrosalicylate by the action of a 5-nitrosalicylate...
Figure 4. Decoupled $^{19}$F-NMR spectra of 5-fluorosalicylate, fluoride, and culture supernatants obtained during the conversion of 5-fluorosalicylate by resting cells of E. coli BL21 (DE3)(pJPH100exN). (A)—(C) Reference compounds: 5-fluorosalicylate (6 mM) (A), KF (6 mM) (B), and mixture of 5-fluorosalicylate and KF (6 mM each). (C) The recombinant E. coli strain was cultivated and the SDO activity induced as described in the materials and methods section. The cells were harvested by centrifugation (15 min, 7000 rpm), washed in 20 mM Na-phosphate buffer (pH 8.0) and finally resuspended in the same buffer to an optical density (OD$_{546}$ nm) of 5. The cell suspension (20 ml) was transferred at room temperature to an open beaker and heavily stirred. The reaction was started by the addition of 5-fluorosalicylate (initial concentration 6 mM) and aliquots (2 ml each) taken every 20 min. The cells were removed by centrifugation (2 min, 15 000 g) and the supernatants analyzed by $^{19}$F-NMR (and HPLC). The $^{19}$F-NMR spectra of the samples taken at $t = 0$ min, (D), $t = 40$ min (E) and $t = 120$ min (F) are shown.

Figure 5. Conversion of 5-fluorosalicylate by the salicylate 1,2-dioxygenase from P. salicylatoxidans (A) 5-fluorosalicylate, (B) 2-oxo-4-fluorohepta-3,5-dienedioic acid, (C) ‘4-pyruvyl-4-florobut-2-en-4-olide’ (D) cis- and trans-2-oxo-3-(5-oxofuran-2-ylidine)propanoic acid and (E) maleylpyruvate.

1,2-dioxygenase (NSDO) to cis- and trans-2-oxo-3-(5-oxofuran-2-ylidine)propanoic acid and nitrite (Qu and Spain 2011). This indicated that the SDO and the NSDO converted their respective substrates to the same organic products. Therefore, the oxidation of 5-fluorosalicylate by the SDO and the conversion of 5-nitrosalicylate by the NSDO were comparatively analysed using the same reaction and HPLC conditions. Thus, it was found that the NSDO converted 5-nitrosalicylate in K-phosphate buffer (20 mM, pH 7) into two main products, which were according to their retention times, in-situ recorded UV/Vis spectra and coinjection experiments identical to the two main products formed from 5-fluorosalicylate by the SDO (‘products X and Y’). The SDO converted 5-fluorosalicylate to almost equimolar amounts of the two products (ratio about 1.3:1). In contrast, the NSDO showed a much stronger preference for the formation of ‘product X’ (ratio X:Y > 5:1).

The SDO and the NSDO convert in addition to the substrates 5-fluorosalicylate and 5-nitrosalicylate, respectively, also 5-chlorosalicylate (Hintner, Reemtsma and Stolz 2004; Qu and Spain 2011). Therefore, the purified SDO and NSDO were incubated with 5-chlorosalicylate in order to test if also this substrate underwent a similar dehalogenation reaction. Thus, it was found that both enzymes indeed formed also from 5-chlorosalicylate the same two major products. The SDO formed
Figure 6. Comparison of the fluoride-releasing reactions in the catabolism of 4-fluorocatechol and 5-fluorosalicylate. The shown pathway for the degradation of 4-fluorocatechol was derived from Schlömann et al. (1990).

in this case preferentially ‘product Y’ (ratio X:Y about 1:6). In contrast, the NSDO converted 5-chlorosalicylate preferentially to ‘product X’ (ratio X:Y about 2:1).

The ability of both enzymes to convert 5-chlorosalicylate indicated that the substrate range of both enzymes could further overlap. Therefore, it was tested if the SDO could convert 5-nitrosalicylate or the NSDO 5-fluorosalicylate. These experiments demonstrated that the SDO could not convert 5-nitrosalicylate. In contrast, a weak activity of the NSDO with 5-fluorosalicylate was detected (<10% of the activity with 5-nitrosalicylate).

**DISCUSSION**

The conversion of 5-fluorosalicylate was characterized by HPLC, mass spectrometry, ion-chromatography and NMR spectroscopy. The results consistently suggested that fluoride was spontaneously removed in the course of the enzymatic reaction from the organic substrate and no evidence could be obtained that in addition to 5-fluorosalicylate any other fluorinated organic compound was immediately formed. This indicated that the postulated ring-fission product 2-oxo-4-fluorohexa-3,5-dienedioic acid (Fig. 5B) is extremely unstable and spontaneously reacts to the ‘dienelactones’ cis- and trans-2-oxo-3-(pent-1-en-3-ylidene)propanoic acid (Fig. 5D). The formation of the two isomeric ‘dienelactones’ can be explained by the spontaneous release of HF from the ‘fluorolactone’ (Fig 5C) and might suggest that both possible enantiomers of the ‘fluorolactone’ are formed in the course of the reaction (assuming a conserved trans-elimination of HF). The slight differences in the in situ recorded absorbance maxima between the two ‘dienelactones’ ($\lambda_{\text{max}} = 292$ and 296 nm) might allow to assign to ‘product X’ the cis-configuration as in many cases the cis-isomers absorb light at slightly shorter wavelengths than the corresponding trans-isomers (Hesse, Meier and Zeeh 2002).

The ability of ring-fission dioxygenases to cleave 5-halosalicylates in the 1,2-position and to somehow couple this reaction with the dehalogenation of the substrate was initially suggested for the metabolism of 5-chlorosalicylate by a Bacillus strain (Crawford, Olson and Frick 1979). The authors of this study reported that the conversion of 5-chlorosalicylate by a partially purified enzyme fraction resulted in the formation of a product with $\lambda_{\text{max}}$ at 292 nm, which slowly disappeared after prolonged incubation. They suggested that this product would probably be 7-carboxy-4-chloro-2-keto-hept-3,5-dieneoic acid (thus, the chlorinated non-cyclic compound which would be the direct product resulting from a 1,2-dioxygenolytic cleavage of 5-chlorosalicylate). In the present study it was shown that the oxidation of 5-fluorosalicylate and 5-chlorosalicylate by the SDO also resulted in the formation of a new (unstable) UV/Vis maximum with a $\lambda_{\text{max}}$ at 292 nm, which was caused by the formation of the ‘dienelactones’ cis- and trans-2-oxo-3-(pent-1-en-3-ylidene)propanoic acid. It is therefore very probable that the product(s) formed from 5-chlorosalicylate by the Bacillus strain studied by Crawford, Olson and Frick (1979) and those identified in the present study are identical and that also the Bacillus strain converted 5-chlorosalicylate to the isomeric ‘dienelactones’.

The conversion of salicylates carrying a halogen substituent in the 5-position clearly resembles the reaction described by
Qu and Spain (2011) for the cleavage of 5-nitrosalicylate by the NSDO, as also in this case the ring-fission resulted in an immediate release of nitrite. Thus, it appears that the oxidative 1,2-cleavage of 5-substituted salicylates carrying a substituent that allows an anionic elimination of the substituent more or less ‘automatically’ results in the removal of the substituent, presumably because the corresponding ring-fission products are chemically unstable and prone to lactonization. In the case of 5-fluorosalicylate, this resulted in an unprecedented release of fluoride, which was clearly different to the enzyme catalysed release of fluoride from the fluorolactone formed in the course of the degradative pathway of 4-fluorocatechol (Fig. 6).

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

REFERENCES
Eppinger E, Ferraroni M, Bürger S, et al. Function of different amino acid residues in the reaction mechanism of gentisate 1,2-dioxygenases deduced from the analysis of mutants of the salicylate 1,2-dioxygenase from Pseudomonas putida salicylatoxidans. Biochim Biophys Acta 2015;1854:1425–37.
Hinpert JP, Reemtsma T, Stolz A. Biochemical and molecular characterization of a ring fission dioxygenase with the ability to oxidize (substituted) salicylate(s) from Pseudaminobacter salicylatoxidans. J Biol Chem 2004;279:37 250–60.