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A chiral high-performance liquid chromatography–tandem mass spectrometry method for the stereospecific analysis of enoyl-coenzyme A hydratases/isomerases

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ABSTRACT

The enzymes catalyzing the stereospecific hydration of 2-enoyl-CoA into the corresponding *S*- or *R*-3-hydroxyacyl-CoA are named enoyl-CoA hydratases (ECH), where the *S*-specific is called ECH-1 and the *R*-specific is called ECH-2. Current ECH assays are mostly based on spectrophotometric methods. Amongst many limitations, these methods do not directly measure the 3-hydroxyacyl-CoA produced, neither do they allow determination of its stereospecific configuration. We have developed a chiral HPLC method coupled with tandem mass spectrometry (MS) for the sensitive, direct, stereospecific and quantitative analysis of ECH-1/-2 reaction products, or *R*-/*S*-3-hydroxyalkanoates in general. The method is based on the reaction of the 3-hydroxyl group on the chiral carbon with 3,5-dimethylphenyl isocyanate, creating a urethane derivative which is then chirally resolved on a chiral HPLC column having 3,5-dimethylphenyl carbamate-derivatized cellulose as the chiral stationary phase. The resolved urethane derivatives are detected using tandem MS in the multiple reactions monitoring (MRM) negative electrospray ionization mode by monitoring the free hydroxy fatty acid fragment ion liberated from its parent urethane derivative. The method resolves the *R*-/*S*-enantiomers of 3-hydroxy fatty acid homologues ranging from C_6 to C_{16}. Using this method, the net ECH activity present in clarified cell lysates of the bacterium *Pseudomonas aeruginosa* cultivated in a rich medium was found to be of both ECH-1 and ECH-2. Interestingly, the clarified cell lysate of *Escherichia coli* cultivated also in a rich medium displayed mainly an ECH-1 (*S*-specific) activity. This method will facilitate the quantification and stereospecific characterization of ECHs, as well as the chiral lipid profiling of bacterial secondary metabolites containing hydroxyalkanoic acid moieties.

1. Introduction

Enoyl-CoA hydratases (ECHs) are a group of enzymes involved in the metabolism of lipids [1]. Typically, ECHs reversibly catalyze the *syn* hydration of trans-2-enoyl-CoA to produce *S*-3-hydroxyacyl-CoA in the second step of β-oxidation [2–4]. These typical *S*-specific (*S*-ECH) ECHs are also known as ECH-1 (crotonases) [5]. ECHs that specifically generate *R*-3-hydroxyacyl-CoA have also been discovered [6] and are known as ECH-2 (*R*-ECH) [5]. ECH-2 are thought to play an important role in the metabolism of lipidic intermediates in eukaryotes [7] as well as in the biosynthesis of polyhydroxyalkanoates, particularly in *Pseudomonas aeruginosa* [8] and in other bacteria [9–11]. Interestingly, both ECH-1 and ECH-2 demonstrate chain length specificity for their substrates [12,13].

Available direct and indirect ECH enzymatic assays are based on spectrophotometric methods [2,12,14] (Fig. 1). The direct ECH activity assay relies on monitoring the decrease in UV absorption of the 2-enoyl-CoA substrates at λ_{max} 263 nm (ε_{263} of 6700 M^{-1} cm^{-1}) resulting from their hydration. The characteristic absorption of enoyl-CoA substrate is due to a conjugation between the α, β double bond and the carbonyl function of the thioester [2,14]. Alternatively, ECH activity is assayed indirectly in a combined enzymatic assay in which *S*-3-hydroxyacyl-CoA, formed by the hydration of 2-enoyl-CoA, is oxidized in presence of NAD^+ and of an *S*-3-hydroxyacyl-CoA dehydrogenase to the corresponding 3-ketoacyl-CoA and $NADH^+$. In this assay, ECH-1 activity is monitored by the increase in UV absorption of $NADH^+$ at 340 nm (ε_{340} of 6220 M^{-1} cm^{-1}) (Fig. 1) [12].

These spectrophotometric methods suffer from many limitations. First, the absorption of 2-enoyl-CoA at 263 nm is largely obscured by the absorption of the adenine residue of the CoA moiety and by that of the hydration product, 3-hydroxyacyl-CoA [12]. This could be partially corrected for by the addition of an appropriate amount of adenylic acid (AMP, ADP or ATP) [15] in the blank cell, to

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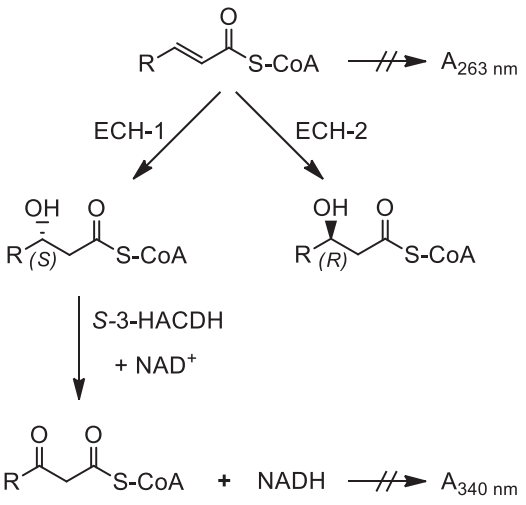

Fig. 1. Different principles of conventional spectrophotometric assays of enoyl-CoA hydratases (ECH). Current ECH assays use spectrophotometric methods based on monitoring the disappearance of 2-enoyl-CoA that absorbs at 263 nm. More indirectly, ECH activity is also estimated via coupling the ECH reaction with an *S*-3-hydroxyacyl-CoA dehydrogenase (*S*-3-HACDH) that, in the presence of NAD^+, reduces the *S*-3-hydroxyacyl-CoA into the 3-ketoacyl-CoA accompanied with the formation of NADH that absorbs at 340 nm.

give a total absorbance not exceeding 2.0 [12] while giving a reading for the experimental cell of 0.4–0.5 [2,14]. However, this approach cannot be standardized and thus this adds to the complexity and cost of the reaction and narrows down the range of substrate concentration at which recording sensitivities are still acceptable at this background noise [15]. Second, the indirect (combined) assay can report ECH-1 activity but it cannot unambiguously report an ECH-2 (*R*-specific) activity because only the hydration product of ECH-1 (*S* configuration) is the substrate of the *S*-3-hydroxyacyl-CoA dehydrogenase provided in the reaction. Although ECH-2 activity can be deduced using the combined assay through the observation of a decrease in A_{263} without a concurrent change (or without an increase) in A_{340} [8,9], this very indirect approach requires appropriate positive controls (standard *S*-3-hydroxyacyl-CoA) that adds to the complexity and cost of this method. Moreover, even though ECH-1 activity can be deduced via the increase in the A_{340} readings in the combined assay, these readings must be interpreted cautiously in terms of hydratase activity, as the net A_{340} is affected by the combined kinetic parameters of both enzymes, unless an excess of dehydrogenase is used to avoid a rate-limiting step [16]. Yet, another drawback on the utility of the combined assay is the chain length specificity of the *S*-dehydrogenase to its substrates [17], this requires a careful choice of the appropriate dehydrogenase according to the chain length of the substrate being used. Moreover, when using substrates with unusual acyl group, the success of the dehydrogenation step is even more doubtful [15]. Furthermore, all spectrophotometric methods require working with purified protein preparations to avoid artefactual results arising from other substances absorbing in the same wavelength, as often observed with complex cell lysates. Finally, both spectrophotometric methods suffer from the necessity of having clear homogeneous samples and from the loss of linearity for concentrated samples. Overall, both spectrophotometric methods are essentially indirect as they do not record the appearance of the reaction products, *S*- or *R*-3-hydroxyacyl-CoA, which possess no useful UV absorption [14].

Because of these limitations and because of the current interest in ECH in general [18] and in ECH-2 in particular [19], we have developed a new direct chiral HPLC method, partially inspired from previous works based on HPLC-UV methods [20], and coupled it with tandem mass spectrometry (MS), for the determination of ECH activity with an exceptional resolution and sensitivity. Previously, an HPLC-UV method for long chain ECH assay has been reported by Tsuchida et al., but was limited to the chiral resolution of *R*-/*S*-3-hydroxyhexadecanoate [21,22]. Our method resolves a much wider range of the *R*-/*S*-enantiomers of 3-hydroxyalkanoates homologues and allows the direct qualitative discrimination between ECH-1 and ECH-2 activities as well as the quantitative determination of their activities.

2. Materials and methods

2.1. Bacterial cultures and preparation of clarified bacterial cell lysates

Bacterial cultures of *P. aeruginosa* strain PA14 in Tryptic Soy Broth (TSB, Difco) were prepared by inoculation of an overnight culture of PA14 into fresh TSB medium at a starting OD_{600} of 0.05 and incubation at 34 °C under rotation until stationary phase (OD_{600} = 3–4).

Bacterial cultures of *Escherichia coli* strain BL21 (DE3) in LB broth (Difco), were prepared by inoculation of an overnight culture of BL21 into a fresh LB medium at a starting OD_{600} = 0.05 and incubated at 34 °C under rotation until OD_{600} = 2–3).

Cells of either strains were harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C, resuspended in lysis buffer (50 mM Tris–HCl, pH 7.7, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) [23] freshly supplemented with Mini Protease Inhibitor Cocktail Tablets (Roche) and then lysed by sonication on ice at maximum power (Branson sonifier 150, USA) for 9 cycles of 30 s interrupted by 30 s on ice. Cell lysates were clarified by centrifugation at 10,000 × *g* at 4 °C for 30–45 min to be used freshly or kept at 4 °C until use.

2.2. Materials and reagents

All solvents used for chromatography were HPLC grades. Methanol was obtained from Fisher, Trinidad; Toluene, chloroform and acetonitrile from J.T. Baker Chemicals (USA); anhydrous pyridine from Sigma (USA); sulfuric acid from Fluka (Switzerland); ammonium hydroxide and glycerol from Fisher (USA); and 3,5-dimethylphenyl isocyanate from Aldrich (USA). Ammonium acetate was obtained from American Chemicals Ltd. (Montréal, Canada). Water was purified using a Milli-Q system from Millipore (Millipack, 0.22 μm, Gamma Gold, USA).

Standard *R*/*S*-3-hydroxyalkanoates homologues: 3-hydroxybutyric acid was obtained from Aldrich, 3-hydroxyhexanoic acid from Santa Cruz Biotechnology, Inc. (USA), 3-hydroxyoctanoic acid from Sigma, 3-hydroxynonanoic acid from Matreya LLC (USA), 3-hydroxydecanoic acid from Sigma (Sweden), 3-hydroxydodecanoic acid from Santa Cruz Biotechnology, Inc., 3-hydroxytetradecanoic acid from Sigma (Sweden), and 3-hydroxyhexadecanoic acid from Santa Cruz Biotechnology, Inc. Polytetrafluoroethylene (PTFE) 0.2 μm membrane filter cartridges were obtained from Chromspec (Canada).

2.3. Preparation of standard mixtures of enantiomerically rich *R*-3-hydroxy fatty acids

Standard mixtures of enantiomerically rich *R*-3-hydroxy fatty acids were prepared by the acid hydrolysis of pure solvent-extracted rhamnolipids (RLs) obtained from *P. aeruginosa* and *Burkholderia glumae* cultures [24,25]. Structurally, their RLs are *R*-3-{*R*-3-[2-*O*-(α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl]oxyalkanoyl}oxyalkanoate, whose *R*-3-hydroxyalkanoic acids have a chain length of $C_{10\pm2}$ and of $C_{14\pm2}$ for RLs of *P. aeruginosa* and *B. glumae*, respectively [26].

A quantity of 100 mg RLs was transferred in a screw-capped glass tube and suspended in 1 mL 15% (v/v) H_2SO_4. One millilitre of $CHCl_3$ was then added and the biphasic mixture heated at 100 °C for 140 min. The $CHCl_3$ layer containing the 3-hydroxy fatty acids was then collected and evaporated to dryness, and finally redissolved in 1 mL of methanol. The percentage of the *R*- relative to the *R*- and *S*-enantiomers was 90% for *R*-3-OH-C_8, 92% for *R*-3-OH-C_{10} and 100% for *R*-3-OH-C_{12} in the standard mixture prepared from RLs of *P. aeruginosa*. The percentage of the *R*- relative to the *R*- and *S*-enantiomers was 100% for *R*-3-OH-C_{12}, 100% for *R*-3-OH-C_{14} and 92% for *R*-3-OH-C_{16} in the standard mixture prepared from RLs of *B. glumae*.

2.4. Chemical synthesis of trans-2-decenoyl-CoA

Trans-2-decenoyl-CoA was synthesized using the mixed anhydride method [27]. Briefly, 252 μmol trans-2-decenoic acid were dissolved in 10 mL of anhydrous diethyl ether containing 315 μmol triethylamine. Then, 315 μmol ethyl chloroformate were added, which was accompanied by formation of salt crystals. The solution was stirred in a screw-capped tube at room temperature overnight. The mixed anhydride was then filtered using 0.2 μm PTFE membrane filter cartridges and the filtered solution was added dropwise to 9 mL of a 1:1:1 solution of 50 mM Na_2CO_3 (pH 8):ethanol:ethyl acetate containing 8.8 μmol Coenzyme A trilithium salt. The solution was stirred at room temperature for about 2 h until no free thiol was detected using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay [28]. Then, the solution was concentrated in vacuo to remove the organic solvent, washed twice with ether to remove excess acid and then purified by HPLC (Waters Delta Prep 4000 preparative chromatography) using a C18 reversed phase analytical column (4.6 mm × 250 mm Partisil 10 ODS-2, 10 μm particle size, Whatman). Elution was performed using 20 mM ammonium acetate/1.75% acetonitrile as eluent A and 95% acetonitrile/5% H_2O as eluent B over the course of 30 min at a flow rate of 2 mL/min. The gradient was set according to the following profile: 0 min, 100% A; 25 min, 50% A + 50% B; 26 min, 100% B; 27 min, 100% A; 30 min, 100% A. Elution was monitored at 260 nm using a Waters 486 tunable UV/VIS absorbance detector system, and fractions containing trans-2-decenoyl-CoA were pooled and lyophilized. The retention time for trans-2-decenoyl-CoA was 17.5 min. To remove all ammonium acetate, the lyophilized solid was redissolved in water and relyophilized.

2.5. Enoyl-CoA hydratase (ECH) enzyme assay

The assay for ECH activity is carried out in the standard assay buffer described previously [12], with some modifications. The assay buffer is composed of 0.1 M potassium phosphate buffer, pH 8.0, 0.1 mg/mL bovine serum albumin, 15 μM trans-2-decenoyl-CoA and 20 μL of clarified cell lysate. The reaction is performed in a total volume of 500 μL in 2-mL microcentrifuge tubes, incubated at 30 °C for 1 h. The resulting solution of enzymatic reaction containing the product *R*-/*S*-3-hydroxydecanote-CoA is then treated with 20 μL 1 N KOH (pH 11.5–12) and heated at 55 °C for 10 min to hydrolyze the thioester bond and liberate the free 3-hydroxydecanoic acid. After this step, a non-physiologic odd-chain length *R*/*S*-3-hydroxyalkanoate is added as internal standard for quantification purposes (e.g. 30 mg/L 3-hydroxynonanoic acid, Matreya LLC, USA). The solution is then acidified to pH 1–2 using 40 μL HCl 3 N prior to the extraction of the 3-hydroxyalkanoates: one millilitre $CHCl_3$ is added, the tube vortexed for 5 s, then centrifuged for 1 min to help phase separation, and finally 900 μL of the $CHCl_3$ layer is transferred to HPLC borosilicate vials and evaporated under a stream of nitrogen. This dried residue of 3-hydroxyalkanoate is then derivatized prior to the chiral HPLC–MS/MS stereospecific analysis, as described below.

2.6. Chiral HPLC–MS/MS analysis of R-/S-3-hydroxyalkanoates

To avoid sample-to-sample variations with regard to derivatization conditions, a sufficient stock of derivatization solution is prepared as follows: 1 μL 3,5-dimethylphenyl isocyanate (Sigma, USA) is dissolved in 500 μL toluene containing 40 μL pyridine. From this stock solution, a volume of 541 μL is added to each vial containing the dried 3-hydroxyalkanoates samples obtained in Section 2.5, and vortexed. The derivatization reaction is left overnight at room temperature. Then, because some toluene-insoluble crystals appear as byproduct at the bottom of the vials, the reaction mixture is filtered using a 1-mL glass syringe fitted with a PTFE membrane filter cartridge (Chromspec, CA) into a new borosilicate vial. Twenty microlitres of this filtered 3,5-dimethylphenyl urethane derivative are injected in a high-performance liquid chromatography (HPLC; Waters 2795, Alliance HT) equipped with a 100 mm × 4.6 mm Lux cellulose-1 (Phenomenex, CA) chiral column (particle size 5 μm). The detector is a triple tandem quadrupole mass spectrometer (qMS/MS, Quattro Premier XE, Micromass) equipped with an electrospray ionization interface. Unless otherwise indicated, all the 3,5-dimethylphenyl urethane derivatives of 3-hydroxyalkanoates are detected and quantified in the negative ionization MRM mode. Data were integrated and processed by MassLynx™ software (version 4.1, Waters).

HPLC separation is performed using a flow rate of 0.4 mL/min with a gradient of water (A) and methanol (B), both containing 10 mM ammonium acetate at pH 8. The gradient is: 0 min, 50% A + 50% B; 1 min, 48% A + 52% B; 25 min, 40% A + 60% B; 26 min, 100% B; 27 min, 50% A + 50% B; 30 min, 50% A + 50% B. The solvent flow is split to 10% through a Valco Tee. The mass spectrometer operating parameters are: capillary voltage 3.0 kV, cone voltage 10 V (unless otherwise specified), source and desolvation temperatures 120 °C and 150 °C, respectively, cone and desolvation gas (N_2) flow at 15 and 100 L/h, respectively. In MRM detection mode, the parent molecular ion is collisionally fragmented and a specific daughter ion is monitored. Argon is used as the collision gas at a pressure of 3.15×10^{-3} mbar, corresponding to a flow of 0.35 mL/min and the collision energy is adjusted at 10 eV.

Each *R*-/*S*-enantiomer is quantified by comparing its surface area with that of the 3-hydroxynonanoates as internal standard using the response factors previously determined for each homologue through calibration curves prepared using pure standards.

3. Results

3.1. Derivatization of 3-hydroxy fatty acids

Since the chiral stationary phase (CSP) of the Lux cellulose-1 column chosen for this study is a cellulose tris(3,5-dimethylphenyl carbamate) coating of silica (Fig. 2A), *R*-/*S*-3-hydroxyalkanoates were derivatized similarly using 3,5-dimethylphenyl isocyanate (3,5-DMPI), generating the 3,5-dimethylphenyl urethane (3,5-DMPU) derivatives (Fig. 2B) to ensure maximum sorbent–solute interaction and therefore high chiral resolution.

The derivatization is performed at room temperature and is accompanied by the formation of crystals of an unidentified toluene-insoluble byproduct. Formation of this crystal is used as a visible indicator of the success of derivatization and is usually seen after 12 h; still, the reaction is left for 24 h to ensure its completion. Equal concentrations (30 mg/L each) of racemic *R*/*S*-3-hydroxyoctanoate (*R*/*S*-3-OH-C_8), nonanoate (*R*/*S*-3-OH-C_9) and decanoate (*R*/*S*-3-OH-C_{10}) were prepared to evaluate the

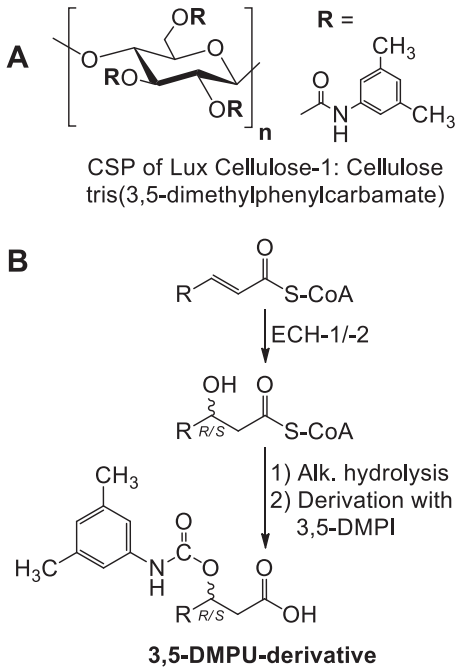

Fig. 2. Stationary phase of the chiral column and derivatization reaction prior to chiral HPLC–MS/MS analysis. (A) The CSP of the Lux cellulose-1 chiral column is a modified-polysaccharide coating of the underlying silica. The modified polysaccharide is cellulose tris(3,5-dimethylphenyl carbamate). (B) After an ECH-1/-2 reaction, the free *S*-/*R*-3-hydroxyalkanoate is liberated by alkaline hydrolysis and derivatized with 3,5-dimethylphenyl isocyanate (3,5-DMPI), forming the 3,5-dimethylphenyl urethane (3,5-DMPU) derivative of 3-hydroxyalkanoates.

chiral resolution of the Lux cellulose-1 column (Fig. 3). 3-OH-C_9 is used as a non-physiologic internal standard (IS) for quantification purposes. This new 3,5-DMPI-mediated derivatization method produces a high enantiomeric resolution of tested racemic 3-hydroxy alkanoates (C_8–C_{10}). This is achieved using a 30-min elution program with a slow gradient of water:methanol starting from 50% and increased up to 60%. Isocratic elution with water:methanol (50:50) results, however, in broadening and long retention times of the three species, especially for the 3-OH-C_{10} enantiomers which eluted at 45 min (data not shown). With the achieved resolving

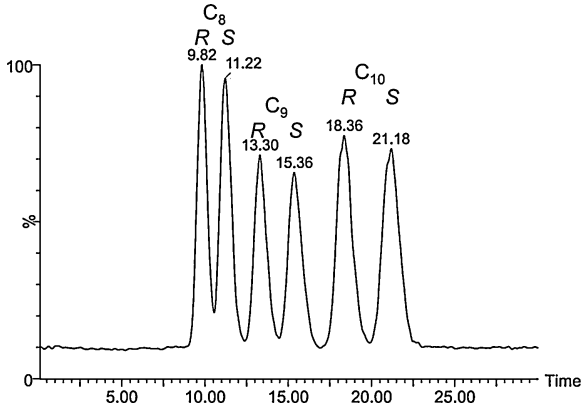

Fig. 3. Chiral HPLC–MS/MS chromatogram of standard racemic *R*/*S*-3-hydroxyoctanoate (3-OH-C_8) and decanoate (3-OH-C_{10}). *R*/*S*-3-hydroxynonanoate (3-OH-C_9) was added as internal standard. The three homologues were added at 30 mg/L. Elution program is a gradient of methanol with water; both solvents containing ammonium acetate. Methanol gradient is as follows: 0 min, 50%; 1 min, 52%; 25 min, 60%; 26 min, 100%; 27 min, 50%; 30 min, 50%. MS detector was set at the MRM negative ESI mode using the following ion transitions: 306 > 159, 320 > 173 and 334 > 187 corresponding to 3,5-DMPU derivatives of 3-OH-C_8, 3-OH-C_9 and 3-OH-C_{10} and their fragment ions, respectively.

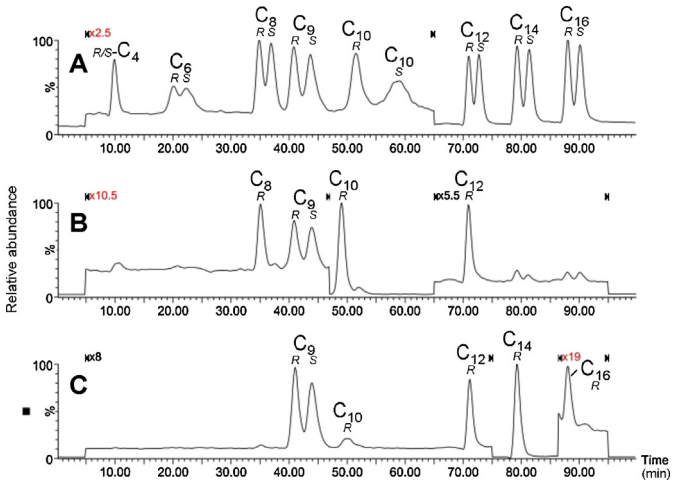

Fig. 4. Chiral HPLC–MS/MS chromatograms of different preparations containing 3-hydroxyalkanoate homologues from C_4 to C_{16} with C_9 added as internal reference of retention time. (A) Standard racemic mixture of *R*/*S*-3-hydroxyalkanoate homologues from C_4 to C_{16} prepared at 60 mg/L. (B) Standard mixture enantiomerically rich in *R*-3-hydroxyalkanoates (C_8–C_{12} with C_{10} as the predominant) prepared by the acid hydrolysis of rhamnolipids (RLs) of *P. aeruginosa*. (C) Standard mixture enantiomerically rich *R*-3-hydroxyalkanoates (C_{12}–C_{16} with C_{14} as the predominant) prepared by the acid hydrolysis of RLs of *B. glumae*. *R*/*S*-3-hydroxynonanoate was added at 60 mg/L as internal reference of retention time. Elution program was a gradient of methanol with water; both solvents contained 10 mM ammonium acetate and adjusted to pH 8. Methanol gradient was as follows: 0 min, 30%; 1 min, 30%; 24 min, 38%; 25 min, 50%; 60 min, 55%; 61 min, 65%; 94 min, 80%; 95 min, 30%; 100 min, 30%. MS detector was set in the MRM negative ESI mode. Some regions of the chromatograms were amplified to facilitate peaks comparison.

power, it is possible to discriminate between enantiomers where the *R*-form always elutes before the *S*-form (see at Section 3.3).

Quantitative determination of enantiomers is achieved using a calibration curve with increasing concentration of each of 3-OH-C_8 and -C_{10}, while maintaining constant the concentration of the 3-OH-C_9 IS, at 30 mg/L. The calibration equations (1) and (2) for the enantiomeric determination of 3-OH-C_8 and -C_{10}, respectively, are:

$$x = \frac{y}{202.8} \quad (1)$$

$$x = \frac{y}{266.4} \quad (2)$$

where x is the concentration of *R*/*S*-3-OH-alkanoate (mM), y is the relative response factor, i.e. the concentration of *R*/*S*-3-OH-C_9 internal standard (0.172 mM) multiplied by the peak area of analyte *R*/*S*-3-OH-alkanoate and divided by the peak area of *R*/*S*-3-OH-C_9 internal standard. These calibration curves are linear with correlation coefficients (R^2) of 0.998 and 0.999, respectively, in the range of concentration tested, starting from 5 mg/L (~0.027 mM) up to 300 mg/L (~1.6 mM) for both 3-OH-C_8 and 3-OH-C_{10}. Lower detection limits down to 0.5 mg/L (~0.0025 mM) can be achieved, demonstrating the high sensitivity of the method. The linearity of the curve illustrates that 6.8 mmol of 3,5-DMPI is sufficient for the derivatization of up to a total of 3.6 μmol of 3-hydroxyalkanoates. The 3,5-DMPU derivatives of 3-hydroxyalkanoates are stable for at least 6 months at room temperature.

3.2. Optimization of chiral HPLC resolution of racemic 3-hydroxyalkanoates

Different ECH-1 or -2 demonstrate chain length specificity to various homologues of 2-alkenoyl-CoA substrates, from C_4 to C_{16}. Thus, ideally, the method should chirally resolve corresponding *R*-/*S*-3-hydroxyalkanoate homologues from C_4 to C_{16} (Fig. 4A).

Generally, enantiomers of the 3,5-DMPU of 3-hydroxyalkanoates from C_8 to C_{16}, are best resolved by HPLC using isocratic elution with 50:50 methanol:water. However, under these conditions, the longer chain homologues (C_{12}–C_{16}) present a much longer retention times (more than 2 h). Therefore, we are instead using a slowly increasing methanol gradient to shorten the elution time of these compounds, while keeping appropriate chiral resolution. We found that the optimal rising methanol gradient slope is 0.3% per minute, and should not exceed 0.4–0.5% per minute, as a higher slope causes complete loss of enantiomeric resolution. Consequently, designing a relatively short elution program that resolves enantiomeric derivatives of all the series of 3-hydroxyalkanoate homologues, C_4–C_{16}, is unattainable because of their vastly different affinities for the stationary phase and the inability of using rapidly rising gradients of methanol. We thus designed a three-step 100-min gradient program which allows acceptable resolution of enantiomers from C_8 to C_{16}, with the C_4 and C_6 not sufficiently resolved (Fig. 4A). Nevertheless, for the purpose of hydratase assays, it is unnecessary to use a long elution program, as normally only one enoyl-CoA substrate is tested, producing only one pair of *R*-/*S*-enantiomers of the resulting 3-hydroxyalkanoates that together with the IS would necessitate a short elution program, like the one used to generate Fig. 3.

3.3. Chiral identification of enantiomeric pairs

To verify the order of elution of the *R*-/*S*-enantiomeric pairs of standard racemic 3-hydroxyalkanoate homologues derivatives, enantiopure standards are required. Because these are not commercially available, we prepared enantiomerically rich *R*-hydroxyalkanoates by acid hydrolysis of the bacterial exoproducts rhamnolipids. The rhamnolipids of *P. aeruginosa* contain *R*-3-hydroxyalkanoates homologues from C_8 to C_{12}, while homologues from C_{12} to C_{16} are found in rhamnolipids of *B. glumae* [26]. Fig. 4B shows that the predominant *P. aeruginosa* homologue is C_{10}, while in *B. glumae*, the predominant one is C_{14} (Fig. 4C).

The RL-derived enantiomerically rich *R*-3-OH-C_8 to C_{16} always coincide with the first eluting peak of each pair of enantiomers of corresponding racemic homologues. Likely, we conclude that the enantiomers of 3-OH-C_4 and C_6 should follow the same trend with the *R*-form eluting first.

3.4. Optimization of MS detection

At concentrations up to 300 mg/L, the 3,5-DMPU of 3-hydroxyalkanoates gave very bad signal-to-noise ratio in full-scan negative ESI mode. However, in single ion recording (SIR) mode where the *m*/*z* of $[M-H]^-$ of the eight species of 3,5-DMPU derivatives of 3-hydroxyalkanoate were monitored (Fig. 5A), we obtain a well enhanced signal-to-noise ratio. However, in SIR mode some little interfering peaks having the same masses as those of some of 3,5-DMPU derivatives of 3-hydroxyalkanoate are co-detected, making the exact identification of each species difficult. This is solved using multiple reactions monitoring (MRM) mode, which enhanced both the sensitivity and the selectivity, with no interfering peaks detected (Fig. 5B). In MRM mode, the fragment ion monitored is the 3-hydroxyalkanoate anion generated upon the collision-induced dissociation of the parent urethane derivative (Fig. 6).

The parent and fragment ion masses defined in the MRM method and the optimum cone voltage and collision energy were determined for each of 3,5-DMPU of the different homologues, with their retention times (Table 1).

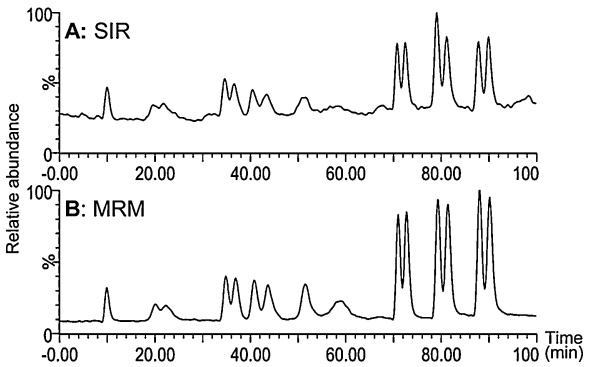

Fig. 5. Chiral HPLC–MS/MS chromatograms of *R*/*S*-3-hydroxyalkanoates in different MS detections modes. (A) Single ion recording (SIR) mode. (B) Multiple reactions monitoring (MRM) mode. *R*/*S*-3-hydroxyalkanoates homologues from C_4 to C_{16} as well as C_9 added as internal standard are all at 60 mg/L.

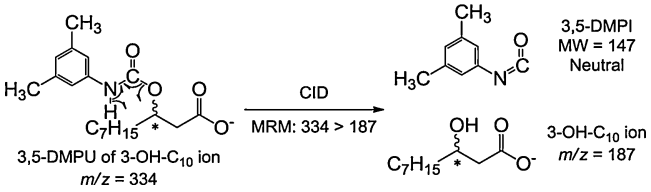

Fig. 6. Mechanism of fragmentation of 3,5-DMPU derivative of *R*/*S*-3-hydroxydecanoate during MS/MS detection at the MRM mode. MRM mode is based on the ion transition occurring during the collision-induced dissociation of 3,5-DMPU derivative of *R*/*S*-3-hydroxydecanoate resulting in the liberation of two fragments: 3,5-DMPI and the free *R*/*S*-3-hydroxydecanoate. The latter fragment is monitored as daughter.

3.5. Determination of ECH activity in cell lysates

We applied our method to characterize the nature of the total ECH activity in clarified cell lysates of *P. aeruginosa* and *E. coli* grown in rich culture media (Fig. 7). The enzymatic assay contained 2-decenoyl-CoA as substrate and the *R*- to *S*-enantiomeric distribution of the formed 3-hydroxydecanoate was monitored. Interestingly, using cell lysates of cells grown in rich

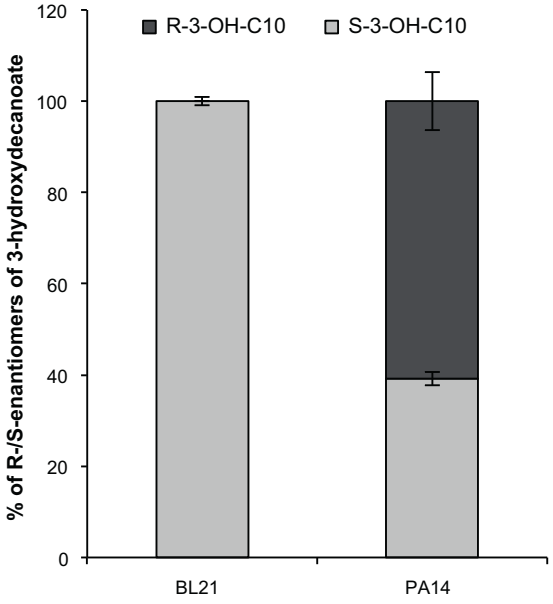

Fig. 7. The type of ECH activity of cell lysates of different bacteria is characteristic. In vitro ECH assay of clarified cell lysate of *E. coli* BL21 (DE3) and *P. aeruginosa* PA14 cultivated in rich media.

Table 1
Ion transitions and optimal conditions of chiral HPLC–MS/MS analysis of 3,5-DMPU derivatives of 3-hydroxy fatty acids.

Species	MW[a] of 3,5-DMPU derivatives	$[M-H]^-$ ion transition (m/z)[b]	Cone voltage (V)	Collision energy (eV)	Retention time window of *R*/*S*-enantiomer (min)[c]	
					R-	*S*-
3-OH-C_4	251.1	250 > 103	10	10	9.9	9.9
3-OH-C_6	279.1	278 > 131	10	10	20.1	22.3
3-OH-C_8	307.2	306 > 159	20	10	34.9	36.9
3-OH-C_9	321.2	320 > 173	20	10	40.8	43.7
3-OH-C_{10}	335.2	334 > 187	20	10	51.6	58.9
3-OH-C_{12}	363.2	362 > 215	20	10	71.0	72.8
3-OH-C_{14}	391.3	390 > 243	20	10	79.4	81.4
3-OH-C_{16}	419.3	418 > 271	20	10	88.1	90.2

[a] Molecular weight calculations are based on the exact masses.
[b] Fragmentation pathway proceeds via the collision-induced breakdown of the urethane bond releasing the 3,5-dimethylphenyl isocyanate from the 3-hydroxy fatty acids.
[c] According to Fig. 4.

media, the distribution of *R*- and *S*-enantiomers of produced 3-hydroxydecanoate is in favour of the *S*-enantiomer in case of *E. coli*, while they are nearly equally abundant in case of *P. aeruginosa*. This entails that the net ECH activity of *E. coli* is mainly of the ECH-1 type (*S*-specific), whereas that of *P. aeruginosa* is of both the ECH-1 and ECH-2 type (Fig. 7).

4. Discussion

This chiral analysis method for 3-hydroxy fatty acids requires a preliminary derivatization of the hydroxyl group carried on the chiral centre using 3,5-dimethylphenyl isocyanate (3,5-DMPI) followed by HPLC enantiomeric resolution using a chiral column prior to tandem mass spectrometry detection in the MRM mode. While 3,5-DMPI has not been previously reported for this purpose, it showed to be an efficient derivatizing agent for 3-hydroxy fatty acids (C_4–C_{16}). It was selected based on the nature of the chiral column used, Lux cellulose-1, whose chiral stationary phase (CSP) is based on 3,5-dimethylphenyl carbamate derivative of cellulose. This approach was inspired from a previous similar work conducted using 3,5-dinitrophenyl isocyanate (3,5-DNPI) as the derivatizing agent with the concurrent use of a chiral column, Sumichiral OA-3100, whose CSP is composed of the 3,5-dinitrophenylurea derivative of *S*-valine [20].

Chiral resolution of 3,5-DMPU derivatives of 3-hydroxy fatty acids (C_8–C_{16}) on Lux cellulose-1 is considerably better than with the 3,5-DNPU derivatives on Sumichiral OA-3100 [20] and is devoid of the enantiomeric overlaps observed in the latter study. With both methods the *R*-enantiomer elutes before the *S*-enantiomer. An advantage in favour of 3,5-DMPI is that it is around 20 times less expensive than 3,5-DNPI, given that both are used at the same concentration. Our method only partially resolved 3-OH-C_6 and did not resolve 3-OH-C_4. Chiral resolution of the latter homologue was untested in the aforementioned study [20] and was problematic in a previous chiral method based on GC [29]. This might be explained by the relatively short alkyl chains of C_4 and C_6 that limit the interactions with the matrix, leading to the loss of enantiomeric resolution.

MS detection sensitivity to 3,5-DMPU-derivatives of hydroxyalkanoates in the full-scan mode is very low. However, the MRM mode provides the required sensitivity and selectivity. Although slightly less sensitive and selective, the SIR mode, that can be used with a simple quadrupole instrument, still provides sufficient sensitivity while being more accessible than the more expensive tandem mass spectrometers required to conduct MRM acquisition.

In ECH assay, usually, only one 2-enoyl-CoA substrate is added to the reaction. As a time saving approach, enantiomeric resolution of the corresponding 3-hydroxyalkanoate can be optimized specifically instead of using the long elution program developed to resolve the whole series of homologues. Interestingly, the amount of 2-enoyl-CoA required to be added in the ECH assay according to our method is much lower than that required with a conventional spectrophotometric assay. Typically in a conventional assay, an amount of 30 μM of 2-enoyl-CoA substrate is added to generate an appropriate initial absorbance at 263 nm [12]. In contrast, our method has a lower limit of detection of 2.5 μM for the 3-OH-C_{10} homologue because of the use of the highly sensitive MRM mode of acquisition.

As a demonstration of the performance of the method, we measured the key difference in ECH activity between two bacteria, *P. aeruginosa* and *E. coli*. Although *P. aeruginosa* demonstrated both ECH-1 and ECH-2 activity on 2-decenoyl-CoA substrates (C_{10}), *E. coli* demonstrated almost exclusively an ECH-1 activity on this substrate. This result was somewhat expected as *P. aeruginosa* accumulates medium-chain-length (C_6–C_{16}) poly-3-hydroxyalkanoates (mcl-PHA) biosynthesized from *R*-3-hydroxyalkanoyl-CoA precursors [30], which are necessarily produced by an mcl-specific ECH-2 (mcl-ECH-2). In contrast, *E. coli* is known to accumulate mainly short-chain-length PHA (scl-PHA) granule composed of *R*-3-hydroxybutyrates (C_4) units [30], and would thus possess instead more scl-ECH-2. This explains why recombinant expression of mcl-specific PHA synthase genes could not lead to accumulation of mcl-PHA in *E. coli* [31,32] unless with co-expression of genes generating mcl-*R*-3-hydroxyalkanoates, like *maoC* (an ECH-2), or mutation of genes like *fadB*, decreasing the accumulation of *maoC* substrate [31,33,34]. This is further corroborated by the findings of Davis et al. who produced more mcl- to scl-PHA upon the simultaneous overexpression of the *P. aeruginosa* mcl-specific PHA synthase gene *phaC1* and mcl-specific ECH genes *phaJ1*/*phaJ2* in *E. coli* BL21 [23].

Our method can be applied not only for the purpose of classification of the type of ECH activity (ECH-1or -2), but also to study the reaction kinetics of ECHs and to characterize their chain length specificity using different homologues of 2-enoyl-CoA substrates. Moreover, this method could be applied for chiral analysis of any hydroxyalkanoates for other purposes. For example, it can be used for the determination of the chiral orientation of hydroxy fatty acids present in certain secondary metabolites which are increasingly demonstrated to be absolutely in the *R*-form, e.g. rhamnolipids [35,36], polyhydroxyalkanoates [30] and sphingoglycolipid [37]. It also provides an additional tool for the study and discovery of the metabolic pathways and enzymes generating hydroxy fatty acids exclusively in the *R*-form.

5. Conclusion

In this work, a new chiral HPLC–MS/MS method has been developed and optimized for the qualitative and quantitative chiral analysis of hydroxy fatty acid enantiomers (C_6–C_{16}). This

method allows the direct qualitative discrimination between ECH-1 (*S*-specific) and ECH-2 (*R*-specific) activities as well as the quantitative determination of the relative proportion of ECH-1 to ECH-2 activities in crude bacterial cell lysates. The sensitivity and selectivity of this method exceeds that of the conventional UV-spectrophotometric assay of ECH and overcomes many of its limitations.

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