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Enzymatic synthesis and surface properties of novel rhamnolipids

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ABSTRACT

New rhamnolipids were obtained via the development of a synthesis procedure consisting of two biocatalyzed steps. In the first step, naringinase was used to introduce a primary alcohol function onto rhamnose by glycosylation of 1,3-propanediol. In the second step, immobilized lipase B from *Candida antarctica* catalyzed the esterification of the primary hydroxyl group with mono- and di-carboxylic fatty acids of increasing chain length (from C8 to C14). For the monoic acids, the initial rate and 24 h yield decreased with increasing chain length. For the dioic acid, the number of carbon atoms of the acid did not influence these parameters. The new rhamnolipid obtained with tetradecanoic acid showed very good surface properties. At pH 5, it had a very low critical aggregation concentration of 1.70 μM and it diminished water's surface tension to 27.6 mN/m. It was also able to form stable insoluble monolayers. On the other hand, the rhamnolipid formed with tetradecanedioic acid showed far less interesting surface properties.

1. Introduction

Biosurfactants which are surface active molecules produced by micro-organisms present a wide structural diversity (glycolipids, lipoaminoacids, lipopeptides, polymers, . . .) and numerous advantages compared to their chemically synthesized counterparts. For example, they can be produced from renewable resources, and are active under extreme conditions (pH and temperature), are highly biodegradable and have low toxicity. On the other hand, they have high production costs due, amongst other things, to low yields and fastidious purification [1–5].

Amongst glycolipids, rhamnolipids which are secondary metabolites produced by strains of *Pseudomonas aeruginosa*, have drawn particular attention as they can be produced in high yields from easily available raw materials such as used oils or wastes from the food industry and have several potential industrial, agricultural and environmental applications (medical, bioremediation. . .) [1,6–10]. The polar head of these compounds consists of one or two rhamnose (Rha) units (linked by an α-1,2-glycosidic bond) and their hydrophobic tail of one or two (very rarely three) β-hydroxy fatty acids linked to each other by an ester bond (Fig. 1). More than fifty congeners have been described in the literature [11]. They differ by their fatty acid chain length and/or the presence of unsaturations. The terminal carboxylic acid function of the fatty part can be either free or, very rarely, methylated [11–13]. The type of rhamnolipid produced depends on the strain and the culture conditions [14]. *P. aeruginosa* has the major disadvantage of being an opportunistic human pathogen causing infections associated with compromised host defenses (AIDS. . .) [1,15]. As rhamnolipids may be valuable for medical and food applications, it would be useful to produce them via other production routes using non pathogenic or safe industrial microorganisms or via a simple method of synthesis. *P. aeruginosa* was the first producer of rhamnolipids which was identified and is still considered the primary producing species [11]. It is also the only bacteria currently used for their industrial production [16]. However, there are increasing numbers of reports of other bacteria being producers of rhamnolipids. Abdel-Mawgoud et al.'s review [11] listed more than 20 such bacteria. Some are from the same phylum and class as *P. aeruginosa* (e.g., *P. fluorescens*. . .), some from other classes (e.g., *Burkholderia thailandensis*. . .) and some even from other phyla (*Renibacterium salmoninarum*. . .). However, the bacteria have not always been conclusively identified and the production of rhamnolipids has not always been confirmed by high precision analytical techniques such as LC–MS. Furthermore, in most cases when a new bacteria species was reported as producing rhamnolipids often only one isolate was actually selected for further studies. Therefore, despite the potential for new sources, only few bacterial species can be considered as confirmed producers

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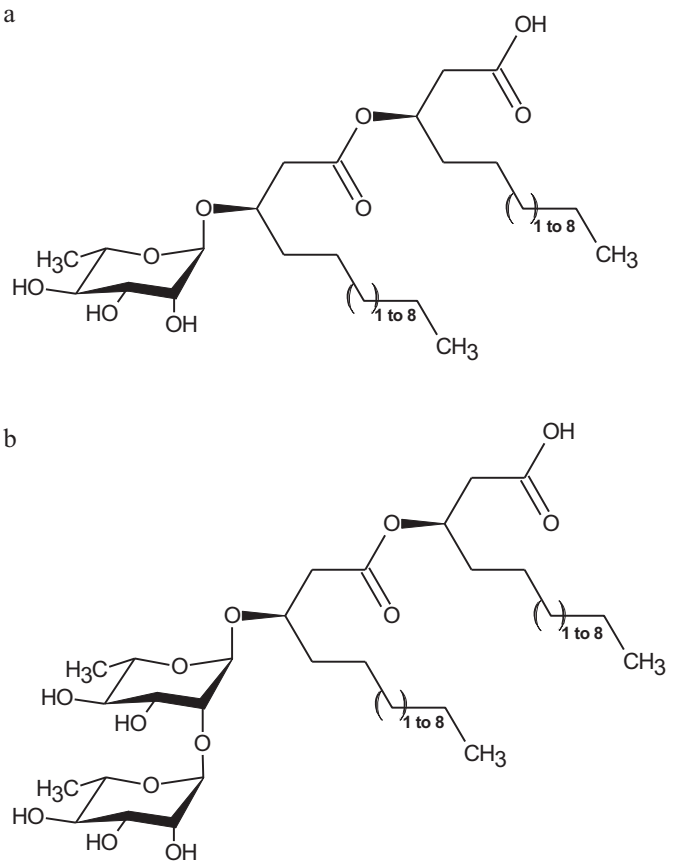

Fig. 1. General structure of the mono- (a) and di- (b) rhamnolipids. There exist also some congeners with only one fatty acid and some with three fatty acid chains. The fatty acids may have one or two unsaturations and the terminal carboxylic function is sometimes methylated. The two most common rhamnolipids are the monorhamnolipid (α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and the dirhamnolipid (α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β- hydroxydecanoyl-β-hydroxydecanoate) (Figure based on [9]).

of rhamnolipids [11]. Examples of studies citing new bacteria sources for rhamnolipids from the *Burkholderia* genus and from thermophilic microorganisms are given below. All seem to require further research before a safe alternative to *P. aeruginosa* is available [11,16].

Several *Burkholderia* species such as *B. glumae*, *B. plantarii*, *B. thailandensis* and *B. pseudomallei* have been shown to produce rhamnolipids. Generally, the fatty acid chains of these rhamnolipids are longer (a majority of 14 carbon atoms) than those produced by *P. aeruginosa* (a majority of 10 carbon atoms) [17,18]. *B. pseudomallei* [19] is pathogenic and responsible for melioidosis, a human infectious disease. *B. thailandensis* is non pathogenic and *B. glumae* and *B. plantarii* are plant pathogens. *B glumae* presents the advantage of already being used at an industrial level for the production of a lipase. However many parameters (pH, temperature, stirring. . .) must still be optimized to improve the production levels of rhamnolipids [17]. Some research has been done on the production of rhamnolipids by thermophilic microorganisms such as *Thermus thermophilus*. It has the advantage of being non pathogenic and of being able to use inexpensive substrates such as sunflower oil. Its culture is carried out at 75 °C, which improves the solubilization of certain substrates and helps avoid contamination [20,21]. Another solution avoiding the use of *P. aeruginosa* is the production of rhamnolipids by recombinant heterologous hosts such as *Escherichia coli*. However, overproduction of rhamnolipids in heterologous host is difficult and further work is needed [22,23]. Duynstee and co-workers developed a chemical synthesis route for the two major glycolipids produced by *P. aeruginosa* [24]. It is not suitable for consideration in an industrial production context as it is a multi-step process, which uses very special and expensive reagents and requires the use of a complex protection/deprotection strategy.

The objective of this work was to investigate a new strategy of synthesis for the production of novel rhamnolipids that could exhibit properties as promising for industrial and environmental applications as their natural counterparts while avoiding the use of the pathogenic gram negative *P. aeruginosa* for their production. An enzymatic approach was preferred to a chemical one which may require more drastic conditions and lead to the generation of side products. It was decided to synthesize amphiphilic compounds from Rha by grafting fatty acids or dicarboxylic acids of increasing chain length and to study the impact of the structure of the acid on the parameters of the enzymatic synthesis such as the initial rate (v_0) and yield. The aggregation behavior of the two longest chain derivatives was studied by determining their critical aggregation concentration (CAC) and their influence on water's surface tension (γ). Their ability to form Langmuir monolayers was also evaluated.

2. Materials and methods

2.1. Reagents and solvents

All reagents used had a minimal purity of 99% unless stated otherwise. Sodium acetate trihydrate, *tert*-butanol (*t*-But), the HPLC grade acetonitrile (ACN), chloroform (stabilized with ca. 0.5% of ethanol), dichloromethane and methanol (MeOH) were from Scharlab (Spain). LC–MS grade ACN and MeOH were from Biosolve (Netherlands). Acetic acid, citric acid (monohydrate, reagent grade >98%), 1,10-decanedioic acid, dimethylsulfoxide (DMSO), 1,12-dodecanedioic acid, formic acid (for mass spectrometry), 2-methyl-2-butanol (2M2B), molecular sieve 3 Å (8–12 mesh, pre-activated by drying 4 h at 250 °C before use), 1,3-propanediol, 1-propanol, L-rhamnose (Rha) (monohydrate), 1,14-tetradecanedioic acid (C14diAc) and tetradecanoic acid (C14Ac) were purchased from Sigma–Aldrich (USA). Decanoic acid (C10Ac), dodecanoic acid (97%) and octanoic acid (98%) were from Fluka (Switzerland). Sodium citrate tribasic dihydrate and sodium tricitrate monohydrate were from SAFC (USA). Hydrochloric acid (AnalaR NormaPur 37%) was from VWR (France). Phosphoric acid (85%) was from Merck (Belgium). Sodium dihydrogenphosphate dihydrate was from UCB (Belgium).

2.2. Synthesis

2.2.1. Enzymes screened for the esterification of rhamnose

Enzymes screened (in parenthesis, the name of the microorganism producers, the synthesis temperature used and chosen on the basis of data given by the suppliers): lipozyme TL IM (EC 3.1.1.3 as all lipases) (*Thermomyces lanuginosus*, 60 °C), lipase Novozym 435 (lipase B from *Candida antarctica*, CALB, 60 °C) (gifts from Novozymes, Denmark); protease N (EC 3.4.24.28) (*Bacillus subtilis*, 55 °C), lipase PS IM (*Burkholderia cepacia*, 60 °C), lipase PS SD (*Burkholderia cepacia*, 55 °C), lipase AS (*Aspergillus niger*, 45 °C), lipase AK 20 (*Pseudomonas fluorescens*, 60 °C) (gifts from Amano, Japan); protease P5380 (EC 3.4.21.14) (*Bacillus licheniformis*, 60 °C) (Sigma–Aldrich).

Esterification protocol: *t*-But was added to 10% (w/v) of molecular sieve 3 Å, L-Rha (0.10 M) and C14Ac (0.25 M). The mixture was magnetically stirred and heated (to the temperature specified above for each enzyme). After 30 min, the reaction was started by adding 1% (w/v) of enzyme. The reaction was carried out for 90 h. Aliquots were withdrawn over time (24, 48 and 90 h), diluted 5 times in ACN/MeOH (1:1, v/v), centrifuged (5 min at 13,000 rpm) and analyzed by high-performance liquid chromatography coupled to an evaporative light scattering detector (HPLC–ELSD) (*cf.* Section 2.3.1).

2.2.2. Introduction of a primary alcohol onto rhamnose by glycosylation

2.2.2.1. Enzyme selection. Almond β-glucosidase (EC 3.2.1.21): ≥2 units/mg solid (one unit liberates 1 μmol glucose from salicin per minute at pH 5 at 35 °C), Ref. G0395 (Sigma–Aldrich).

Naringinase from *Penicillium decumbens*: α-L-rhamnosidase activity (EC 3.2.1.40) ≥ 300 units/g solid (one unit liberates 1 μmol of reducing sugar from naringin per minute at pH 4 at 40 °C), secondary β-glucosidase (EC 3.2.1.21) activity ≥ 10 units/g solid, Ref. N1385 (Sigma–Aldrich).

Conditions tested with almond β-glucosidase

- 1st set of reaction conditions: 3% (w/w) of Rha monohydrate, 87% (w/w) of an alcohol and 10% (w/w) of distilled water were mixed and stirred magnetically at 50 °C in a water bath for 30 min. 0.5% (w/w) of enzyme were then added.

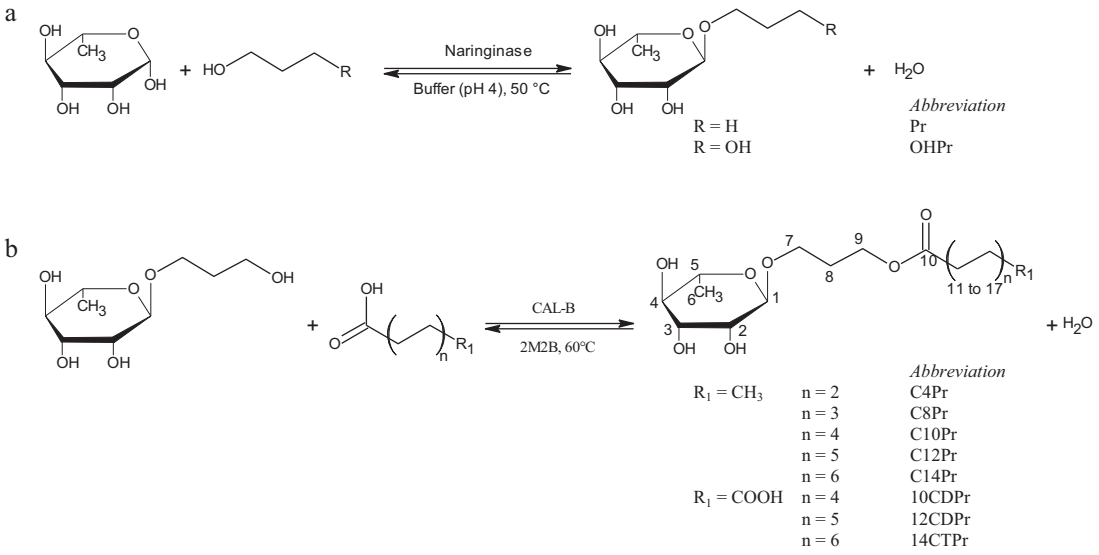

Fig. 2. Synthesis route developed to obtain new rhamnolipids by two successive enzymatic steps. (a) Step 1: introduction of a primary hydroxyl function by rhamnosylation of 1,3-propanediol catalyzed by a glycosidase (naringinase). (b) Step 2: esterification by fatty acids catalyzed by a lipase (CALB). The abbreviation used for each derivatives are defined and the numbering used for the NMR attribution are indicated on the developed formula of the OHPr esters.

- 2nd set of reaction conditions: the alcohol was first equilibrated for 16 h at 40 °C with a phosphate buffer 90 mM at pH 5.7 (15 g alcohol for 5 g buffer). The alcohol was then added to Rha to obtain a concentration of sugar of 0.10 M (or 0.20 M), then 0.5% (w/v) (or 2%) enzyme were added.

Conditions tested with naringinase

- 3% (w/w) of Rha, 10% (w/w) of alcohol and 87% (w/w) of buffer at 0.05 M and pH 4 (citrate or phosphate or formate) were mixed and stirred magnetically at 50 °C for 30 min. 0.5, 1.4 or 2% (w/w) of naringinase were then added.

After addition of the enzyme, the reaction media were heated at 50 °C with a water bath and stirred magnetically. To check that no reaction occurred in the absence of biocatalyst, blanks were performed for each reaction condition tested. Both 1-propanol and 1,3-propanediol were tested as alcohol for the acetalization of Rha with each enzyme. Each reaction was repeated at least twice. The reactions were carried out for 7 days. Aliquots were withdrawn over time, diluted 5 times in DMSO, centrifuged (5 min at 13,000 rpm) and analyzed by HPLC–ELSD (*cf.* Section 2.3.2). For the synthesis with almond β-glucosidase, aliquots were also withdrawn over time and filtered on a 0.45 μm PTFE membrane without dilution before injection.

2.2.2.2. Production of propyl-α-rhamnoside (Pr) and hydroxypropyl-α-rhamnoside (OHPr). Naringinase was selected to rhamnosylate 1-propanol and 1,3-propanediol (Fig. 2a). The conditions used are those reported for the selection of the enzyme. Although, the three buffers tested gave good results, the formate buffer (0.05 M pH 4) was chosen as its constituents were separated more easily from the products of interest by preparative HPLC (citric acid for example contaminated the first fractions of OHPr collected). A concentration of 1.4% (w/w) of enzyme was used and the time allowed for the synthesis was 24 h.

2.2.3. Esterification of OHPr catalyzed by CALB

The esterification reaction is shown in Fig. 2b. A stock solution of the purified OHPar (see Section 2.4.1 for purification procedure) was prepared in milliQ water. A precise volume (and thus mass of rhamnoside) was introduced in a screw-capped tube and lyophilized. This procedure was developed because the rhamnoside was viscous (difficult to transfer and weigh) and poorly soluble in the solvent used for the esterification. 2M2B was added to the dried compound (apparent concentration of 0.050 M). Fatty acid (0.30 M) and molecular sieve 3 Å (50 g/l) were introduced in the tube and the mixture was magnetically stirred and heated at 60 °C in a water bath. The amount of sieve used was more than 50 times the amount necessary to absorb the water produced by the reaction if its yield was 100%. After 16 h, the reaction was started by adding 0.75 g/l of CALB (blanks without enzyme were also done). The reaction was carried out for 48 h. Aliquots were withdrawn over time, diluted in ACN/MeOH (1:1, v/v), centrifuged (5 min at 13,000 rpm) and analyzed by HPLC–ELSD (*cf.* Section 2.3.3). Each reaction was repeated at least three times.

For the competition assays (with a mixture of two fatty acids), the global acid concentration was initially fixed at 0.30 M and each acid was introduced at a concentration of 0.15 M.

2.3. High performance liquid chromatography

Solvents: A (milliQ water with 0.1%, v/v, of formic acid), B (ACN with 0.1%, v/v, of formic acid).

Columns: no. 1 Zorbax Eclipse XDB C18 (150 mm × 4.6 mm, 5 μm, 0.8 ml/min) (Agilent); no. 2 Zorbax Eclipse XDB C8 (150 mm × 4.6 mm, 3.5 μm, 0.8 ml/min) (Agilent); no. 3 Halo C18 (75 mm × 4.6 mm, 2.6 μm, 1.5 ml/min) (Advanced Materials Technology). The columns were thermostated at 30 °C.

HPLC–ELSD: The analyses were performed on an Agilent Technologies 1200 series HPLC coupled to an evaporative light scattering detector (ELSD). The ELSD parameters were: 40 °C and 3.5 bar N_2.

HPLC–RI (HPLC coupled to a refractive index detector): The analyses were performed on an Agilent Technologies 1260 series HPLC. The RI was thermostated at 30 °C.

2.3.1. Enzymes screened for the esterification of rhamnose: analyses of the reaction media

HPLC–ELSD was used with column no. 1. The method started at 70% of B (30% of A). The percentage of B was then increased linearly to 100% in 10 min. The column was cleaned with 100% of B for 3 min.

2.3.2. Introduction of a primary alcohol function by rhamnosylation of 1,3-propanediol: Pr and OHPr analyses

HPLC–ELSD was used with column no. 1. The method started at 0% of B. Its percentage was then increased linearly to 30% in 10 min. The column was then cleaned with 100% of B for 5 min.

HPLC–RI was used with column no. 3: isocratic elution with 100% of A (method used to check the purity of OHPar, the propanediol being undetected by ELSD).

2.3.3. Esterification of OHPr catalyzed by CALB: analyses of the reaction media

HPLC–ELSD was used with column no. 1 for C4Pr and column no. 2 for all other ester derivatives of OHPr: isocratic elution for 6 min followed by 3 min cleaning with 100% B. The percentage of B for the isocratic elution varied from 25% to 80% depending on the acid chain length and of the number of acid functions of the reagent.

Standards for external calibration consisted of the purified derivatives (see below). The HPLC–ELSD quantification allowed determination of the reaction's 24 h yield (η, in %, equilibrium was reached for all the syntheses) and v_0 ($mM_{ester}\ l^{-1}\ h^{-1}\ g_{Enz}{}^{-1}$). v_0 corresponds to the slope of the graph representing the ester concentration as a function of reaction time within the first five hours (or less if non linear).

HPLC–RI was used in addition to HPLC–ELSD for the determination of the purity of the fractions collected during the flash chromatography for the esters obtained from fatty acids undetected by ELSD (C4Pr, C8Pr and C10Pr).

2.4. Purification of the products

2.4.1. Purification of Pr and OHPr by preparative HPLC

After synthesis, the medium was centrifuged and stored at 4 °C until purification.

The rhamnosides were purified on a Waters Atlantis dC18 OBD column (100 mm × 30 mm, 5 μm) on a Waters preparative system Prep 4000. Pure milliQ

H_2O and ACN were used as mobile phase. Before injection, the media were filtered through a 0.45 μm PTFE membrane.

Purification of Pr: For the first 5 min, pure milliQ H_2O was used. The percentage of ACN was then increased linearly to 45% in 20 min. The column was cleaned for 4 min with 100% of ACN. The flow was 20 ml/min.

Purification of OHPr: For the first 7 min, pure milliQ H_2O was used then the percentage of ACN was increased to 100% in 2 min. The column was cleaned for 1 min with 100% of ACN. The flow was 40 ml/min. The global yield after purification was 25%.

The purity of the collected fractions was checked by HPLC–ELSD and HPLC–RI.

The pure fractions were then pooled and dried with a speed vac apparatus (10 mbar, 40 °C).

2.4.2. Purification of the OHPr esters by flash chromatography

Until purification, the reaction media were stored at 4 °C. A liquid/liquid extraction was done prior to the flash chromatography on silica gel (60 Å/40–63 μm). One volume of milliQ H_2O and one volume of CH_2Cl_2 were added to the supernatant. The organic phase was dried under vacuum in a rotavapory evaporator and the residue was dissolved in the flash chromatography eluent consisting of a mixture of $CHCl_3$ and CH_3OH, their proportions depending on the length of the fatty chain of the derivative. The fractions which were shown to be pure by HPLC–ELSD and HPLC–RI were pooled. The solvent was eliminated in a rotavapory evaporator. The compound was then dry–frozen.

2.5. Chemical characterization of the purified products

The MS spectra of the purified products were acquired by direct infusion (3 μl/min) into a HCT mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI). The samples were diluted in MeOH with 0.1% formic acid in order to obtain a total concentration of 5 μg/ml. The ESI parameters were 10 psi (N_2), 4 l/min (N_2) and 300 °C. Data are reported as follows: MS mode (observed *m/z* for the ion, [attribution]).

The nuclear magnetic resonance (NMR) spectra (^1H, ^{13}C, COSY, HSQC, HMBC) of the purified products were recorded at 600 MHz (^1H) and 125 MHz (^{13}C) with a Varian instrument. Data are reported as follows: chemical shift in ppm (multiplicity (bs: broad singlet, dd: double doublet, t: triplet, quint: quintuplet, m: multiplet), number of H, coupling constants in Hertz, attribution).

The HPLC–ELSD (HPLC–RI when pertinent) purity of all purified products were superior to 99%.

2.5.1. Chemical characterization of Pr and OHPr

Pr derivative: ESI-MS: negative mode (*m/z* 251, $[M+HCOOH-H]^-$) and positive mode (*m/z* 229, $[M+Na]^+$ and *m/z* 435 $[2M+Na]^+$).

^1H NMR (DMSO-d6, 600 MHz): 0.83 (t, 3H, 7.8 Hz, H-9); 1.10 (d, 3H, 6 Hz, H-6); 1.48 (sextuplet, 2H, 7.2 Hz, H-8); 3.14 (t, 1H, 9.6 Hz, H-4); 3.25 (m, 1H, H-7A); 3.35 (m, 1H, H-5); 3.38 (m, 1H, H-3); 3.45 (m, 1H, H-7B); 3.55 (bs, 1H, H-2); 4.49 (bs, 1H, H-1).

^{13}C NMR (DMSO-d6, 150 MHz): 11.04 (C-9); 18.32 (C-6); 22.79 (C-8); 68.43 (C-7); 68.78 (C-5); 71.02 (C-2); 71.21 (C-3); 72.43 (C-4); 100.34 (C-1).

OHPr: ESI-MS: no spectra obtained under the analytical conditions used probably due to inadequate ionization conditions.

^1H NMR (DMSO-d6, 600 MHz): 1.10 (d, 3H, 6 Hz, H-6); 1.61 (quint, 2H, 6.6 Hz, H-8); 3.13 (t, 1H, 9 Hz, H-4); 3.33 (m, 1H, H-7A); 3.34 (m, 1H, H-5); 3.36 (m, 1H, H-3); 3.41 (m, 2H, H-9); 3.54 (bs, 1H, H-2); 3.57 (m, 1H, H-7B); 4.48 (s, 1H, H-1).

^{13}C NMR (DMSO-d6, 150 MHz): 18.34 (C-6); 32.99 (C-8); 58.18 (C-9); 63.99 (C-7); 68.75 (C-5); 70.99 (C-2); 71.19 (C-3); 72.44 (C-4); 100.38 (C-1).

2.5.2. Chemical characterization of the OHPr esters

C14Pr: ESI-MS: negative mode (*m/z* 477, $[M+HCOOH-H]^-$) and positive mode (*m/z* 455, $[M+Na]^+$).

^1H NMR (CD_3OD, 600 mHz): 4.64 (bs, 1H, H-1), 3.77 (bs, 1H, H-2), 3.61 (dd, 1H, 3.0 et 9.6 Hz, H-3), 3.35 (t, 1H, 9.0 Hz, H-4), 3.54 (m, 1H, H-5), 1.24 (d, 3H, 6.6 Hz, H-6), 3.45 (m, 1H, H-7A), 3.75 (m, 1H, H-7B), 1.90 (m, 2H, H-8), 4.15 (m, 2H, H-9), 2.30 (t, 2H, 7.2 Hz, H-11), 1.59 (m, 2H, H-12), 1.28 (m, 20H, H-13, H-22), 0.88 (t, 3H, 7.2 Hz, H-23).

^{13}C NMR (CD_3OD, 150 MHz): 100.30 (C-1), 70.08 (C-2), 71.01 (C-3), 72.50 (C-4), 68.43 (C-5), 16.64 (C-6), 63.41 (C-7), 28.50 (C-8), 61.02 (C-9), 174.08 (C-10), 33.65 (C-11), 24.65 (C-12), 22.29–31.63 (C-13, C-22), 13.01 (C-23).

14CTPr: ^1H NMR (CD_3OD, 600 MHz): 4.64 (bs, 1H, H-1), 3.77 (m, 1H, H-2), 3.61 (dd, 1H, 3.0 et 9.6 Hz, H-3), 3.35 (t, 1H, 9.0 Hz, H-4), 3.54 (m, 1H, H-5), 1.24 (d, 3H, 6.0 Hz, H-6), 3.45 (m, 1H, H-7A), 3.75 (m, 1H, H-7B), 1.89 (m, 2H, H-8), 4.15 (m, 2H, H-9), 2.30 (t, 2H, 7.8 Hz, H-11), 1.58 (m, 2H, H-12), 1.29 (m, 16H, H-13 to H-20), 1.58 (m, 2H, H-21), 2.25 (t, 2H, 7.8 Hz, H-22).

^{13}C NMR (CD_3OD, 150 MHz): 102.95 (C-1), 73.50 (C-2), 73.67 (C-3) 75.17 (C-4), 71.09 (C-5), 19.28 (C-6), 66.08 (C-7), 31.16 (C-8), 63.69 (C-9), 176.77 (C-10), 36.31 (C-11), 27.36 (C-12), 31.42–31.88 (C-13 to C-20), 27.31 (C-21), 36.31 (C-22), 179.07 (C-23).

2.6. Study of the surface active properties

2.6.1. Determination of critical aggregation concentration (CAC) and surface tension at that concentration (γ_{CAC})

A Wilhelmy plate (platinum Plate 21 mm × 10 mm × 0.5 mm) tensiometer (Tensimat N3, Prolabo) was used to determine the surface tension (γ) at 20 °C of solutions of C8Pr, C14Pr and 14CTPr. The γ was followed during time. The γ at equilibrium (γ_{eq}) were determined by linear regression of the curves $\gamma = f(t^{-1/2})$, only the data from the meso-equilibrium region of the curve were used for the extrapolation $t^{-1/2} \rightarrow 0$ (t tends towards infinity).

To dissolve the pure Rha derivatives in the buffer, ultrasound was applied for 5 min at room temperature, and then the solutions were heated at 40 °C and magnetically stirred for 30 min. Once the solutions returned to room temperature, they were filtered on a 1 μm nylon membrane. Dilutions were prepared from at least 2 stock solutions. The solutions were analyzed by HPLC–ELSD. Calibration curves done with solutions of the purified products in ACN/MeOH (1:1, v/v) allowed quantification. Each injection was repeated three times.

Aqueous solutions of the derivatives were studied at two pHs: pH 5 with an acetate buffer 25 mM and pH 3 with milliQ H_2O adjusted at the desired pH by concentrated HCl diluted 10 times.

2.6.2. Study of the Langmuir monolayers

The Langmuir film balance (KSV instrument, Helsinki, Finland) used consisted of a rectangular Teflon trough (364 mm × 75 mm × 5 mm) with a central well (37 mm × 37 mm × 70 mm), two mobile barriers, a Wilhelmy plate (19.62 mm × 10 mm), a temperature probe and a thermostatization system linked to a water bath Julabo F12-MV (Julabo, Labortechnik GmbH, Seelbach, Germany). Before each compression isotherm, the trough was filled with the subphase (acetate buffer 25 mM at pH 5 or milliQ H_2O adjusted at pH 3 with concentrated HCl diluted 10 times) and after compression, the surface was cleaned by suction. The cleanliness of the system was checked by recording a compression isotherm of the pure subphase. If the surface pressure varied less than 0.5 mN/m for the entire compression, the system was considered clean. The temperature of the subphase was stabilized at 25 °C. The pure rhamnose derivatives were dissolved in a mixture of CH_2Cl_2/CH_3OH (9:1, v/v) at a concentration of 2 mM. A precise volume (varying between 10 and 100 μl) of the sample was deposited drop by drop at the surface of the aqueous phase with a Hamilton syringe (Bonaduz AG, Switzerland). 15 min was allowed for solvent evaporation and dispersion of the molecules at the surface. Then, the film was compressed with the mobile barriers at a speed of 10 mm/min. The surface pressure was recorded during the compression. Each isotherm was repeated at least three times. After each analysis, the trough, the barriers, the temperature probe and the Wilhelmy plate were cleaned with pure isopropanol and rinsed thoroughly with milliQ H_2O.

The compression modulus was calculated from the $\pi = f$ (molecular area) isotherms via the equation: $C s^{-1} = -A_\pi(d\pi/dA)$ with A_π the area occupied by a molecule at the considered π [24].

3. Synthesis: results and discussion

3.1. Enzymes screened for the esterification of rhamnose and choice of the synthesis route

Under the experimental conditions used, among the 6 lipases and 2 proteases tested, only CALB led to the formation of new products detected by HPLC–ELSD. The other enzymes did not seem to recognize Rha as a substrate or were deactivated under the conditions used. To confirm either hypothesis, the reaction conditions must be varied. For example, different solvents, acyl donors and concentrations of enzyme and reagent need to be tested. With CALB, 4 new products with very close retention times were detected by HPLC–ELSD. LC–MS analysis confirmed that they were 4 regioisomers of rhamnose mono-esters (detection of the $[M+Na]^+$ and $[M-H_2O+H]^+$ ions). The synthesis was not regioselective although CALB has often been shown to be highly regioselective for the esterification of sugars [26–28]. This is mostly the case when a unique primary alcohol function is present. Rhamnose has only 4 secondary hydroxyl functions.

The absence of a marked selectivity towards an hydroxyl of rhamnose can be surprising view the results obtained by Duan et al. [29] and Mellou et al. [30] when studying the esterification catalyzed by CALB of rutin, a flavonoid where the aglycone part is linked to a disaccharide (α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranose). Indeed, its acylation was selective for the position 4 of the rhamnose moiety. This selectivity was explained by a

molecular modeling study [31]. It established that when rutin docks itself into CALB, the aglycon part binds itself at the entrance of the binding site and the sugar moiety points towards the bottom of the pocket and only the hydroxyl at position 4 of rhamnose is close to the catalytic residues of the lipase. The difference in selectivity observed in our study may be due to the absence of the aglycon part, which changes the way the rhamnose interacts with the binding site.

For further syntheses, CALB was selected. To obtain a unique Rha derivative for physico-chemical and biological studies, a two-step synthesis route was developed (Fig. 2). 1,3-Propanediol was first rhamnosylated to introduce a primary alcohol function onto Rha and then the introduced alcohol was selectively acylated with CALB.

3.2. Introduction of a primary alcohol function by rhamnosylation of 1,3-propanediol

To catalyze the acetalization of Rha with 1,3-propanediol or 1-propanol, two glycosidases, almond β-glucosidase and naringinase were tested. With β-glucosidase, no new product was detected by HPLC–ELSD with either alcohol for reaction times up to seven days. The two sets of reaction conditions tested were chosen on the basis of previous studies having led to good yields for the almond β-glucosidase catalyzed synthesis of β-glucosides by reverse hydrolysis ([32] for the 1st set and [33] for the 2nd set of conditions). It thus seems that almond β-glucosidase, one of the most studied glycosidase for glycosides synthesis and known to recognize β-glucose, β-galactose and β-fucose [34], does not accept L-Rha as substrate.

Naringinase, the second glycosidase tested, is an enzyme complex whose main activity is an α-rhamnosidase (Rase). The synthesis conditions used were based on those developed by Martearena et al. [35] for the production of alkyl-α-rhamnosides (-methyl, -ethyl, -propyl and -isopropyl) from the corresponding alcohols and Rha or naringin. Under these conditions, a new product was detected by HPLC–ELSD, it was named Pr with 1-propanol and OHPr with 1,3-propanediol.

The identities of Pr and OHPr were confirmed by MS and NMR (*cf.* Section 2). OHPr exhibits several characteristic signals in NMR such as a doublet ($J = 6.0$ Hz) at δ 1.10 typical of methyl-6, a singlet at δ 4.48 corresponding to the anomeric proton and 2 triplets at δ 3.33/3.45 and δ 3.41 assigned to CH_2-7 and CH_2-9, respectively. Moreover, irradiation of H-1 (δ 4.48) in a ^{1}D NMR NOE experiment showed a clear enhancement of H-2 (δ 3.54) and CH_2-7 (δ 3.33/.357), demonstrating that those protons were all located on the same face of the molecule.

The present study shows that the conditions developed by Martearena et al. (2003) [35] may be used with a diol. However, the citrate buffer was replaced by a formate buffer to facilitate the OHPr purification by preparative HPLC.

3.3. Esterification of OHPr with tetradecanoic acid

HPLC–ELSD analysis showed that CALB catalyze the esterification of the purified OHPr with C14Ac. A unique new product (named C14Pr), not synthesized in the absence of enzyme, was detected. MS and NMR spectroscopy confirmed that it resulted from the acylation of the primary hydroxyl function introduced onto Rha by glycosylation of 1,3-propanediol. The structure of C14Pr was fully proved by ^{1}D and ^{2}D NMR. Most noteworthy were the HMBC correlations observed between H-1 and C-7, and between CH_2-9 and C-10. Esterification of OHPr was highly regioselective, which is not the case for native Rha. This confirms the preference of CALB for primary alcohols rather than secondary ones [26–28].

The esterification conditions were chosen on the basis of our previous study which aimed at optimizing the v_0 and η of mannosyl myristate synthesis by (trans)esterification catalyzed by CALB [36]. The esterification of OHPr with C14Ac confirmed the positive effect of the presence of molecular sieve, which has already been highlighted in numerous studies [26,37,38]. As for mannosyl myristate synthesis by esterification [36], the use of 50 g/l sieve did not significantly influence the v_0 but resulted in an increase of the 24 h η of 35% compared to the reaction carried out without sieve. Water is a by-product of the esterification and can also be initially present in small amounts in the reactants, the enzyme and the solvent. Water affects the equilibrium of the reaction and its elimination favors synthesis rather than hydrolysis. Initially, the amount of water produced (that is trapped by the molecular sieve) is too small to have an influence on v_0 but as water affects the equilibrium; the 24 h η is influenced by the addition of sieve. Blanks with no lipase confirmed that the sieve did not catalyze the esterification of the rhamnoside. Furthermore, the addition of sieve did not lead to a qualitative change of the HPLC–ELSD chromatogram of the reaction media whatever the time of synthesis and no higher esters (di- or tri- for example) were detected.

As in our previous study, the molar ratio acid/alcohol was 6/1 [36]. Such excess favors the lipase catalyzed esterification of saccharides [37,38].

3.4. Esterification of OHPr with tetradecanedioic acid

Esterification of OHPr with a diacid should lead to a derivative with a free terminal carboxylic acid function which is the case for most of the natural rhamnolipids produced by *P. aeruginosa* (Fig. 1). Furthermore, this new function may be used for additional modifications.

Tetradecanedioic acid (C14diAc) was selected to esterify OHPr under the same conditions as used with C14Ac. The HPLC–ELSD chromatogram showed that a single new product was formed. The NMR analysis confirmed that it corresponded to the monoester with a free carboxylic function, named 14CTPr. No bolaform with two Rha was detected.

Lipase catalyzed acylations of sugars or their derivatives with free or activated dicarboxylic acids have not been extensively studied in the literature. The esterification of butyl α-D-glucopyranoside with diacids has been studied in the presence of the lipase from *Mucor miehei*, [39] or porcine pancreatic lipase [40]. CALB is more specific than the latter lipase which led to the formation of di- and tri-esters in addition to the monoester. The effect of the chain length in our study and in these two previous studies is discussed in Section 3.5.

Biocatalyzed transesterification of sugars with activated esters of dicarboxylic acids (usually divinyl and more rarely diethyl esters) has been more widely studied than their esterification with free diacids [41–44]. As in our case with free diacids, only the monosubstituted compound was generally obtained with proteases or lipases.

3.5. Esterification of OHPr with mono- and di- carboxylic acids: influence of the chain length and of the number of carboxylic acid functions on v_0 and 24 h η

For the monocarboxylic acids (Fig. 3a), v_0 diminished with increasing chain length of the fatty acid from 8 to 12 carbon atoms and then increased for 14 carbon atoms. There exists a dual optima (C8 and C14) in the chain length range studied, v_0 being clearly maximal for C8Pr. The 24 h η (Fig. 3b) were very close for all chain lengths but tending to decrease slightly with increasing chain length.

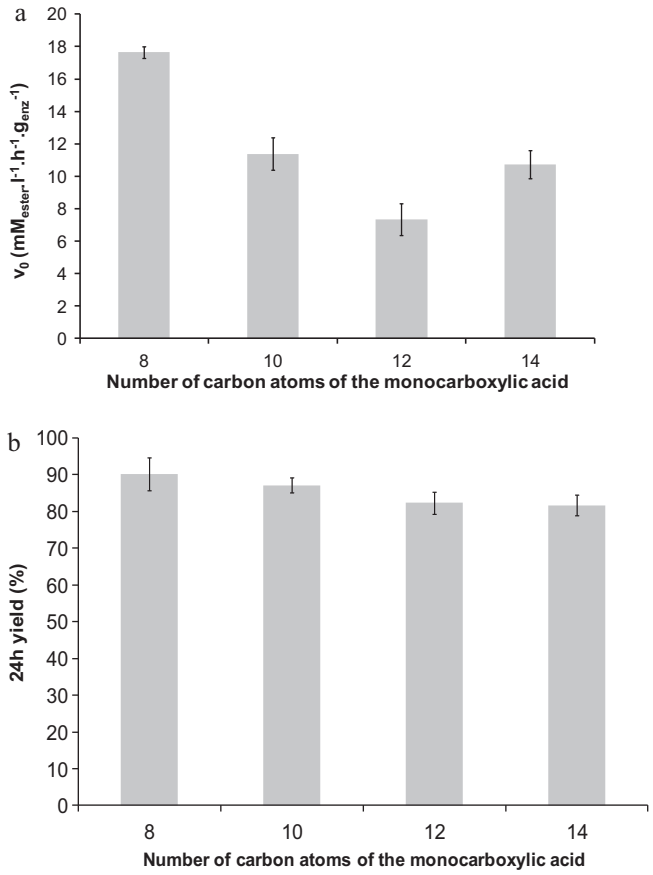

Fig. 3. Influence of the chain length of the monocarboxylic fatty acid on the v_0 (a) and 24 h η (b) of the OHPr esterification catalyzed by Novozym 435. 0.05 M rhamnoside, 0.30 M fatty acid, 50 g/l molecular sieve 3 Å, 0.75 g/l enzyme, 60 °C in 2M2B.

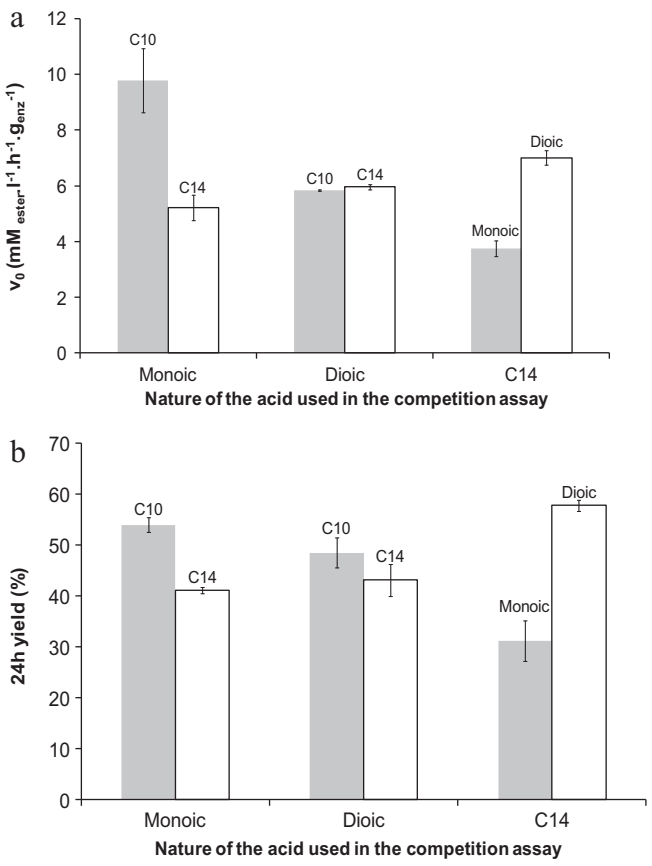

Fig. 4. Competition assays for studying the selectivity as function of chain length (C10 and C14) for the mono- and dioic acids or as a function of the number of carboxylic acid functions (mono- or di-) for the C14 acids: (a) v_0 and (b) 24 h η. 0.05 M rhamnoside, 0.15 M fatty acid n°1, 0.15 M fatty acid n°2, 50 g/l molecular sieve 3 Å, 0.75 g/l enzyme, 60 °C in 2M2B.

OHPr was also esterified in the presence of an equimolar mixture of C10 (C10Ac) and C14 monoic acids. This competition assay confirmed the tendency observed when using the pure acids. The v_0 of the shortest acid (C10Ac) is approximately twice the value of the longest (C14Ac) when the two acyl donors are used simultaneously (Fig. 4a). The v_0 of C10Ac in the competition assay and alone are similar whilst the v_0 of C14Ac used in a mixture with C10Ac was half of that when used alone. The global 24 h η in esters in the competition was similar to that of the pure acids, the 24 h η of each ester decreased and that of C10 was approximately 30% higher than that of C14.

The results regarding the chain length selectivity of the esterification catalyzed by CALB of OHPr by monocarboxylic acids are consistent with other studies using the same lipase. The rate of glucose esterification by fatty acids of various chain lengths (C12, C14 and C16) increased with diminishing chain length [26]. For the esterification of propanol by a mixture of linear fatty acids of various chain lengths (from 4 to 16 carbon atoms) a dual optimum was observed for v_0 for chain lengths of 6 and 10 carbon atoms [45]. Several hypotheses were formulated to explain the existence of this dual optimum. Firstly, the acyl binding pocket could consist of subdomains that could optimally host distinct chain lengths. Secondly, as the selectivity depends on both the acylation and deacylation steps, a different chain length could be optimal for each step. The optimum for the deacylation step could also be linked to the nature of the alcohol. Finally, there could exist several binding sites for the acyl donor, some being scissile acid binding sites and others not. The latter would not lead to the formation of the product but may play a role by increasing the concentration of certain substrates close to the active site. Pederson et al. [46] showed that the v_0 and η increased when the chain length diminished for the esterification of maltose with fatty acids (4–12 carbon atoms) in *tert*-butanol/pyridine 9:11 (v/v). The same tendency was shown by Ward et al. [47] for the esterification of 1,2-*O*-isopropylidene D-xylofuranose with fatty acids (12 to 18 carbon atoms). These observations are in accordance with the modelization studies of the fatty acid binding sites of six lipases, including CALB, undertaken by Pleiss et al. [48]. They concluded that CALB should have a high activity for short and medium chain length fatty acids and decreasing activity for longer ones.

However there are also examples in the literature where an opposite chain length selectivity was reported for CALB. A higher conversion of butanol was noticed with the longer fatty acids (4, 8, 10 and 12 carbon atoms) used pure or in a mixture [49]. For the esterification of ethyl D-glucopyranoside with acids having 8 to 18 carbon atoms, the lowest conversion was obtained for the shortest and the v_0 increased with the chain length [50]. Vaysse et al. [51] obtained a formation rate of methyl octanoate close to zero but it increased for the longer chain length acids (C10–C18), the optimum length being of 12 carbon atoms. It was also shown that CALB activity towards long and short chain fatty acids depends on the molar ratio sugar/fatty acid used. For long chains, the rate is higher with low excess of acyl donor; for short chains, it is the opposite [52]. This tends to demonstrate that the chain length specificity of a lipase depends on intrinsic factors (lipase origin, structure of the binding and active sites, free or immobilized form, nature of the immobilization matrix. . .) but also on numerous extrinsic factors such as the use of bi-phasic media [51], the presence of solvents [50] and

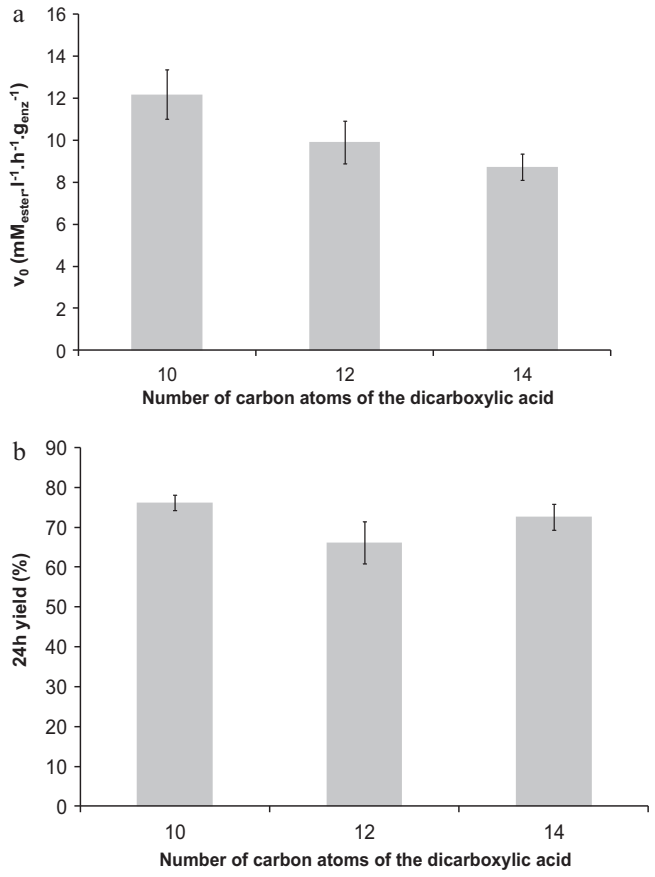

Fig. 5. Influence of the chain length of the dicarboxylic fatty acid on the v_0 (a) and 24 h η (b) of the OHPr esterification catalyzed by Novozym 435. 0.05 M rhamnoside, 0.30 M dicarboxylic fatty acid, 50 g/l molecular sieve 3 Å, 0.75 g/l enzyme, 60 °C in 2M2B.

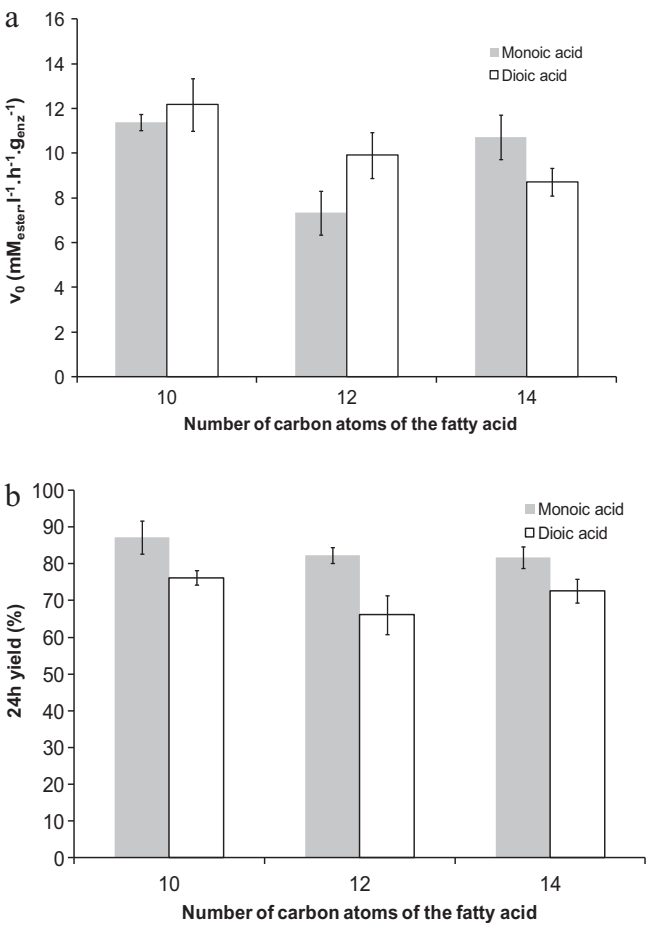

Fig. 6. Influence of the number of carboxylic acid function (mono- or di-) on the v_0 (a) and 24 h η (b) of the OHPr esterification catalyzed by Novozym 435 for fatty acids of increasing chain length. 0.05 M rhamnoside, 0.30 M carboxylic fatty acid, 50 g/l molecular sieve 3 Å, 0.75 g/l enzyme, 60 °C in 2M2B.

of co-substrates [53], the temperature [50,51], the substrates concentrations and ratios [52]. . .. These extrinsic factors may explain the contradictory results found in the literature.

Our results for the dicarboxylic acids (Fig. 5a) show a slight decrease of v_0 with increasing chain length. For the 24 h η (Fig. 5b), no significant effect of the number of carbons was observed. This is contrary to the results of Fabre et al. [39] and Boyat et al. [40] when studying the esterification of butyl α-D-glucopyranoside with diacids of increasing chain length. They observed higher yields for the longest acids and no reaction with the short ones (C4 and C6). However they did not use CALB but immobilized lipase of *Mucor miehei* (Lipozyme ®) and free porcine pancreatic lipase, respectively.

The competition assay (Fig. 4a and b) confirmed that the number of carbon atoms (10 or 14) of the diacid chain did not influence the v_0 nor the 24 h η of the esterification. Compared to the experiments with the pure diacids, the v_0 and 24 h η of each acid diminished in the competition assay but the global yield was higher.

Compared to the monocarboxylic acids where selectivity towards the shortest chain fatty acids was highlighted, no such preference was observed for the dioic acids. The presence of two carboxylic acid functions seems to mask the hydrophobicity dissimilarity caused by the difference in the number of carbon atoms of the fatty chain. However, two studies with CALB showed a decrease of reaction rate with increasing chain length for the transesterification with divinyl dicarboxylates (with chain length ranging from 4 to 10 carbon atoms) of nucleoside derivative (5-fluorouridine) [54], or of 1,2 diol drugs (mephenesin and chlorphenesin) [55]. The activation of the acid function may explain the differences observed with our study.

Fig. 6a and b compare the v_0 and 24 h η obtained for mono- and di- carboxylic acids having 10, 12 and 14 carbon atoms. The concentrations used were of 0.30 M for both mono- and dioic acids. In the latter case, twice the number of acid functions is present in the medium. The v_0 for the C10 acyl donors were similar. For the C12, the v_0 of the dioic acid was larger than that of the monoacid and for C14, it was the opposite. For all chain lengths, the 24 h η of the monocarboxylic acids were higher than those of the corresponding dioic acids. Fig. 4 shows the results obtained when both the mono- and di- carboxylic acids having a chain length of 14 carbon atoms were used in an equimolar mixture to esterify the rhamnoside. The v_0 and 24 h η were clearly higher for the dioic acid, which is the opposite of what was observed when each acid was used separately. The simultaneous presence of both types of acids seems to modulate the selectivity. They may have neighboring or shared binding sites [45].

4. Physico-chemical study: results and discussion

4.1. Influence of Rha derivatives on water's surface tension

The influence of the longest chain length derivatives of each type (C14Pr with a simple alkyl chain and 14CTPr with an alkyl chain with a terminal carboxylic acid function) on water's surface tension was studied. As 14CTPr is a weak acid, it was decided to

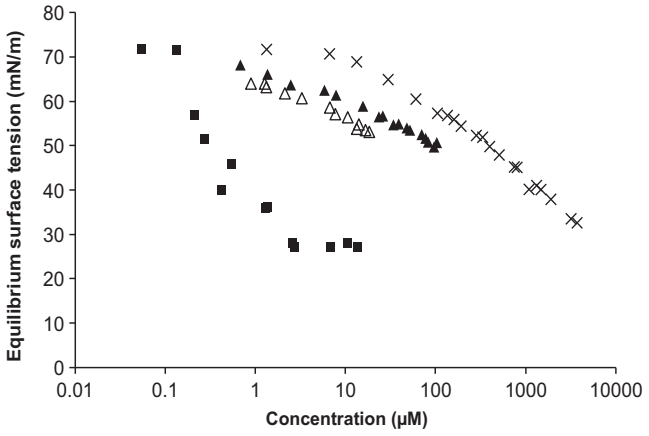

Fig. 7. Evolution of the equilibrium surface tension as a function of the concentration of the rhamnose derivatives in the acetate buffer 25 mM at pH 5: C14Pr (■), 14CTPr (▲) and compound C8Pr (x) or in milliQ water adjusted at pH 3 with HCl: compound 14CTPr (△). The equilibrium surface tensions were obtained by extrapolation to an infinite time by linear regression of the curves of the surface tension at meso-equilibrium as a function of the inverse of the square root of time ($t^{-1/2}$).

solubilize the molecules in aqueous buffers so that the pH of the solutions remained constant over the full range of concentrations considered. As one of the final objectives for synthesizing these new rhamnolipids is their potential use in cosmetic or dermatological formulations, both products were studied in an acetate buffer at pH 5 which is considered to correspond to the pH of the outer layers of the skin [56,57]. Rhamnolipids are particularly interesting for cosmetic and dermatological applications as they are effective surfactants and have good antimicrobial activities against fungi and bacteria for example. They have been used in insect repellents, acne pads, antidandruff products, deodorants, nail care products. . .[58–60].

Fig. 7 shows the influence of the Rha derivatives on the equilibrium surface tension (γ_{eq}) of their aqueous solutions. At pH 5, γ_{eq} decreased with the increase of C14Pr's concentration until reaching a minimum value. Thereafter, the γ_{eq} stabilized and further increase of the concentration had little effect on the γ_{eq}. The intersection between the tangents to the descending part of the curve and to its horizontal region gives C14Pr's γ_{CAC} and CAC. They are respectively of 27.6 ± 0.6 mN/m and of 1.70 ± 0.16 μM. Both values are extremely small. C14Pr is thus strongly surface active. The CAC of classical surfactants such as sodium dodecylsulfate (SDS), cetyltrimethylammonium bromide (CTAB), 3-[(3-cholamidopropyle)dimethylammonio]-1-propanesulfonate (CHAPS) and triton X-100 in milliQ water are respectively of 8190, 980, 6660 and 350 μM and their γ_{CAC} are of 38.5; 37.6; 45.2 and 31.0 mN/M [61]. It is difficult to compare the values obtained with C14Pr to those reported in the literature for natural rhamnolipids as they are often studied as mixtures of congeners. The proportions of the congeners significantly influence the physico-chemical properties of the mixtures. Furthermore, the experimental conditions vary. For example, some authors use pure water and other buffers to solubilize the rhamnolipids, some add NaCl. Generally in water or in NaCl (10 mM or 500 mM), irrespective of the proportions of the congeners and the producer microorganisms, the γ_{CAC} lie between 27 and 31 mN/m [17,62–65]. C14Pr's γ_{CAC} is in the same range. The values of the CAC differ more, depending on the proportion of the congeners and the solvent (pure water, buffers, or salt solutions). The CAC vary from 20 μM to 300 μM (17–18, 62–65]. C14Pr's CAC is significantly smaller. *P. aeruginosa* produces mixtures of mono- and di- rhamnolipids with fatty acids chain lengths varying mostly between C8 and C12, the most abundant congeners are the mono- and di- rhamnolipids with 2 chains of 10 carbon atoms (RhaC10C10 and RhaRhaC10C10 respectively). The CAC of pure RhaC10C10 and pure RhaRhaC10C10 were determined in pure water by Helvaci et al. and they were respectively of 100 and 150 μM [62]. They are at least 50 times higher than the CAC determined for C14Pr. Several reasons may explain this difference. Although there are two fatty acid chains on the natural rhamnolipids, they are shorter (10 carbon atoms) than the one of C14Pr (14 carbon atoms to which we can add the 3 carbon atoms of the propanediol spacer). Furthermore, they have a terminal carboxylic acid function which is probably ionized under the experimental conditions and the RhaRhaC10C10 possesses 2 polar Rha units whilst C14Pr has no terminal carboxylic acid function and has only one Rha unit. Helvaci et al. also studied the CAC of these rhamnolipids in 500 mM NaCl. In that case, their CAC was halved. This is probably due to the fact that the Na^+ cations can mask, at least partially, the negative charge of the terminal carboxylic acid. However, even under these conditions, the CAC are still much higher than that of C14Pr [62]. Several species of *Burkholderia* bacteria have been shown to produce rhamnolipids. In general, the chain lengths of the fatty acid parts of these rhamnolipids are longer than those produced by *P. aeruginosa* [16–18]. Costa et al., for example, have determined the CAC (in water) of a mixture of rhamnolipids produced by *B. glumae* consisting of 63% dirhamnolipids with 2 fatty acid chains of 14 carbon atoms (RhaRhaC14C14), 19% of RhaRhaC12C12 and 18% of RhaRhaC12C14. The CAC is 33 μM which is still 20 times greater than that determined for C14Pr. Once again, this may be explained by the presence of a terminal carboxylic acid function and of two Rha units on the natural rhamnolipids. Compared to the rhamnolipids produced by *P. aeruginosa*, the CAC of those obtained from *B. glumae* is significantly smaller. The addition of CH_2 groups in the fatty chains facilitates micelle formation.

14CTPr had much less influence on the γ than C14Pr. The lowest γ reached was approximately of 50 mN/m for concentrations around 100 μM which corresponded to the maximum concentration that could be reached (saturation of the solution). No break in the slope of the curve $\gamma = f$ (concentration) was observed. Under the experimental conditions chosen, no aggregation of 14CTPr occurred contrary to what was observed with C14Pr. The only structural difference between these two molecules is the presence of a carboxylic acid function at the end of the fatty chain of 14CTPr. This function thus has a very important impact on the surface properties of the Rha derivatives: 14CTPr has two polar heads (the rhamnose and the carboxyl) separated by a chain of 17 carbons including an ester link. The molecule does not seem to be able to organize itself so as to put these heads in contact with the aqueous phase whilst "protecting" its hydrophobic part from that environment. It is important to note that at pH 5 and taking into consideration the pKa of the C14diAc, approximately 60% of the carboxylic functions are ionized (COO^-) and 40% are not (COOH). There are thus two types of molecules which possibly have different behaviors. Ionization for example increases the polar character of the second polar head. The effect of 14CTPr on water's γ was also studied at pH 3, where around 98% of the acid functions should be protonated. Overall, it can be considered that there is a large majority of one type of molecule. Fig. 7 shows that the γ_{eq} reached are slightly lower than those reached at pH 5 for the same concentrations and that no break in the curve was observed. The decrease in the number of COO^- probably decreases the electrostatic repulsions thus giving rise to higher surface concentrations and thus lower γ. Saturation of the solution occurred at a lower concentration at pH 3 than at 5.

The impact of the shortest rhamnoside derivative (with no terminal carboxylic function), C8Pr, on the acetate buffer's γ was also studied. As expected, it led to a smaller decrease of the γ_{eq} than C14Pr. For a concentration of 4000 μM, the γ_{eq} was around 33 mN/m. The lower γ reached for the longer chain derivative may

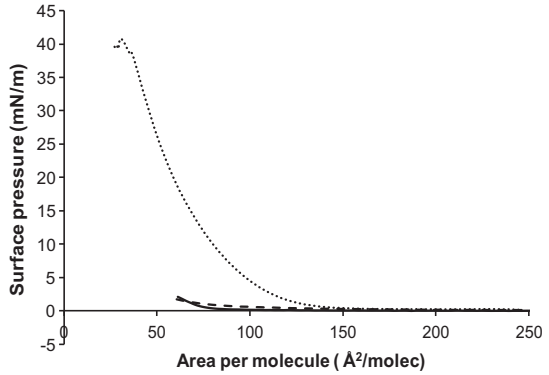

Fig. 8. Compression isotherms of the rhamnoside derivatives (compression speed 10 mm/min, 25 °C): (. . .) C14Pr and (—) 14CTPr on the acetate buffer 25 mM at pH 5, (- -) 14CTPr on milliQ water adjusted at pH 3 with HCl.

be due to an increase in the attractive hydrophobic interactions between the alkyl chains which allow a more compact organization of the molecules at the interface leading to an increase in their surface concentration which is directly linked to the γ. Similar tendencies have been reported in the literature for other surface active molecules [66–69].

4.2. Study of the Langmuir film monolayers

A representative compression isotherm obtained for C14Pr at the acetate buffer (25 mM pH 5)/air interface is shown in Fig. 8. Under the experimental conditions chosen, C14Pr is able to form an insoluble monolayer at the interface. The compression modulus ($C\,s^{-1}$) of the monolayer was calculated for the entire isotherm. Generally, it is considered that the monolayer is: in a gaseous state when $C\,s^{-1}$ is smaller than 12.5 mN/m, in a liquid expanded state when its value is between 12.5 and 100 mN/m, in a liquid condensed state for values in the 100–250 mN/m range and in a solid state when $C\,s^{-1}$ is superior to 250 mN/m. This parameter approaches zero at transition stages and at the collapse of the monolayer [25]. For C14Pr, $C\,s^{-1}$ indicates that the monolayer is in a gaseous state then in a liquid expanded state before collapsing. The minimum area occupied by the molecules in the gaseous state ($C\,s^{-1} < 12.5$ mN/m) is 118 ± 5 Å^2/mol and the maximum surface pressure (π) in that state is 2.2 ± 0.3 mN/m ($n = 4$). The monolayer collapses at 39 ± 2 mN/m and the minimum area occupied by a molecule of C14Pr at the interface is 33 ± 3 Å^2 ($n = 4$). Under the conditions used for the experiment, the C14Pr molecules do not manage to arrange themselves sufficiently close so as to form a monolayer in a more condensed state than the liquid expanded one. Contrary to C14Pr, 14CTPr does not seem to be able to form a stable monolayer at the acetate buffer (25 mM pH 5)/air interface. The $C\,s^{-1}$ is inferior to 12.5 mN/m during the entire compression and π remains very low. As mentioned above, at pH 5, more than half of the terminal carboxyl groups are ionized and this may favor their solubilization in the subphase during the course of the compression or render the organization of the molecules at the interface difficult due to intermolecular repulsion between COO^-. It may be impossible to put the two polar heads in contact with the aqueous phase and the repulsion between the molecules may be important. 14CTPr was also spread at the surface of milliQ water adjusted to pH 3, pH at which most of the molecules should be protonated. However, this did not favor the formation of a stable monolayer.

5. Conclusions and perspectives

The new synthesis strategy developed has allowed the obtention of novel rhamnolipids via a biocatalytic route in two steps. The first uses naringinase which has the advantage of being an industrial catalyst, inexpensive, easily available and resistant to industrial conditions. It is currently used in the food industry for the debittering of grapefruit juices. This enzyme was used to catalyze the rhamnosylation of 1,3-propanediol to introduce a primary hydroxyl function onto Rha. The synthesis strategy was also applied with success to naringine instead of pure L-Rha (data not shown). This flavonoid presents the advantage of being readily available and cheaper than the pure sugar as it can be easily extracted from numerous fruits. However, a mixture of hydroxypropyl glucoside and rhamnoside is then obtained which can be difficult to purify. A solution could be to use the mixture of the two for the second step catalyzed by the lipase. The mixture of surfactant obtained may have interesting synergistic activities. Furthermore in a context of green chemistry it is interesting to note that the reagent used to introduce the primary hydroxyl function onto Rha, 1,3-propanediol can be obtained by fermentation from glucose (starch sector) or from glycerol (vegetal oil sector). The first path has been developed by Genencor in collaboration with DuPont (a modified strain of *E. coli* was elaborated to convert glucose into propanediol) and the second one by Metabolic Explorer.

In the second step of the synthesis route, the lipase B from *Candida antarctica* (Novozym 435) was used to selectively esterify the primary hydroxyl function introduced onto Rha. Both mono- and di-carboxylic acids were used successfully. The esterification catalyzed by CALB with the dioic acid has rarely been studied in the literature which mainly focused on the use of activated divinylesters of these acids. The effect of the chain length of the fatty acid and its number of carboxylic acid functions was studied on the v_0 and 24 h η of the esterification catalyzed by CALB. For the monocarboxylic acids tested, the two parameters had a tendency to decrease with increasing chain length whilst for the dicarboxylic acids no clear influence of the chain length was noticed. The influence of the number of carboxylic acid functions seems to depend on the separate or simultaneous use of the different types of acids.

C14Pr shows good surface properties. Its CAC and γ_{CAC} are particularly low and it can form insoluble monolayers. The fundamental surface properties of shorter and longer chain derivatives will be the subject of a further study. Adding one terminal carboxyl function drastically decreases the surface properties as was observed with 14CTPr. It may be interesting in the future to study rhamnosides obtained with longer chain dicarboxylic acids. A minimum chain length may be required for the two polar heads (the Rha and the carboxyl group) to allow compact packing of the molecules.

As natural rhamnolipids have several interesting biological properties such as antimicrobial, antiphytoviral, zoosporicidal and plant defense elicitor activities [7,8,10] and as it is generally recognized that these activities must be linked to the interaction of these molecules with constituents of biological membranes [70], the study of the interaction of C14Pr with model membranes, Langmuir monolayers and liposomes of characteristic lipids, is currently underway using the Langmuir film balance and isothermal titration calorimetry.

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