Enzymatic Production and Physicochemical Characterization of Uncommon Wax Esters and Monoglycerides

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Wax esters from fatty alcohols and uncommon fatty acids were synthesized in yields up to 90% when commercially available microbial lipases from *Rhizomucor miehei* (Lipozyme™) and *Candida antarctica* (SP 435) were used with limited water content in nonpolar solvents under mild conditions. The corresponding fatty acids were prepared by chemical conversion of naturally occurring resources (agricultural surpluses). Also, when phenylboronic acid was added as solubilizing agent in a nonpolar solvent, the direct enzymatic monoacylation of glycerol with uncommon fatty acids was successful. The measurement of π/A-isotherms by means of a Langmuir film balance indicated medium film pressures, medium or large molecular areas, and interesting phase behavior. The monolayer of a wax ester at the air/water interface could be directly visualized by Brewster angle microscopy.

KEY WORDS: π/A-isotherms, Brewster angle microscopy, film pressure, glycerol, lipase, monoacylation, nonaqueous conditions, uncommon wax esters.

Wax esters based on common long-chain fatty acids and alcohols are applied in industry because of their wetting behavior at interfaces, especially in cosmetics, pharmaceuticals, dyes/lacquers and lubricants. Methods to produce wax esters include acidic catalysis, metal catalysis and the conversion of acylchlorides at high temperatures (100 to 300°C) and/or at high pressure (1,2). The resulting high production costs, as well as the low reaction rates and undesired side reactions, have now focused attention on biotechnological methods.

Suitable biocatalysts for direct esterification of fatty acids with fatty alcohols at low water content are microbial lipases. They are active under mild reaction conditions, e.g., normal pressure, temperatures below 100°C and physiological pH values. Long-chain wax esters, identical to naturally occurring products, were synthesized (3–6) with enzyme preparations from fungi, yeasts or bacteria.

On account of improved interfacial behavior of their monolayers on top of aqueous subphases, our team tries to develop wax esters with structures that include uncommon moieties. For example, uncommon (poly)hydroxy fatty acids and mono-, di- or triols from microbial origin or purchased commercially served as reactants (7–10). The measurement of film pressure by means of a Langmuir film balance indicated high stability of their monolayers.

Uncommon fatty acids and their methyl esters were tested for the lipase-catalyzed synthesis of wax esters and the monoacylation of glycerol. Physicochemical measurements were employed to study the monolayer stability on aqueous subphases.

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EXPERIMENTAL PROCEDURES

Materials. Rhizomucor miehei lipase (Lipozyme™, immobilized on Duolite) was purchased from Novo Industrie (Mainz, Germany)—activity: 23.2 batch interesterification units/g (μmol fatty acid/min·g); water content: 15.8%. Immobilized lipase SP 435 from *Candida antarctica* was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark).

Uncommon fatty acids or their methyl esters were obtained by different chemical modifications or naturally occurring fatty acids as follows: (*E*)-9(10)-(hydroxymethyl)-octadec-10(8)-enoic acid was synthesized by Dr. U. Biermann (11,12) (Fachbereich Chemie, Universität Oldenburg, Germany) and was a gift. 9(10)-Acetonyl-octadecanoic (methyl ester) and 9(10)-dimethoxy carbonylmethyl octadec-8(10)-enoic acid methyl ester were gifts from Dr. U. Linker (13) (Fachbereich Chemie). Tridecafluoroheptadecanoic acid was synthesized by tin(II) chloride/silver (I) acetate-initiated free radical addition of perfluoralkyl iodide to 10-undecenoic acid, followed by tributylstannane reduction of the addition product (14). Mixtures of 9,12- and 10,13-dihydroxy-octadecanoic methyl esters were kindly prepared by Dr. T. Lucas and Dr. R. Quermann (15) (Organisch-Chemisches Institut, Westfälische Wilhelms-Universität, Münster, Germany). The methyl esters of 9,10-epoxy- and 9-oxo-decanoic acids were obtained from Dr. M. Rüsch gen. Klaas, P. Bavaj and B. Wolff (H.P. Kaufmann-Institut, Münster, Germany), who produced them by ethylene-metathesis of oleic acid ester to 9-decenoic ester with the catalysis of B_2O_3-Re_2O_7/Al_2O_3-SiO_2 and $SnBu_4$ (16,17), followed by functionalization of the double bond (17,18).

Fatty alcohols and glycerol were from Fluka (Neu-Ulm, Germany) and Merck (Darmstadt, Germany).

Wax ester synthesis and detection. Bioconversions were achieved alternatively in the presence or in the absence of nonpolar solvents and at a molar ratio of fatty alcohol/fatty acid of 3:1. Erlenmeyer flasks (50 mL) served as reaction vessels and were shaken at 140 rpm. Other conditions, such as biocatalyst concentration and incubation temperature, are shown in the figure legends. The conversions were determined by quantitative thin-layer chromatography (TLC) after spraying with coloring agents. Stationary phases included (i) Silica gel 60_{F254} (No. 5554; Merck) and (ii) Silica gel RP-8 (No. 15424; Merck). Developing systems were (A) $CHCl_3/CH_3COCH_3$ = 96:4 (vol/vol); (B) $CHCl_3/CH_3OH/NH_3$ (25%) = 90:15:2 (vol/vol/vol); and (C) CH_3OH/H_2O = 85:15 (vol/vol). Coloring reagents were (I) H_2SO_4 (conc.)/CH_3OH = 1:1 (vol/vol) at 150°C; (II) Vanillin/H_2SO_4 (conc.) = 1:100 (wt/vol) at 150°C; and (III) o-Dianisidine (acetic acid solution) at 150°C. For quantitative measurements, color reactions were detected by light absorption at 500 nm with a TLC-scanner, Model CD 60, Desaga (Heidelberg, Germany).

Purification and identification of wax esters. The immobilized biocatalyst was separated by filtration, and the solvent was evaporated. For purification of the remaining crude product, the following methods were used alternatively: (a) Thick-layer chromatography (Silica gel plates SIL G-200 UV_{254}/F, 20 × 20 cm, 2 mm; Macherey and Nagel, Düren, Germany); developing system; $CHCl_3/CH_3COCH_3$ = 96:4 (vol/vol); detection, ultraviolet (254 nm) or H_2SO_4/CH_3OH = 1:1 (vol/vol); elution of the wax esters was performed by using CH_2Cl_2/CH_3OH in a ratio of 9:1 (vol/vol); (b) liquid chromatography (Lobar prepacked column, size C, 440-37, LiChroprep RP-8, 40–63 μm; No. 10629, Merck); developing system, CH_3OH/H_2O = 85:15 (vol/vol); detection, refractive index detector; (c) crystallization from CH_3OH or CH_3OH/H_2O mixtures. The molecular structures of purified wax esters were elucidated by means of ^1H and ^{13}C nuclear magnetic resonance, gas chromatography/mass spectroscopy and fast atomic bombardment measurements (Multzsch, R., W. Lokotsch, B. Steffen, S. Lang, V. Wray, L. Witte, H.M. Schiebel and F. Wagner, unpublished results).

Synthesis, detection, purification and identification of monoglycerides. The Lipozyme-catalyzed synthesis of monoglycerides was achieved as described (10). Parameters are given in the corresponding figure legend. TLC coupled with densitometer was needed for analysis; silica gel 60, $CHCl_3/CH_3OH/NH_3$ (25%) = 65:15:2 (vol/vol/vol) as developing system, vanillin/H_2SO_4 as coloring reagent. After separation of immobilized biocatalyst and cooling to 25°C, the nonconverted glycerol phenylboronic acid ester was separated by filtration, and the solvent was evaporated from the residual solution. The next steps included thick-layer chromatography (see method a), hydrolysis in acetone/water (2:3, vol/vol; with 30 min, reflux) and extraction with ligroin. For identification, the methods given above were used.

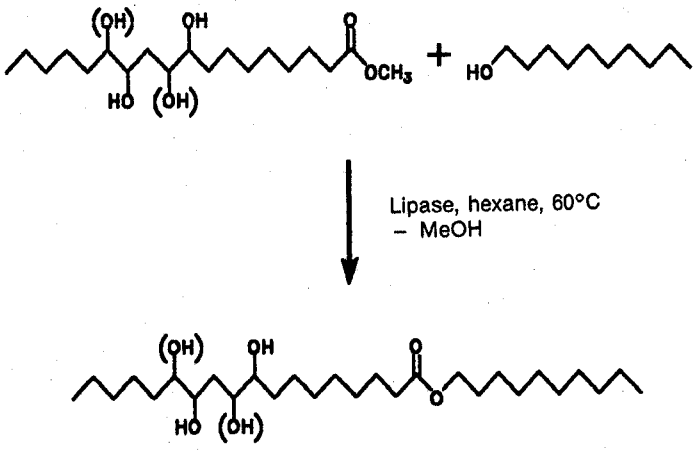
Determination of film (surface) pressure. The film (surface) pressure area (π/A) isotherms were measured with a Langmuir film balance (model FW2; MGW Lauda, Königshofen, Germany). A 25-μL solution of wax ester or monoglyceride in chloroform (1 mg/mL) was distributed on a neutral subphase (water, bidistilled), and the monolayer was compressed during a period of 30 min at 25°C.

Microscopy of monolayers. Special equipment, described by Hönig and Möbius (19), was used after distribution of a wax ester sample on an aqueous subphase (Langmuir film balance); the monolayer was irradiated with laser-light at the Brewster angle at 53° for air/water interfaces. The light reflected by the surface molecules could be observed by a microscope connected to a computer-controlled video camera.

RESULTS AND DISCUSSION

Uncommon wax esters and monoglycerides were produced by the enzymatic combination of the following substrates: (i) Functionalized long-chain fatty acids and fatty alcohols, both mono- and diols [reaction Scheme 1 is for the lipase-catalyzed acylation of decanol with 9(10), 12(13)- dihydroxyoctadecanoic acid methyl ester in *n*- hexane]; (ii) functionalized medium-chain fatty acids/fatty alcohols; and (iii) functionalized long-chain fatty acids/glycerol.

From subsequent physicochemical testing of these biotransformation products in a Langmuir film balance,



SCHEME 1

we hoped to find monolayers of good stability at areas larger than 18.5 Å², which is the smallest possible area of an *n*-alkyl chain. These properties could be advantageous for technical applications, e.g., in cosmetics.

Wax esters from functionalized and branched fatty acids. (*E*)-9(10)-(Hydroxymethyl)octadec-10(8)-enoic acid and 9(10)-acetonyl octadecanoic acid (methyl ester) were esterified enzymatically with decanol, leading to yields of more than 60% based on the less concentrated substrates (Fig. 1). Figure 1 indicates that at the beginning of the lipase-catalyzed reaction, free fatty acids were more suitable for such biotransformations than methyl esters. Method a (see Experimental Procedures section) was used for purification of the crude product.

The physicochemical behavior of such uncommon wax esters on aqueous subphases in a Langmuir film balance is presented in Figure 2; molecular areas are 50 to 70 Å² at the collapse points of both monolayers. The location of possible polar anchors within the wax ester molecules could be the reason for these large areas. Nevertheless, the maximum film pressure seems to be disadvantageously low

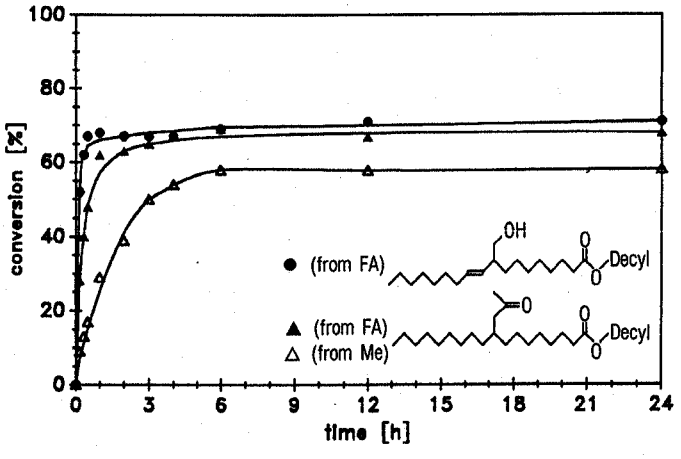


FIG. 1. Acylation of decanol with 9(10)-hydroxymethyl-octadec-10-enoic acid and 9(10)-acetonyl-octadecanoic acid [methyl (ME) ester] with Lipozyme™ at 60°C. Conditions: 0.9 mmol decanol, 0.3 mmol fatty acid (FA) (ME), 0.2 g Lipozyme, 20 mL *n*-hexane; TLC: stationary phase 1, developing system A, coloring reagent I.

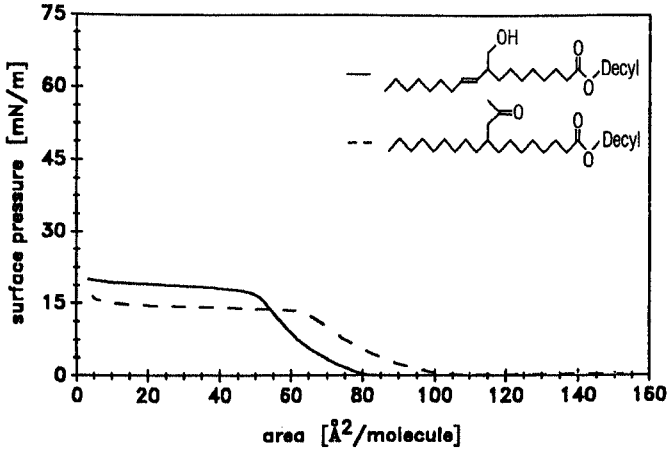


FIG. 2. π/A-isotherms of wax esters from centrally functionalized fatty acids at 25°C.

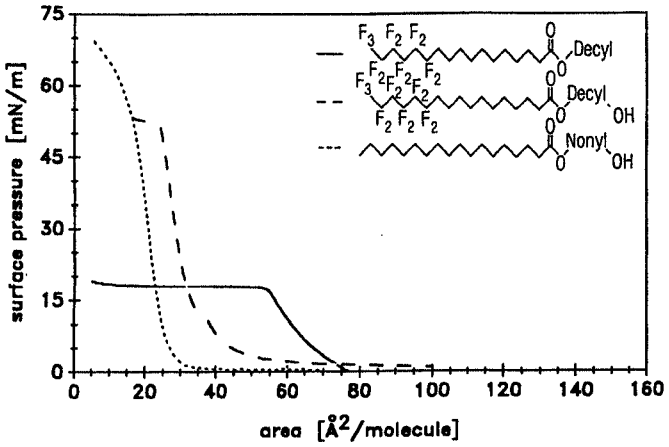


FIG. 3. π/A-isotherms of wax esters from partly fluorinated fatty acids at 25°C. Comparison with a similar wax ester without fluor atoms (15).

and indicates less monolayer stability. Two wax esters, centrally functionalized in the C-18 fatty acid moiety, 9(10)-dimethoxy-carbonylmethyl-octadecanoic acid decyl ester and 3(carboxydecyl)-heptyl-4-octyl-butyrolactone, were also prepared by coupled chemical (12) and biochemical methods and showed similar π/A isotherms of 15 mN/m at 70 and 50 Å² per molecule.

Wax esters from partly fluorinated fatty acids. For wax ester production, 0.16 mmol tridecafluoroheptadecanoic acid and 0.5 mmol 1-decanol were incubated in the presence of 0.1 g Lipozyme™ in 20 mL *n*-hexane at 60°C. The yield was 83% after 6 h. With 1,10-decandiol as acyl acceptor, the yield of monoacylated product amounted to 70%. Silica gel-TLC with developing system B and coloring reagent II was performed before the densitometric measurement. Pure products could be isolated after crystallization from methanol of the decyl ester (melting point 30°C) and from methanol/water = 5:1 (vol/vol) of the hydroxy decyl ester (melting point 33°C).

In view of the monolayer studies, especially of the 1,10-diol-based wax ester, a high film pressure was expected because one free hydroxy group acts as a strong anchor in the aqueous subphase. A value higher than 45 mN/m confirms the results of similar compounds without fluor atoms (20). Another finding of this experiment was that the final arrangement of a partly fluorinated alkyl chain requires an area of about 25 Å² (Fig. 3), which is in agreement with theoretical and experimental results on 17-(perfluoropentyl)-heptadecanoic acid and similar compounds obtained by Bernett and Zisman (21).

Wax esters from centrally dihydroxylated fatty acids. Mixtures of 9,12- and 10,13-dihydroxy-octadecanoic methyl esters were incubated with a threefold excess of 1-decanol under lipase catalysis in *n*-hexane for 24 h. A wax ester mixture could be isolated at 85% yield. The product was crystallized from methanol/water = 1:1 (vol/vol). Its melting point was 47°C.

Because of the distribution of hydrophilic parts within the molecule, a molecular area of more than 100 Å² was reached at a film pressure of 20 mN/m (Fig. 4). An unusual linear course of transition from liquid-expanded to liquid-condensed phase was observed; a second increase of film pressure to more than 60 mN/m was reached at a substantially lower area.

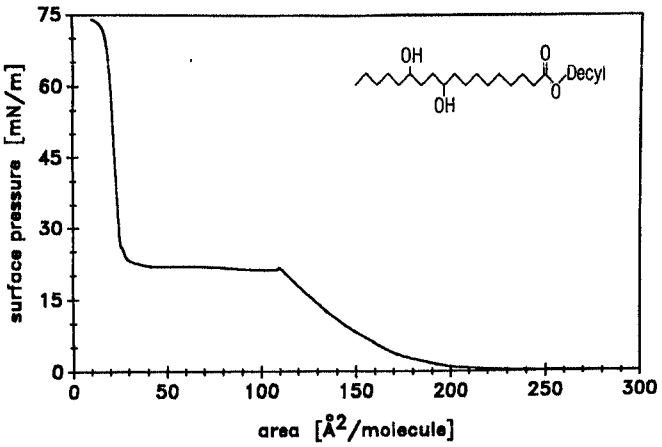


FIG. 4. π/A-isotherm of a wax ester from centrally dihydroxylated fatty acids at 25°C.

The monolayer of the 1-decanol-based wax ester at the air/water interface could be directly visualized by Brewster angle microscopy (19). The linear course of the π/A-isotherm is confirmed by the transition from liquid-expanded to liquid-condensed phase (bright spots) (Fig. 5A). A condensed bright film can be observed at the collapse point (Fig. 5B).

Wax esters from decanoic acid derivatives. Using octanol as acyl acceptor, the Lipozyme-catalyzed reaction with the methyl esters of 9,10-epoxy- and 9-oxo-decanoic acids were performed without additional organic solvent at 60°C. Figure 6 shows the course of wax ester productions after 3 h with yields higher than 80%. The conversion of substrates was determined on silica gel RP-8 with developing system C and coloring reagents II,III; method b was chosen for product purification. Similar successful experiments were also carried out in combining octanol with other C-10 acids, such as 10-OH-decanoic acid or decanoic acid.

Figure 7 summarizes the π/A-isotherms of five octyl esters of decanoic acid and decanoic acid derivatives. With the monolayers of 9,10-epoxy- and 9-oxo-decanoic acid-based wax esters, film pressure values in the range of 20

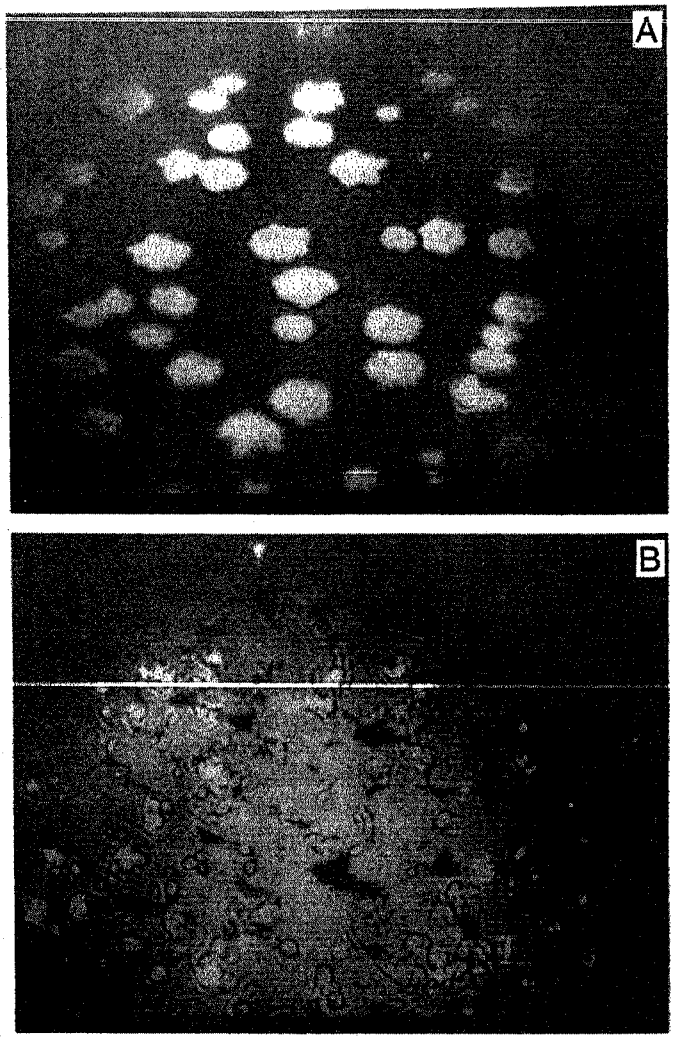


FIG. 5. Brewster-angle microscopy photos before (A) and after (B) the film collapse of 9(10),12(13)-dihydroxy-octadecanoic acid decyl ester.

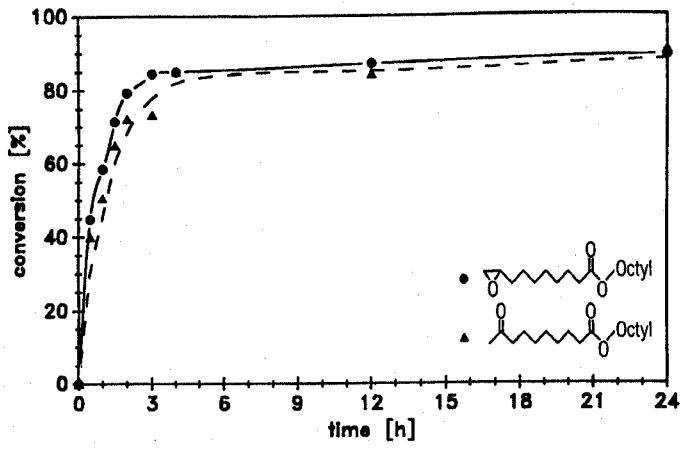


FIG. 6. Acylation of octanol with the methyl esters of 9,10-epoxydecanoic acid and of 9-oxo-decanoic acid with Lipozyme™ at 60°C without solvent. Conditions: 12 mmol octanol, 4 mmol fatty acid (methyl ester), 0.4 g Lipozyme; TLC: stationary phase 2, developing system C, coloring reagents II,III.

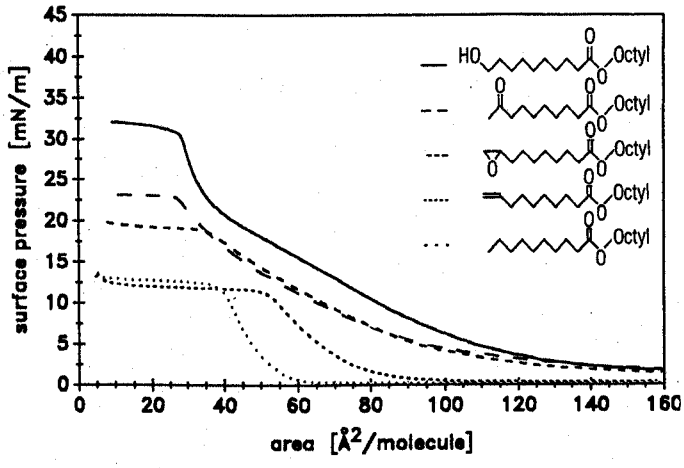


FIG. 7. π/A-isotherms of decanoic acid octyl esters at 25°C.

to 23 mN/m were observed, while the values of more-hydrophobic compounds were lower, and that of a more-hydrophilic hydroxy wax ester was higher.

The curves also show that functionalization at the outer position of wax esters leads to collapse points at relatively small areas, compared to those obtained by centrally functionalized compounds. The large values for molecular areas could be explained as follows: (i) Under low pressure, the more hydrophobic wax esters could be anchored *via* their carboxylic group, which is centrally positioned in the molecule; (ii) The more hydrophilic compounds with the functional group at the end should give minimum areas of an alkyl chain. However, our experience with similar waxes indicated that the short-chain wax esters (18 C-atoms) do not resist the outer pressure and collapse at larger areas compared to long-chain wax esters with 26 C-atoms and more (7).

Monoglycerides from centrally functionalized fatty acids. Based on successful studies with 17-hydroxy-octadecanoic acid (10), glycerol was monoacylated with the abovementioned 9(10)-acetonyl-octadecanoic acid and 9(10)- dimethoxy-carbonylmethyl-octadec-8(10)-enoic acid methyl ester. By means of a "one-pot" reaction (Fig. 8) with phenylboronic acid as a solubilizing agent for glycerol in *n*-hexane, the bioconversion with the first uncommon acid led to 80% yield after 6 h (Fig. 9). Methyl ester protection of the acid group could be responsible for the lower yield in the case of the second one.

Figure 10 presents the corresponding π/A-isotherms. They show good stability, which is caused by polar anchors of the remaining free hydroxy-groups of glycerol.

Through cooperation of chemical, biotechnological and physicochemical research teams, a lot of uncommon wax esters and monoglycerides were prepared. The interesting film pressure behavior of their monolayers on aqueous subphases offers an opportunity to test these products for their wetting properties in real application systems (cosmetics, dyes and lacquers, etc.)

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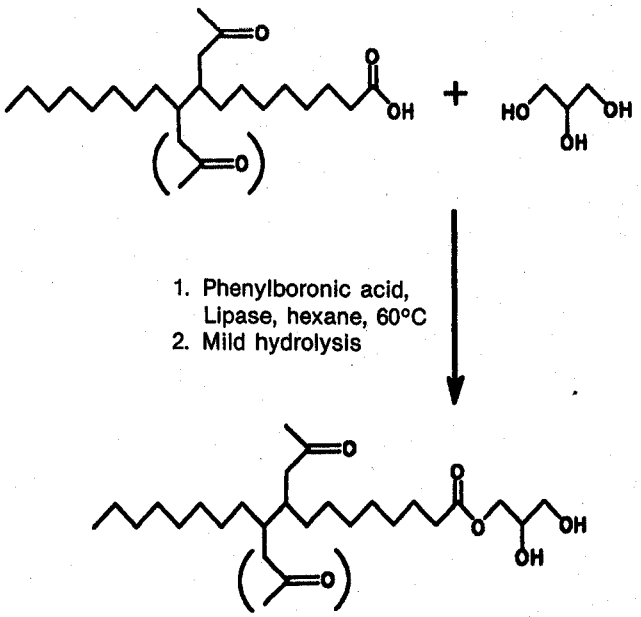

FIG. 8. Reaction scheme for the lipase-catalyzed monoacylation of glycerol with 9(10)-acetonyl-octadecanoic acid.

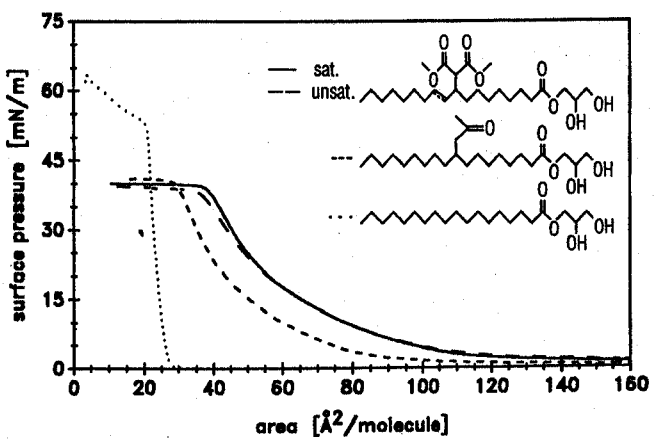

FIG. 9. Monoacylation of glycerol with 9(10)-acetonyl octadecanoic acid and 9(10)-dimethoxy carbonylmethyl octadec-8(10)-enoic acid methyl ester at 60°C. Conditions: 1.5 mmol glycerol, 0.3 mmol fatty acid (methyl ester), 0.2 g lipase SP 435, 20 mL *n*-hexane.

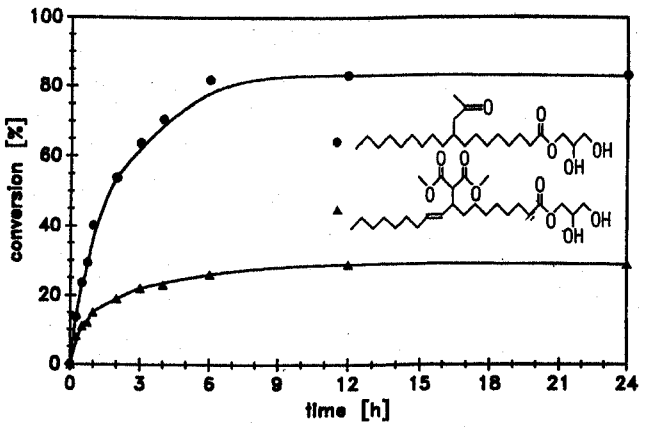

FIG. 10. π/A-isotherms of monoglycerides from centrally functionalized fatty acids at 25°C; Sat. = saturated; unsat. = unsaturated.

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