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Aminolysis of linoleic and salicylic acid derivatives with *Candida antarctica* lipase B: A solvent-free process to obtain amphiphilic amides for cosmetic application

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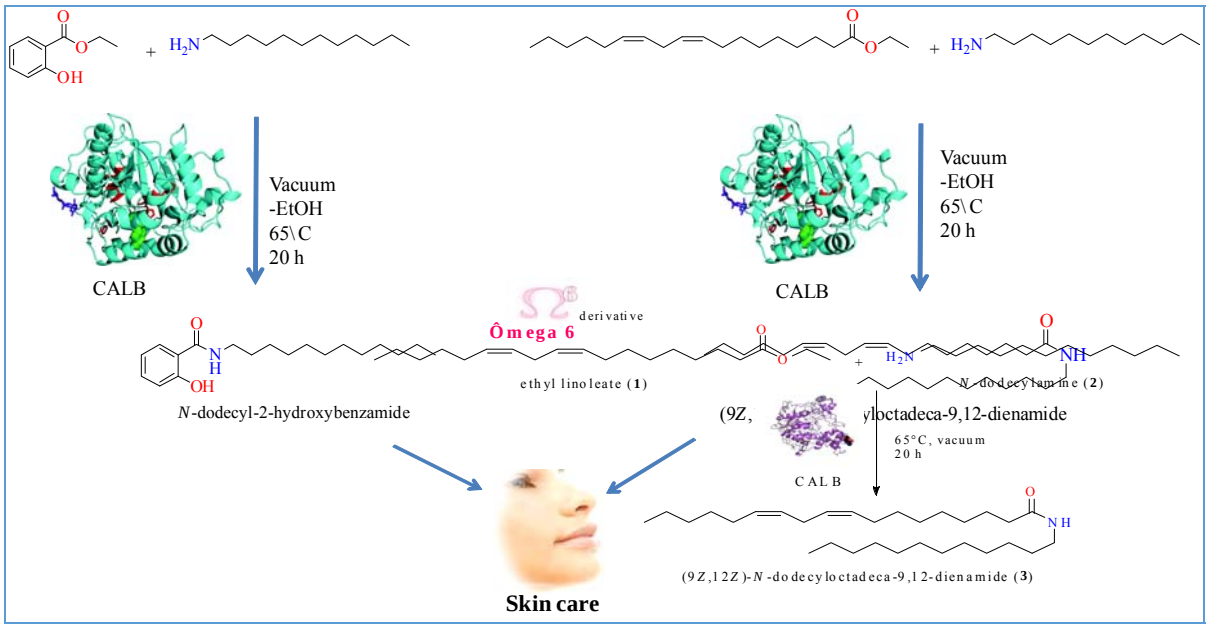
Graphical abstract:



Highlights

- **In this work, an enzymatic and solvent-free process was developed aiming the production of amphiphilic molecules for cosmetic application.** The fatty amides (9*Z*,12*Z*)-*N*-dodecyloctadeca-9,12-dienamide (**3**) and *N*-dodecyl-2-hydroxybenzamide (**5**), were synthesized through aminolysis reactions catalyzed by *Candida antarctica* lipase B. The reactions were carried out in a solvent-free process, at 65°C and reduced pressure (50 mbar). The products were monitored by HPLC analysis. The conversion rates were measured through disappearance of ethyl linoleate and ethyl salicylate. The best conversion rates (up to 95%) were obtained by adding an enzyme amount of 5.0 g/mol of acyl donor group substrate and an equimolar substrates ratio (1:1). This work reveals that enzymatic synthesis provides an attractive way for the cosmetic industrial production of fatty amides.

Abstract

In this biotechnological process, the fatty amides (9*Z*,12*Z*)-*N*-dodecyloctadeca-9,12-dienamide (**3**) and *N*-dodecyl-2-hydroxybenzamide (**5**), respectively derived from linoleic acid and salicylic acid were synthesized through aminolysis reactions catalyzed by *Candida antarctica* lipase B. These amphiphilic compounds receive great attention from cosmetic industry due to a range of beneficial properties for skin. The aminolysis reactions were performed with the esters ethyl linoleate (**1**) and ethyl salicylate (**4**) as acyl group donors and the fatty compound *N*-dodecylamine (**2**) as the nucleophilic substrate. The aminolysis reactions were carried out in a solvent-free process, which is beneficial from an environmental and economical perspective, at 65°C and reduced pressure (50 mbar). Parameters as enzyme amount and substrates molar ratios were investigated and the products were monitored by HPLC analysis. The conversion rates were measured through disappearance of ethyl linoleate and ethyl salicylate. The best conversion rates (up to 95%) were obtained by adding an enzyme amount of 5.0 g/mol of acyl donor group substrate and an equimolar substrates ratio (1:1). The products characterization was performed by High Resolution Mass Spectrometry, Infrared spectroscopy and Nuclear Magnetic Ressonance. This work reveals that enzymatic synthesis provides an attractive way for the cosmetic industrial production of

fatty amides, wich may represent key ingredients to maintain and/or restore a healthy skin.

Keywords: Biocatalysis, Solvent-free process, Lipase, Skin care, Antioxidants, Green chemistry..

1. Introduction

Over the last decades, the application of enzymes as catalysts has expanded horizons in several fields of biotechnology. This expansion may be related to a 'greener' way to produce fine chemicals with high degree of selectivity, none or minimal side reactions and relatively milder conditions compared to the chemical counterparts [1].

In this scenario, lipases (triacylglyceride hydrolases; EC 3.1.1.3) constitute the most important enzymes for biotechnological applications [2]. They present commercial availability, stability and usually do not require expensive cofactors [3,4]. These biocatalysts are capable of reacting with several substrates, catalyzing not only the hydrolysis and synthesis of long-chain acylglycerols but also alcoholysis, acidolysis, interesterification and the transfer of acyl groups from esters to amines (nucleophiles), promoting aminolysis reactions [5,6]. The use of lipases in aminolysis of esters in anhydrous media has been successfully described for the synthesis of peptides, fatty acid amides, polymers, surfactants and low cost detergents [7, 8].

From the cosmetic sector standpoint, lipases represent great biocatalysts in the synthesis of fine chemicals such as specialty esters, aroma compounds and active ingredients [9]. Notedly, the lipase B from *Candida antarctica* (CALB) has proven to be a versatile catalyst to synthesize cosmetic products for skin care, such as retinol (vitamin A) and ascorbic acid (vitamin C) derivatives, widely used as natural antioxidants in the treatment of photoaging and skin disorders [10].

In a previous work, CALB catalyzed aminolysis reactions in a solvent-free process, leading to the production of ceramides analogous molecules, which are essential for epidermal permeability barrier function and overall condition of the skin [11-13]. In this work, we proposed the employment of CALB as catalyst in aminolysis reactions between the esters ethyl linoleate (**1**) and ethyl salicylate (**4**), respectively derived from linoleic and salicylic acids, and the fatty compound *N*-dodecylamine (**2**).

The interest in the application of these compounds as cosmetic ingredients can be described as follows; firstly, the presence of a linoleic acid (LA) derivative is interesting in cosmetic formulations since it represents an important skin constituent, with specific functions in the production and maintance of epidermal permeability barrier [14-16]. LA has received increased attention in cosmetic market due to its anti-inflammatory, acne reduction and moisture retention properties [12]. Furthermore, this polyunsaturaded fatty acid (PUFA) is featured with 18 carbon atoms and two *cis* double bonds at position 6 (*omega*-6 family), representing an essential fatty acid. Whereas the

human body can not synthesize double bonds at position 6, LA must be necessarily obtained from the diet or topical applications [14].

With respect to ethyl salicylate (**4**), salicylic acid (SA) derivatives composes the phenolic acid family, recognized by the antioxidant activity against the highly reactive free radicals, which are involved in adverses skin effects such as photoaging, wrinkling, general loss of elasticity and cancer [17-20]. SA is frequently used in the treatment of *Acne vulgaris* due to its keratolytic propertie and is also known as an ultraviolet B sunscreen, reducing sunburn and melasma production [21-23].

Lipophillic derivatives of salicylic acid also have been proven to present anti-aging, photoprotective, keratolytic and antibacterial properties. In fact, it has been shown that the addition of fatty chains to SA molecule confers superior efficacy on the original molecule [24]. The greater efficacy is related to the skin permeability, which is lower for hydrophilic compounds, since the outermost layer of skin represents a highly lipophilic barrier constituted of ceramides, fatty acids and cholesterol [25]. In this context, the addiction of fatty chains can be extended to LA derivatives, where the increasing of fatty chains in unsaturated fatty acids, particularly those of *cis* configuration, confers greater protective effect against cutaneous dehydration and xenobiotics [26]. Thus, this structural mofication represents an attractive way to improve the skin-product compatibility.

Additionally, this reactions may lead to the production of amphiphilic molecules, which presenting an hydrophobic region (soluble in lipophilic medium) and a hydrophilic head (solubility in aqueous environment), represent ideal stabilizers for emulsion formulations, being recognized as emulsifiers or surfactants [27]; once there is a concern about the classical synthesis of surfactants derived from petroleum [28, 29], biosurfactants containing amide bonds are potential substitutes, presenting skin tolerance, good biological degradability and significantly lower toxicological effects [30].

Therefore, the structural modification of LA and SA esters (**1** and **4**) via aminolysis reactions catalyzed by CALB with the fatty *N*-dodecylamine (**2**), in a solvent-free process, can be used as a tool to produce amphiphilic amides, which may atract great interest from cosmetic industry and differentiated applications for skin cosmetic formulations.

2. Materials and methods

2.1 Materials

The lipase B from *C. antarctica* (Novozym 435, immobilized) was kindly provided by Novozymes A/S, Bagsvaerd, Denmark. Ethyl linoleate (**1**) was purchased from Stearinerie Dubois (Ciron, France), while *N*-dodecylamine (**2**) and ethyl salicylate (**4**) were purchase from Sigma-Aldrich (St. Louis, MO).

2.2 The reaction parameters

It is important to consider that to run enzymatic reactions under solvent-free conditions, at least one of the reactants has to be kept in the liquid state; thus, the reaction temperature has to be high enough (65°C) to shift the equilibrium in the right sense. Also, to limite the possible reverse reaction (hydrolysis), the reactions were carried out under vacuum, evaporating thus, the ethanol (by-product) formed during the reaction.

2.3 *Enzymatic reactions*

In order to obtain the amphiphilic amides **3** and **5** in great yields, two studies were perfomed to evaluate the effect of substrates molar ratio and the biocatalyst amount. In the effect of substrates molar ratio study, the reactants acyl donor groups **1** or **4** and the nucleophile *N*-dodecylamine (**2**) were added in 50 mL flasks in the following values: 0.8: 1.0; 1.0:1.0 and 1.0:2.0 (acyl donor **1** or **4**: nucleophile **2**). Then, after 5 min under vacuum (50 mbar) for gas removal of the system, 5.0 g of *Candida antarctica* B/ mol of ethyl esters (**1** or **4**) were added to the reaction mixtures.

Also, one sequence of reactions was performed by changing the biocatalyst amount (CALB) in order to find the higher yields. This study was carried out in 50 mL glass reactors, wherein equimolar substrates concentrations [1 acyl donor groups (**1** or **4**) : 1 nucleophile (**2**)] were added. After 5 min under vacuum (50 mbar) for gas removal of the system, different CALB amounts were added to the reaction mixtures (0.6; 1.2; 2.5; 5.0; 7.5; 10 g/mol acyl donor reactants **1** or **4**).

All the reactions were conducted under stirring (250-300 rpm) using an overhead stirrer IKA RW 16 Basic, Staufen, Germany, equipped with a plastic propeller, at 65°C. After 20 h, the products were purified through *flash* cromatography and the rates of conversion of the reactions were monitored through the disappearance of ethyl linoleate (**1**) or/and ethyl salicylate (**4**) using HPLC analysis.

3. Analytical and characterization methods

3.1 High Performance Liquid Cromatography (HPLC)

The aminolysis reactions were monitored by High-Performance Liquid Chromatography (HPLC analysis) carried out in a system (Alliance-Waters) composed of a column (Xterra MS C18 5M, 150 mm × 2.1 mm), a column oven (temperature 40 $^{\circ}$C), an autoinjector and UV/vis detector (PDA, W2996= 210 nm). The compounds were evaluated with an eluent system of methanol (A) and water (B), both containing 0.1% of trifluoroacetic acid, as shown in **Table 1**.

3.2 Nuclear Magnetic Resonance (NMR)

The NMR specters of proton and carbon (^{1}H and ^{13}C NMR) of the chemical structure of the products were obtained using a Bruker AM 500 at the Analytical Center of the Institute of Chemistry of São Carlos (IQSC/USP), operating in 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR), using $CDCl_3$ as the deutered solvent and TMS as the internal standart unless otherwise noted. The chemical shifts are given in ppm and the coupling constants (*J*) in Hz.

3.3 High Resolution Mass Spectrometry (HRMS)

The analyses were performed on a High Resolution Mass Spectrometer quadrupole / flight time, operating in the MS mode in the unit: microTOF-QII, detector Daltonis Bruker (Bremen Germany). In these analyzes, the samples were ionized in the positive mode using atmospheric pressure chemical ionization (APCI). The analyses

were performed at the Institute of Chemistry of São Carlos, in the Chromatography Laboratory (Prof. Dr. Fernando Mauro Lanças, FAPESP Grant n°2004/09498-2).

4.Results and Discussion

4.1 Effect of molar reactants concentrations in the yield reactions

The study concerning the effect of substrates molar ratio was performed with the addition of different values of the substrates (0.8: 1.0; 1.0:1.0 and 1.0:2.0 – wherein acyl donor: nucleophile) and 5.0 g CALB/mol of the acyl donor group **1** or **4** in all reactions (Scheme 1).

The reactions were carried out following the described procedures and the synthesized products were monitored by HPLC analysis. The results are summarized in **Table 2**.

The reactions 1-6 were performed as a preview study, in order to find the proper molar ratios of the reactants. As can be seen in the analysed reactions 1-3, by adding a equal molar ratio of the substrates **1** and **2** and 5.0 g CALB/mol of acyl donor group, it was possible to obtain the high yield of 99% of the fatty amide **3**. The results were similar in the reactions 4-6, where a yield of 96% of the fatty amide **5** was obtained by adding a equimolar ratio of the reactants **4** and **2**. These results opened the way for the standardization of the equal molar ratio of reactants for the subsequent reactions.

4.2 The effect of the biocatalyst amount in the yield reactions

In sequence, the aminolysis reactions among ethyl linoleate (**1**) and *N*-dodecylamine (**2**) with different enzyme amount catalyzed by CALB were carried out following the described procedures and the synthesized product was monitored by HPLC analysis. The results are summarized in **Table 3**.

The results showed that the fatty amide **3** was obtained with 30% yield by adding the lower enzyme amount selected (0.6 g/ mol ethyl ester **1**). The HPLC analysis exhibited that this yield was raised to 73% after duplicating the added enzyme amount (1.2 g/mol ethyl ester **1**). The addiction of 2.5 g CALB/mol ethyl linoleate (**1**) to the mixture reaction led to a 88% yield after 20 h.

A great yield of 99% was obtained by adding 5.0 g CALB/mol ethyl linoleate (**1**) and therefore, this enzyme amount was standarzided for further reactions. The reactions carried out with 7.5 g and 10.0 g CALB/mol ethyl linoleate (**1**) led to the production of the fatty amide **3** with similar yields (99-100%).

Sequentially, aminolysis reactions were carried out with the reagents ethyl salicylate (**4**) and *N*-dodecylamine (**2**) in order to produce the amphiphilic *N*-dodecyl-2-hydroxybenzamide (**5**). The aminolysis reactions of ethyl salicylate (**4**) and *N*-dodecylamine (**2**) catalyzed by CALB were carried out following the described procedures and the synthesized products were monitored by HPLC analysis (**Scheme 2**). The results are summarized in **Table 4**.

In this case, the fatty amide **5** was obtained with 25% yield by adding the lower enzyme amount selected (0.6 g/ mol ethyl ester **4**). The HPLC analysis exhibited that this yield was raised to 68% after duplicating the added enzyme amount (1.2 g/mol ethyl ester **4**). The reaction 3 achieved a 85% yield of the fatty amide **5** with the addiction of 2.5 g CALB/mol ethyl salicylate (**4**). The results showed that by adding 5.0 g CALB/mol ethyl salicylate (**4**) it was possible to obtain a 96% yield of the fatty amide **5** and the results with the addiction of 7.5 and 10 g CALB/ mol ethyl ester **4** were approximated, with 97% yield. Considering this proximity between the reaction yields, the experimental conditions of the reaction 4 (5.0 g CALB/mol ester) may be standardized for the production of *N*-dodecyl-2-hydroxybenzamide (**5**).

Amide compounds are of great value in different fields, including cosmetic, pharmaceutical and agrochemical [31, 32]. Due to this interest, the syntheses of these compounds has been conducted either by chemical and enzymatic pathways. Conventional chemical syntheses of amides may be carried out under high temperatures and extended reaction times, involving for example alkoxides, anhydrides, alkyllithium, acyl chlorides, metallic catalysts or Grignard reagents [33-36]. Amides syntheses have also been reported via microwave radiation [31, 37].

Related to enzymatic syntheses, CALB has proven to be the most efficient catalyst for aminolysis reactions and its environmentally benign character is desirable for large scale industrial aplications [38]. Kaushik et al., 2015 performed studies were a serie of N-alkyl-substituted amides have been synthesized in the presence of CALB at 60-90°, in a solvent-free system with yields between 75-83% [39].

Studies have demonstrated that CALB can be useful for direct formation of ethanolamides of various long-chain fatty acids, optically pure amides and lactams through aminolysis [33, 38, 40]. Phenolic compounds such as caffeic acid amide can also be obtained by CALB aminolysis reactions, as demonstrated in the study performed by Xiao et al., 2013 [41].

CALB also catalyzed the direct amidation of fatty and hydroxy fatty acids (RA: ricinoleic acid, LQA: lesquerolic acid and TOD: 7,10,12-trihydroxy-8-(*E*)-octadecenoic acid) to their primary amides with ammonia in organic solvent. At 55 °C and a 2:1 ammonia to fatty acid substrate ratio, the transformation reactions of LQA, RA and TOD approached completion (over 95%) within 24 h, while a decreased in the ratio to 1:1 reduced the transformation to approximately 80% [42]. Studies investigated the direct amidation of the carboxylic acid 7,10-dihydroxy-8(*E*)-octadecenoic acid with *N*-methylethanol amine catalyzed by CALB. After 72 h, in organic solvent medium, CALB mediated the production of a novel secondary amide *N*-methyl-7,10-dihydroxy-8-(*E*)-octadecenoylethanolamide with 95% yield, which displayed antimicrobial and antioxidative activities [43]. Furthermore, CALB catalyzed the synthesis of optically enriched α-haloamides and N-alkyl substituted amides with potential antinemic activity [44, 45].

In this work, the fatty amides (9*Z*,12*Z*)-*N*-dodecyloctadeca-9,12-dienamide (**3**) and *N*-dodecyl-2-hydroxybenzamide (**5**) were synthesized through a solvent-free biotechnological process, where products yields over 96% were obtained after 20 h in reactions carried out in equimolar substrates ratio and 5.0 g CALB/mol of acyl group donors substrates. The amphiphilic compounds **3** and **5** are currently passing through studies, exhibiting efficacy in their biological activities so far. Since amides derived from fatty acids are important for the maintenance a healthy skin and their long hydrophobic chains constitute a barrier against evaporation of water and the resulting dry skin [46], these amphiphilic compounds represent potential candidates for skincare cosmetic formulations, agregatting as well the beneficial properties previous described.

5. Conclusion

The cosmetic market is renowned for moving a huge financial capital, attracting the attention of academic and research centers to the development of new skin care chemicals. In this sense, compounds which aggregate cutaneous beneficial properties with economic and environmentally friendly routes are of major interest.

Fatty amides are of great interest due to their ranging of industrial applications not only in cosmetics, but also as surfactants and detergents formulations. In this way, enzymatic synthesis of fatty amides in milder conditions are interesting from an economical and enviromental aspect. In this work, two amphiphilic amides (**3** and **5**) with potential for skin care application were synthesized through an enzymatic biotechnological process. The syntheses of the cosmetic chemicals were achieved due to the application of CALB enzyme as biocatalyst, which provided high yields (96-99%), under mild conditions and in a solvent-free system. This work shows that CALB and its acylating activity exhibits high catalytic efficiency for implementation in the production of cosmetic ingredients.

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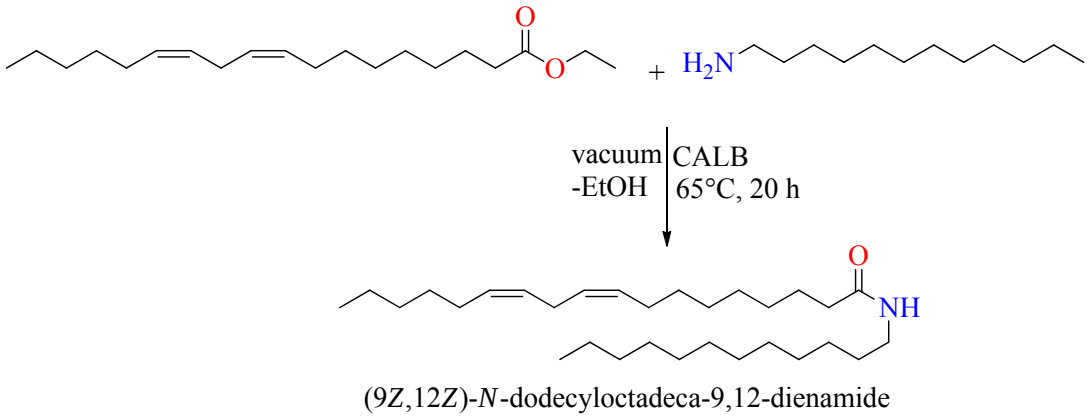
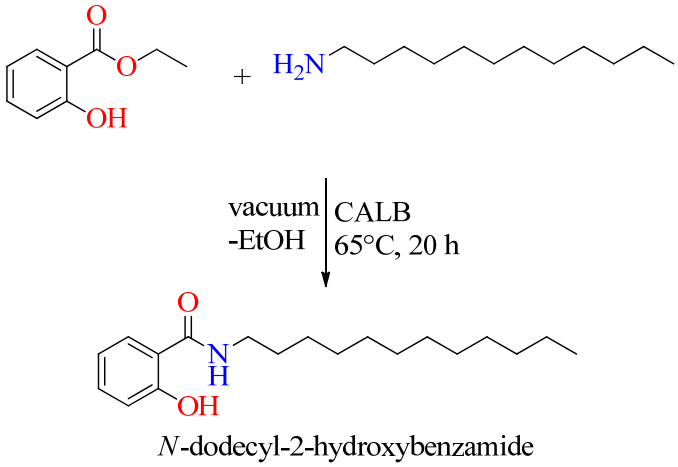


(9*Z*,12*Z*)-*N*-dodecyloctadeca-9,12-dienamide

Scheme 1. Aminolysis reaction catalyzed by CALB for the synthesis of (9*Z*,12*Z*)-*N*-dodecyloctadeca-9,12-dienamide (**3**).



N-dodecyl-2-hydroxybenzamide

Scheme 2. Aminolysis reaction catalyzed by CALB for the synthesis of *N*-dodecyl-2-hydroxybenzamide (**5**).

Table 1. Chromatography conditions for analysis of aminolysis reactions by HPLC

Fatty amides	Time (min)	Flow (mL/min)	(A)% MeOH	(B)% H_2O	t_r (reactant)	t_r (fatty amide)
3	40	0.27	80	20	14.5	22.2
5	30	0.27	50	50	9.2	21.0

t_r: retention time, column: X terra MS C18 5M.

Table 2. Aminolysis reactions performed with different molar ratios of reactants for the synthesis of (9*Z*,12*Z*) -*N*-dodecyloctadeca-9,12-dienamide (**3**) and *N*-dodecyl-2-hydroxybenzamide (**5**).

Reaction	Ethyl linoleate (**1**): *N*-dodecylamine (**2**) (Mols)	Remaining ester **1** (%)	Fatty amide **3**[a] (%)
1	0.8: 1.0	27	73
2	1.0:1.0	1	99
3	1.0:2.0	1	99
Reaction	Ethyl salicylate (**4**): *N*-dodecylamine (**2**) (Mols)	Remaining ester **4** (%)	Fatty amide **5**[a] (%)
4	0.8:1.0	34	66
5	1.0:1.0	4	96
6	1.0:2.0	1	99

Experimental conditions: T=65 °C, p=50 mbar, CALB: 5 g/mol of esters **2** or **4**, time: 20 hours, [a]: yields values obtained by HPLC analysis.

Table 3. Aminolysis reactions of ethyl linoleate (**1**) and *N*-dodecylamine (**2**) for the synthesis of (9*Z*,12*Z*) -*N*-dodecyloctadeca-9,12-dienamide (**3**) catalyzed by CALB.

Reaction	CALB (g/mol ethyl ester **1**)	Remaining ethyl linoleate (**1**) (%)	(9*Z*,12*Z*) -*N*-dodecyloctadeca-9,12-dienamide (**3**)[a] (%)
1	0.6	70	30
2	1.2	27	73
3	2.5	12	88
4	5.0	1	99
5	7.5	1	99
6	10.0	0	100

Experimental conditions: T=65 °C, p=50 mbar, CALB: g/mol of ethyl linoleate (**1**), equimolar concentration of reactants (1:1), time: 20 h, [a]: yields obtained by HPLC analysis.

Table 4. Aminolysis reactions among ethyl salicylate (**4**) and *N*-dodecylamine (**2**) for the synthesis of *N*-dodecyl-2-hydroxybenzamide (**5**).

Reaction	CALB (g/mol ethyl ester **4**)	Remaining ethyl salicylate (**4**) (%)	*N*-dodecyl-2-hydroxybenzamide (**5**)[a](%)
1	0.6	75	25
2	1.2	32	68
3	2.5	15	85
4	5.0	4	96
5	7.5	3	97
6	10	3	97

Experimental conditions: T=65 °C, p=50 mbar, CALB: g/mol of ethyl salicylate (**4**), equimolar concentration of reactants (1:1), time: 20 hours, [a]: yields obtained by HPLC analysis.