Combined structural and biological activities for new polyunsaturated fatty derivatives obtained by biotechnological process

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Synopsis

The objective of this study is to demonstrate the use of new polyunsaturated fatty derivatives for cutaneous applications. These new compounds present an analogue structure of cutaneous lipid, stabilize the polyunsaturated fatty acids (face to oxidation) and demonstrate specific biological activities. Three molecules described are Omega 6 fatty acid stabilized compound (O6FASC), the O3FASC and the O9FASC. The derivatives are synthesized via the same biotechnological process. This work describes the choice of final structure, the design of the biotechnological process and the free solvent enzymatic synthesis used for the synthesis of these three cutaneous lipid analogues. The restructuring effect of such analogues has been demonstrated with an in vivo study on volunteers. The stabilization of the O3FASC and O6FASC, and the biological activities of these three compounds are presented. The O6FASC shows very good results in anti-inflammatory effects; the O3FASC has anti-stress activities, whereas the O9FASC presents interesting results in improving elasticity and firmness. All these activity tests are presented in this work.

Introduction

The skin has a large number of important functions. It provides an interface between a hostile external environment and the host. Thus the major function of the skin is to provide a barrier between the body and the outside environment [1]. There
are numerous barrier functions, and the skin has unique structures that provide for these various barriers. For example, the permeability barrier which resides in the extracellular lipid membranes in the stratum corneum (SC) prevents the loss of water and electrolytes. In addition, it prevents the entry of toxic compounds. The uppermost skin layer, the SC, in particular, is responsible for said functions. This layer is composed of cells in the terminal stage of keratinization, the corneocytes deposited in a complex lipid mixture [2]. The corneocytes are very poorly permeable; they are completely filled with keratin and their cells walls are thickened with deposition of highly cross-linked proteins into a corneocyte envelope. Intercellular lipids of SC consist mostly of a mixture of ceramides, fatty acids and cholesterol and are organized in multiplayer lamellae [3]. The high content of ceramides and very tight packing of the lipid lamellae are the basis of exceptional resilience of the skin against chemical as well as mechanical damage and its low permeability both outwards, for body’s intrinsic substances, and inwards for xenobiotics [4–6].

A lower amount of any changes in the composition of SC lipids, especially ceramides, are encountered in skin diseases such as, for example, atopic dermatitis or psoriasis [7]. In the case of atopic dermatitis, a lower content of ceramides in the SC leads to higher permeability of the skin for allergens, toxic and irritating substances or microbes [8, 9]. These substances subsequently cause or worsen the inflammation which further degrades the barrier function of the skin and closes the so-called vicious circle of atopic dermatitis. Lower content of ceramides is also encountered in dry or ageing skin of people exposed to stresses [10, 11]. Based on the current knowledge of biochemistry of these abnormalities, it is a logical correction and therapeutic procedure to supply missing ceramides by topical administration, i.e. by relipidization of the SC. This approach allows restoring of intercellular equilibriums between the lipid and aqueous phases in the SC and helps subsequent regeneration and optimization of physiological processes going on in the SC and lower layers of the epidermis. Topically, administered formulation containing lipids with the structure similar to skin lipids, especially ceramides, improves homeostasis and overall condition of the skin [12, 13].

In addition to the importance of ceramide molecules, observations have led to the concept that a polyunsaturated fatty acid (PUFA)-containing lipid must play a direct and critical role in formation or maintenance of the epidermal water barrier [14–18]. When orally administered, the linoleic and α-linolenic acids present a lot of favourable properties for human physiology [19, 20]. The importance of linoleic and α-linolenic acids in the improvement of psoriatic symptoms has been demonstrated [21]; linoleic acid has anti-inflammatory effects [22, 23], whereas the biological interest of α-linolenic acid is more focused on anti-stress and anti-acne activities [24–26]. In the field of cutaneous biology, a dietary deficiency of these PUFAs results in characteristic scaly skin disorder and excessive epidermal water loss [27].

Taking the importance of cutaneous lipids into account, we present in this study a new design of structural analogue of ceramides, including PUFAs. The new derivatives obtained by biotechnological process demonstrate very good restructuring effects, stabilization of fatty acids and their biological efficacy has been demonstrated.

Materials and methods

Enzyme and chemicals

Novozym 435 (immobilized Candida antarctica lipase B) and Lipozyme RM IM (immobilized Rhizomucor miehei lipase) were kindly provided by Novozymes A/S, Bagsvaerd, Denmark. Linoleyl ethyl ester, α-linolenyl ethyl ester and oleyl ethyl ester were purchased from Stearinerie Dubois (Ciron, France). Specific oils were purchased from Polaris (Pleuven, France). Aminoglycerol was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Biosynthesis of the omega fatty acid stabilized compounds

The aminoglycerol was added to the acyl donor (linoleyl, α-linolenyl, or oleyl ethyl ester concentration was adjusted to have a aminoalcohol/ethyl esters molar ratio equal to 1) in a glass reactor. Then, the temperature mixture was brought to 65°C under stirring (about 300 rpm) using an overhead stirrer IKA RW 16 Basic, equipped with a plastic propeller; IKA, Staufen, Germany). After 5 min under vacuum (50 mbar), the lipase Novozym 435 (5 g mol⁻¹ of substrate) was added to...
the mixture to remove the gazes of the system. The reaction was then run under vacuum.

For all experiments, the reaction was stopped after 20 h. The intermediate was recovered by filtering off the enzyme preparation except for the O9FASC. Disappearance of ethyl esters and formation of products were monitored using high performance liquid chromatography (HPLC) to determine the rate of conversion of the reaction.

The second acyl donor was added to the intermediate (palmitoyl, or oleyl ethyl ester – in the O9FASC case – to have a molar ratio equal to 1) in the glass reactor. The temperature mixture was increased at 65°C under stirring. After 5 min under vacuum (50 mbar) to remove the gazes of the system, the lipase Lipzyme RM IM (30 g mol⁻¹ of substrate) was added to the mixture (except for the O9FASC where no enzyme is added again). The reaction was then run under vacuum for 20 h. The final product was recovered by filtering off the enzyme preparation. Disappearance of ethyl esters and formation of products were monitored using HPLC to determine the rate of conversion of the reaction. Products obtained were analysed by ¹H and ¹³C nuclear magnetic resonance (NMR) to confirm their structure.

Analytical methods

High performance liquid chromatography
The aminolysis reaction was monitored by HPLC analysis carried out in a system (Alliance – Waters, Saint Quentin, France) composed of a column (Xterra MS C18 5 μM, 150 x 2.1 mm), a column oven (temperature, 40°C), an autoinjector, a UV/Vis detector (PDA, W2996, λ = 210 nm). The compounds were eluted by gradient elution with an eluent system of methanol (A) and water (B), both containing 0.1% of trifluoroacetic acid (see Table I).

Table I Gradient elution for HPLC analysis

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml min⁻¹)</th>
<th>% Methanol</th>
<th>% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.27</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>0.27</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>0.27</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0.27</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>55</td>
<td>0.27</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

HPLC, high performance liquid chromatography.

Nuclear magnetic resonance analysis.
The chemical structures of the synthesized products were determined by ¹H NMR and ¹³C NMR in CDCl₃ using a Brucker AM 400 spectrometer at 400 MHz (Brucker, Paris, France).

Stabilization of PUFA
The determination of the oxidation stability of oils and fats was carried out using 743 Rancimat® apparatus (Metrohm AG, Herisan, Switzerland). The resulting induction time characterized the resistance of oils and fats to oxidation. During the measurement a stream of air was passed through the oil or fat sample contained in a sealed and heated reaction vessel. This treatment resulted in oxidation of the oil or fat molecules in the sample, with peroxides initially being formed as the primary oxidation products. After some time the fatty acids were completely destroyed; the secondary oxidation products formed included low-molecular organic acids in addition to other volatile organic compounds. These were transported in the stream of air to a second vessel containing distilled water. The conductivity in this vessel was recorded continuously. The organic acids can be detected by the increase in conductivity. The time that elapses until these secondary reaction products appear is known as the induction time, induction period, or Oil Stability Index.

Biological activity tests on FASC

In vitro test.

Evaluation of the effect of the O3FASC on the inhibition of 5α-reductase activity:

Principle:
The study consists in evaluating the effect of O3FASC on the inhibition of 5α-reductase activity (Bioalternatives, Gençay, France) following application of radiolabelled testosterone on reconstructed human epidermises (SkinEthic®, Nice, France) was carried out.

Simplified protocol:
Tests were carried out on 15 SkinEthic® epidermises (0.50 cm², 17 days):
- The epidermises were incubated at 37°C for 24 h in the presence of 5% CO₂.
Epidermises were treated by topical application of emulsions containing different concentrations (0.50%, 1% and 2%) of the O3FASC or a solution containing 10–4 M finasteride, a pharmacological reference molecule (Merck Laboratories, Darmstadt, Germany). A test was also performed on untreated control skin. Three epidermises were used for each test product.

After 24 h of incubation, the epidermises were retreated with the test products and a radioactive testosterone solution (Amersham CFA, 129.57 mCi mmol⁻¹, 2.25 nmole per epidermis; Amersham, UK) was applied topically at the same time.

Recovery of the medium beneath the epidermises after 24 h of incubation.

Extraction and separation of steroid molecules [particularly testosterone and dihydroxytestosterone (DHT)] by thin layer chromatography (TLC).

 Autoradiography of TLC plates and quantification of the radioactivity using a Phosphorus Imager (Packard Instrument Co., Meriden, CT, USA) and its specific software.

**Results:**

The results are expressed as the percentage of residual radioactive testosterone (non-converted T⁰⁺) in the medium and as a percentage of converted radioactive DHT (Scheme 1).

**Effect of the O3FASC in association with BSA on the electrophysiological activity of sensory neurons:**

The study consists in evaluating the effects of the compound on the release of calcitonin gene-related peptide (CGRP) by sensory neurons and on the calcium mobilization in sensory neurons after capsaicin stimulation. Sensory neurones were prepared according to the method described by Hall et al. (1997) [28].

The first step consisted in solubilizing the test compound in the culture medium. The O3FASC compound was transferred into the culture medium in presence of delipidated bovine serum albumin (BSA). Determination of the optimal solubilization conditions with respect to the neurons viability was achieved by the following steps:

- Testing a range of delipidated BSA to determine its non-cytotoxicity limit choosing two BSA concentrations for the compound solubilization assay.
- Testing a range of compound concentrations solubilized by the concentrations of BSA selected in the previous step.

The second step consisted in the actual electrophysiological activity assay on sensory neurons (stressed by capsaicin, and treated with 0.1% of O3FASC vs. control), evaluated by the following parameter:

- CGRP release
  The quantities of CGRP released spontaneously and after stimulation by capsaicin in the supernatants were measured by an ELISA test (Rat CGRP enzyme immunoassay SpiBio Kit A05482: Medcorp Inc., Montreal, Canada). Data were transferred and analysed with the Statview software (Abacus Concepts Inc., Berkeley, CA, USA). The comparisons of each group were carried out by ANOVA using the unpaired t-test.

- Mobilization of calcium into cell bodies
  At the end of the incubation, the supernatants were changed by incubation solution containing the fluorescence calcium specific probe, Fluor-4 Acetethylmethyl, at 3 μM and 1% delipidated BSA alone or with O3FASC. After incubation, the cells were transferred on the stage of an inverted microscope (Nikon Diaphot 300: Nikon, Tokyo, Japan) and observed in epifluorescence. During the analysis of 40 s, photographs were taken every 333 ms with a camera (DMX 1200 Nikon) controlled with software (Lucia 6.0 Nikon). After observation during 10 s, neurons were stimulated by capsaicin at 10⁻⁶ M. The activity status of the neurons at T0 could be determined for each cell and the mean fluorescence per condition was calculated. Data

% of DHT⁺ inhibition = \[\frac{\text{% of control converted DHT⁺} - \text{% of converted DHT⁺}}{\text{% control converted DHT⁺}} \times 100\]

% of conversion inhibition T⁺/DHT⁺ = \[\frac{\text{% control T⁺/DHT⁺ conversion - %T⁺/DHT⁺ conversion}}{\text{% control T⁺/DHT⁺ conversion}} \times 100\]

**Scheme 1** Calculation of the inhibition percentage of converted DHT and of the T⁺/DHT⁺ conversion. T, testosterone; DHT, dihydroxytestosterone.
were transferred and analysed with the PRISM software.

**Ex vivo test.**

**Anti-inflammatory effect of the O6FASC:**

**Principle:**
The experimental model used consisted of two human skin explants to which a cream containing 1% O6FASC (Sephra, Puteaux, France) and a placebo cream were applied (need for a pre-formulation considering the solid state of the product at 37°C) and stimulated by an aggressive molecule, phorbol myristate acetate (PMA).

**Protocol:**
Explants were kept alive in an appropriate culture medium at 37°C, then incubated for 24 h with PMA after topical application of the creams containing O6FASC or not. An untreated, non-stimulated control was performed in parallel. The quantification of the release of the interleukin (IL1β) secreted in the culture supernatant has been realized using the ELISA method (Spibio; Ref 583311).

**Anti-elastase activity study for the O9FASC:**

The anti-elastase activity (Bio-EC, Longjumeau, France) has been realized on human explants. The study consists of observation of general morphology and visualization of the matrix of elastic fibres of the dermis. This matrix has been observed in all parts of the dermis and measured in picture analysis. Explants were prepared from abdominal plasty from 40-year-old woman.

For each biopsy, the elastic fibres matrix surface has been measured regarding to the surface of dermis to determine the percent of elastic fibres matrix surface. The test has been realized with pure oleic acid, the placebo cream and the cream (same as placebo cream) with 1% O9FASC.

**In vivo test.**
For each in vivo test, main inclusion criteria are defined. Moreover, non-inclusion criteria defined are as follows: pregnant or nursing woman, cutaneous pathology on the studied zone, use of topical or systemic treatment during the previous weeks liable to interfere with the assessment of the cutaneous tolerance of the studied product, excessive exposure to sunlight or UV rays within the previous month and subject enrolled in another clinical trial during the study period. Compliance assessment is defined by following criteria: only usual hygiene products were authorized; no excessive exposure to sunlight or UV rays during the study was authorized.

**Restructuring activity (Dermscan, Lyon, France):**

The study was performed, involving twice daily application (usual conditions of application) for 14 days of a cream containing 1% the O6FASC vs. a placebo cream on the forearms of two groups of 15 volunteers (43 to 55 years old Caucasian women) suffering from very dry, rough skin (one group using the cream containing the O6FASC and the other using the placebo) (Table II). Microscopic observation was carried out after stripping the forearm of each volunteer. Scanning electron microscopy is a very complex method of measurement, so it was decided to analyse the results of only three volunteers (the choice of three volunteers was made at random).

The formulation of placebo and the O6FASC cream are detailed in Table II.

The ambient conditions for measurement were set at:
- Ambient temperature: 24 ± 2°C;
- Relative humidity between 30% and 40%

**Protocol:**
On D0:
- The volunteers came to the laboratory, not having applied any cosmetic product on their forearm since the previous evening.
- The application zone on the forearm was defined.
- Stripping took place in the defined application zone so that intercellular spaces and number of cellular layers could be observed by scanning electron microscope.

D0–D13: Each group of volunteers applied the cream containing O6FASC or twice-daily (morning and evening) placebo cream.

On DJ14:
- The volunteers returned to the laboratory, not having applied any product on their forearm since the previous evening.
- Stripping took place in the defined application zone defined on D0, so that intercellular spaces and number of cellular layers could be observed by scanning electron microscope.
The objective of this study is to evaluate on subjects, the soothing effect of product O3FASC (Dermscan, Lyon, France) vs. placebo after 28 days of use, by a test with capsaicin. The experimental plan was a comparative, double blind and intra-individual study; each subject was his/her own control. The evaluation of the anti-itching test with O3FASC was carried out according to the reference ‘stinging-test’ which involves measuring the reactivity of the skin to an irritant agent using capsaicin as the irritant substance (Dermscan).

### Protocol:
- Determination of the capsaicin concentration triggering irritation in volunteers presenting with sensitive/reactive facial skin and allowing skin reactivity to be assessed.
- Cleansing of/removal of make-up from the nose wings by five successive applications of a 10% ethanol hydro-alcoholic solution.
- Three minutes later, simultaneous application to each nose wing. The test was stopped if the subject complains of uncomfortable sensations.
- Three minutes later, simultaneous application of solution C1 (hydro-alcoholic solution containing $1.25 \times 10^{-4}$% capsaicin) to the nose wings in accordance with the randomization list stipulating the sides of application. The test was stopped if the subject complains of uncomfortable sensations and level C1 was attributed to the volunteer as a capsaicin detection threshold. If the subject has not reported specific sensations, the test was continued with the same randomized application procedure but on this occasion using solution C2 (hydro-alcoholic solution containing $1.10^{-3}$% capsaicin).

At 30 s, 1 min 30 s and 5 min, the sensations of the subjects were evaluated with the following scale:
- 0: no sensation
- 1: dubious to hardly perceptible sensation
- 2: slightly perceptible sensation
- 3: moderately perceptible sensation
- 4: markedly perceptible sensation
- 5: painful sensation

A reactivity score of reactivity was calculated according to the following formula:

$$\text{Reactivity score} = \frac{\sum \text{scores of capsaicin side}}{\sum \text{scores of hydro-alcoholic solution side}}$$

The study was carried out on 40 subjects (2 groups of 20 subjects) with specific criteria: female between 18–60 years old and photoype I to IV, presenting a sensitive and reactive skin on the face.

**On D0:**
- The volunteers ($n = 14$) came to the laboratory without having applied any cosmetic products to the face since the previous evening (except the morning toilet).
- Implementation of the capsaicin test.
- Distribution of the 1% O3FASC and placebo product to volunteers who applied it twice a day (in the morning and in the evening) on the whole face.

**On D28:** (the last application of the product was carried out in the previous evening).

### Table II Composition of placebo and O6FASC creams

<table>
<thead>
<tr>
<th>Cream type</th>
<th>INCI name</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo cream</td>
<td>Montane 60 Sorbitan stearate</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Montanox 60 Polysorbate 60</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Miglyol 812 Caprylic/capric triglyceride</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>Demineralized water Aquav</td>
<td>qs 100</td>
</tr>
<tr>
<td></td>
<td>Sépicide CI Imidazolidiny lurea</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Phénonip Phenoxethanol/methylparaben/ethylparaben/buty]paraben/propylparaben/isobutylparaben</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Ultrez 10 Carborner</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>NaOH solution (10%) Sodium hydroxide</td>
<td>0.25</td>
</tr>
<tr>
<td>O6FASC cream</td>
<td>Montane 60 Sorbitan stearate</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Montanox 60 Polysorbate 60</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Miglyol 812 Caprylic/capric triglyceride</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>O6FASC Safflower oil/palm oil aminopropanediol esters</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Demineralized water Aquav</td>
<td>qs 100</td>
</tr>
<tr>
<td></td>
<td>Sépicide CI Imidazolidiny lurea</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Phénonip Phenoxethanol/methylparaben/ethylparaben/buty]paraben/propylparaben/isobutylparaben</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Ultrez 10 Carborner</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>NaOH solution (10%) Sodium hydroxide</td>
<td>0.25</td>
</tr>
</tbody>
</table>
The volunteers returned to the laboratory without having applied any cosmetic product to the face since the previous evening (except the morning toilet).

A second new test of capsaicin was carried out by the technician in charge of the study in the same conditions that of D0.

The formulation of O3FASC cream are detailed in Table III (the formula of the placebo is the same without the O3FASC).

**In vivo elasticity – firmness test for the O9FASC:**

The firmness and restructuring activity has been realized on 20 volunteers for 56 days. Volunteers were women, 40–70 years old, with a dry and damaged skin. Evaluation of non-inclusion and compliance have been defined and controlled before and during the test. The test has been realized on cheekbones, one side with the placebo (cream without the O9FASC: Bio-EC, Longjumeau, France) and the other side the same cream with the O9FASC (Bio-EC) (Table IV).

### Table III Composition of O3FASC cream

<table>
<thead>
<tr>
<th>O3FASC</th>
<th>%</th>
<th>INCI name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tégo care PS</td>
<td>4.00</td>
<td>Methyl glucose sesquistearate</td>
</tr>
<tr>
<td>Nacol 22/98</td>
<td>2.00</td>
<td>Behenyl alcohol</td>
</tr>
<tr>
<td>Miglyol 812</td>
<td>4.00</td>
<td>Caprylic/capric triglyceride</td>
</tr>
<tr>
<td>DC 200 V 100</td>
<td>2.00</td>
<td>Dimethicone</td>
</tr>
<tr>
<td>O3FASC</td>
<td>1</td>
<td>Linseed oil/palm oil aminopropanediol esters</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>qs 100</td>
<td>Aqua</td>
</tr>
<tr>
<td>Glycéline</td>
<td>2.00</td>
<td>Glycerin</td>
</tr>
<tr>
<td>Ketrol CG-SFT</td>
<td>0.30</td>
<td>Xanthan gum</td>
</tr>
<tr>
<td>Phénonip</td>
<td>0.50</td>
<td>Phenoxyethanol/ethylparaben/butylinparaben/propylparaben/isobutylinparaben</td>
</tr>
<tr>
<td>Sépiplus 305</td>
<td>1.00</td>
<td>Polycrylamide/C13-14 isoparaffin/Laureth-7</td>
</tr>
<tr>
<td>Acide lactic</td>
<td>0.10</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>Sépidie CI</td>
<td>0.20</td>
<td>Imidazolidinyurea</td>
</tr>
<tr>
<td>Parfum Pamplest</td>
<td>0.15</td>
<td>Parfum</td>
</tr>
</tbody>
</table>

**O3FASC, Omega 3 fatty acid stabilized compound.**

| Table IV Composition of O9FASC cream

<table>
<thead>
<tr>
<th>O9FASC</th>
<th>%</th>
<th>INCI name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulium delta</td>
<td>5.00</td>
<td>Cetyl alcohol/glyceryl stearate/PEG-75 stearate/ceteth-20/steareth-20</td>
</tr>
<tr>
<td>Nacol 22/98</td>
<td>2.00</td>
<td>Behenyl alcohol</td>
</tr>
<tr>
<td>Isofil 20</td>
<td>3.00</td>
<td>Octyldecenol</td>
</tr>
<tr>
<td>Miglyol 812</td>
<td>3.00</td>
<td>Caprylic/capric triglyceride</td>
</tr>
<tr>
<td>DC 200 V 100</td>
<td>2.00</td>
<td>Dimethicone</td>
</tr>
<tr>
<td>O9FASC</td>
<td>1.00</td>
<td>Olive oil/aaminopropanediol esters</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>qs 100</td>
<td>Aqua</td>
</tr>
<tr>
<td>Phénonip</td>
<td>0.50</td>
<td>Phenoxyethanol/ethylparaben/butylinparaben/propylparaben/isobutylinparaben</td>
</tr>
<tr>
<td>Butylène glycol</td>
<td>3.00</td>
<td>Butylen glycol</td>
</tr>
<tr>
<td>Ketrol CG-SFT</td>
<td>0.30</td>
<td>Xanthan gum</td>
</tr>
<tr>
<td>Sépidie CI</td>
<td>0.20</td>
<td>Imidazolidinyurea</td>
</tr>
<tr>
<td>Sépiplus 265</td>
<td>0.20</td>
<td>Ammonium acrylate and acrylamide copolymer/Polysobutene/Polysorbate 20</td>
</tr>
<tr>
<td>Parfum 40416-01</td>
<td>0.20</td>
<td>Fragrance</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.013</td>
<td>Lactic acid</td>
</tr>
</tbody>
</table>

**O9FASC, Omega 9 fatty acid stabilized compound.**

The study was performed using the Cutometer® MPA 580 (Courage & Khazaka, Cologne, Germany). The measuring principle was based on the suction method. Negative pressure was created in the device and the skin was drawn into the cylindrical aperture of the probe (2 mm in diameter).

Inside the probe, the penetration depth was determined by an optical measuring system. Each suction phase was followed by a relaxing phase (Fig. 1). The resistance of the skin to be sucked up by the negative pressure and its ability to return to its original position are displayed as curves at the end of each measurement (Fig. 2). From these curves, these parameters can be calculated.

- During the suction phase, the deformation of the skin occurs in two steps: an instantaneous path measuring sheer elastic forces (Ue) and a delayed path corresponding to the viscoelastic...
displacement i.e. to tissue fluidity (Uv). R6 represents the ratio Uv/Ue; it decreased when the skin elasticity improved, and increased when the viscous component increased.

- The same was observed during the relaxation step.

R7 represents the ratio Ur/Uf. R7 increased when the immediate recovery came close to the starting point, i.e. when skin firmness improved.

In addition, microscopic observation of the SC has been realized to get images of the cutaneous microdepressionary network. Rapid polymerization of cyanoacrylate on the surface of the skin (on cheekbones) allows to take without pain the external layers of SC (four to six corneocytes layers). Each sample was observed with optical microscopy (2×, 5 objective), lighted laterally with cold light; the observation led to define a score from 1 to 12 depending on the parameters of microdepressionary network (Fig. 3).

**Results**

**Biosynthesis**

A ceramide is composed of sphingosine or phytosphingosine and a fatty acid. The most ceramides found in the skin are presented below (Fig. 4). All have an amide function with non-hydroxylated, \(\alpha\)-hydroxylated or \(\omega\)-hydroxylated acids. A modelling system has been realized on the natural ceramide structure and revealed the presence of three key points in the molecule: a polar head and two lipophilic carbon chains, one of which is linked by amide function to the polar section of the molecule (Fig. 5).

Moreover, previous works realized in the laboratory indicate that PUFAs can be stabilized on an amide form. We thus expected that Omega 6 and Omega 3 could be stabilized on the lipophilic carbon chain, and to mime ceramide structure, palmitic acid can be integrated on the above lipophilic chain. For this synthesis, we suggested an original biotechnological solvent-free process (Fig. 6) [29, 30].

Starting from an oil rich in Omega 6 or Omega 3 fatty acid, the first step consists of an amidation using a lipase. Better results were obtained if the oil was transformed in fatty acid ethyl esters before proceeding. The intermediate was engaged in the second step for transesterification with a lipase.

The two-step process was realized under vacuum without any solvent at 65°C. The reduced pressure led to the removal of water or alcohol, formed progressively during the condensation, and thus significantly accelerated the kinetics of the
reaction. Moreover, the reduced pressure limited the degradation of the oxidizable fatty acid as well as the low temperature used. The use of immobilized enzymes (absorbed on a macroporous resin) allowed them to be easily removed from the reaction medium and to be then recycled. Conversion rate was greater than 99% with perfect selectivity (over 99%). (Table V).

The use of lipase as biocatalytic enzyme revealed to be a partner of choice for the synthesis of this type of sensitive molecule and offered the possibility to act in wild conditions. The easiness of this two-step synthesis, starting from vegetal raw materials, without any protection step, any solvent, or purification step, made these molecules partners of choice for new interesting ceramide analogues. The biotechnological process being successful, we focused our work in studying the possible stabilization of linoleic (Omega 6) and α-linolenic (Omega 3) acids on this amide form.

Figure 4 Natural cutaneous ceramides.

Figure 5 Modelling system of ceramide.

Figure 6 Synthesis of Omega 9 fatty acid stabilized compound.
and the biological activities of each of the new derivatives.

Stabilization of polyunsaturated fatty acids

It is known that linoleic acid and α-linolenic acid are polyunsaturated molecules, very sensitive to free radicals (coming from the environment). The stability of linoleic acid and α-linolenic acid which are fragile molecules was investigated by accelerated oxidation using the Rancimat test and excellent stabilization of the linoleic acid and α-linolenic acid was observed in the linoleyl and linolenyl fatty acid stabilized compound form (Table VI). If the modelling system is correct, the synthesis of these new derivatives should give some interesting physical properties linked to the structure of the molecule.

Structural activities (physical properties)

The restructuring effect was observed by scanning electron microscopy. On D0, the samples collected after strippings comprised numerous cell layers (at least 4 to 5, but often more) and relatively large interleaved spaces. This result was anticipated given the condition of the forearms selected for this study. On D14, a substantial improvement in cell cohesion was observed in 100% of volunteers treated with the cream containing the FASC. The samples were considerably less dense than on D0 and some slides tended to show single layers unlike the placebo cream. In addition, the interleaved

Figure 7 $^1$H NMR spectra of Omega 9 fatty acid stabilized compound.
spaces had totally disappeared in most cases. Volunteers treated with the cream containing the FASC presented with considerably improved cell cohesion, contrary to the placebo cream. Given his/her experience with this type of test, the evaluator concluded that the cream containing the O6FASC has a restructuring effect that the placebo cream does not have (Fig. 8). After having demonstrated the restructuring effect of these new derivatives, biological activities linked to the fatty acid structure have been studied for each of the O6FASC, O3FASC and O9FASC.

Biological activities

Anti-inflammatory effect of O6FASC
Linoleic acid also possesses anti-inflammatory activity [31–33]. It is therefore interesting to check whether these properties are preserved in the synthesized molecule. The results are presented in the presence of a non-stimulated control and also with the PMA as the positive control. Two placebos were used because one of them triggered an increase in IL1β release which was certainly linked to an irritant effect of one of the cream components. The results nevertheless illustrate a substantial reduction in IL release in the presence of the O6FASC. Similar results were observed with the second cream, the only exception being that the placebo did not trigger any initial irritation (Table VII).

Attenuation of cutaneous stress of O3FASC
The role of O3FASC in the attenuation of stress in cutaneous view has been demonstrated. To our knowledge, no studies has been realized in this field by any omega 3 compound. The O3FASC can decrease electrical activity of sensitive neurones after stress simulation (see Fig. 9). This activity has been measured by visualization of calcic flashes with fluorescence (Fig. 10). Moreover, for neurones sensitive to CGRP liberation, the O3FASC is able, with stress conditions, to inhibit the aggressive liberation of CGRP (Fig. 11). Without stress, the presence of O3FASC leads to the restoration of basal neuronal communication disturbed by the vehicle BSA (Fig. 12). To complete these results, and as cutaneous problems can occur because of stress, our studies have been focused then on acne. When applied in a cosmetic formula, the O3FASC is able to reduce the activity of 5α-reductase (the study has been as well realized with linseed oil, rich in omega 3 fatty acid, as a positive control; see Table VIII). Moreover, com-

<table>
<thead>
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<th>Table VII</th>
<th>Attenuation of cutaneous stress of O3FASC</th>
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<tbody>
<tr>
<td></td>
<td>Average IL1β, pg ml⁻¹</td>
</tr>
<tr>
<td>Non-stimulated control</td>
<td>63.3 ± 40</td>
</tr>
<tr>
<td>PMA</td>
<td>198.3 ± 16</td>
</tr>
<tr>
<td>Placebo 1</td>
<td>303 ± 19</td>
</tr>
<tr>
<td>O6FASC</td>
<td>126 ± 43</td>
</tr>
<tr>
<td>Placebo 2</td>
<td>166.8 ± 77</td>
</tr>
<tr>
<td>O6FASC</td>
<td>99.8 ± 25</td>
</tr>
</tbody>
</table>

IL, interleukin; PMA, phorbol myristate acetate; O6FASC, Omega 6 fatty acid stabilized compound; O9FASC, Omega 3 fatty acid stabilized compound.
pared with an oil rich in omega 3 fatty acid (linseed oil), the derivative was more active in low concentrations. *In vivo* test has been realized on the O3FASC to visualize the irritation limitation for skin under stress. After irritation with capsacin at J28, there was a decrease of 53% of the reactivity score to the irritation for the formula with 1% O3FASC (whereas for the placebo the decrease was only 9%) (see Fig. 13).

| Placebo | 100 |
| 0.25% Linseed oil (rich in Omega 3) | 104 |
| 0.5% O3FASC | 76 |

O3FASC, Omega 3 fatty acid stabilized compound. NB: O3FASC 0.5% has the same concentration of α-linolenic acid as linseed oil 0.25%.

**Figure 9** Visualization of electric activity of sensitive neurons.

**Figure 10** Percent of fluorescence variation linked to visualization of calcic flashes.

**Figure 11** Measurement of CGRP released after capsacin stimulation. CGRP, calcitonin gene-related peptide.

**Figure 12** Measurement of spontaneous CGRP released. CGRP, calcitonin gene-related peptide.

**Table VIII** Activity of 5α-reductase with O3FASC

<table>
<thead>
<tr>
<th>Activity of 5α-reductase (%)</th>
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<tbody>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>0.25% Linseed oil (rich in Omega 3)</td>
</tr>
<tr>
<td>0.5% O3FASC</td>
</tr>
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</table>

**Figure 13** Reactivity score to capsacin.
Anti-elastase activity of O9FASC

Following this general biotechnological process with the use of other oils, we developed an O9FASC, starting from an oil rich in oleic acid (for example, olive oil). In this case, we decided to replace the palmitoyl oil used in the second step by another oil rich in oleic acid. Figure 14 shows the two-step enzymatic synthesis. Once O9FASC is synthesized, the role of this molecule in the inhibition of elastase activity has been demonstrated. Pre-treatment of the O9FASC (1% has been applied), and the 24 h contact with elastase, the O9FASC can inhibit the activity of elastase, with 80% protection regarded to the placebo; and the inhibition was greater than the one observed for the oleic acid (62% protection regarded to the positive reference; see Fig. 15) the visualization of elastic fibers on the deep reticular dermis of explants, at day 6, 24 h after elastase addition. Figure 16 gives the interpretation of the results in percentage. Thanks to this original structure of the O9FASC, the Omega 9 was clearly vectorized through the epidermis and through the dermis to reach the deep reticular dermis. Moreover, the efficacy of the O9FASC in firmness and restructuring effect has been studied on 20 volunteers. After 56 days, the R6 parameter was measured. This parameter represents the skin elasticity. The more the value was low, the better the elasticity of your skin. If 4% decrease was observed for the placebo, regarding to T0, the O9FASC presents 14%
decrease, which means 10% improvement in elasticity of O9FASC regarding to the placebo (Fig. 17). After 56 days, the R7 parameter was measured. This parameter represents as well the skin elasticity. The more the value increased, the better the firmness of your skin. If 15% increase was observed for the placebo, regarding to T0, the O9FASC presented 27% increased, which means 12% improvement of firmness of O9FASC regarding to the placebo (Fig. 18). After 56 days, the elasticity and the firmness have been improved using the cream containing O9FASC regarding to the placebo. In addition, microscopic observation of the SC samples has been realized to get image analysis of the cutaneous microdepressionary network.

Comparing the improvement of the cutaneous microdepressionary network of the placebo formula and the one with 1% O9FASC, better results were obtained for the O9FASC formula (+14% improvement of cutaneous microdepressionary network) (See Fig. 19). The images of volunteer 13 (on the same skin area) illustrate the improvement of cutaneous microdepressionary network after 56 days of application of the formula containing 1% of the O9FASC (Fig. 20). This report clearly shows a general strategy for the synthesis of new fatty acid derivatives with cutaneous application linked (i) to the structure of the molecule and (ii) to the fatty acids biological activity.

**Conclusions**

Thanks to molecular modelling, new interesting molecules for cutaneous applications were
designed using biotechnological process from vegetal raw materials. As expected with the molecular modelling, these new compounds show physical skin property (restructuring effect of the SC cement). For cosmetic applications, the use of PU-
FAs (linoleic, ω- linolenic) in different forms as oils, ethyl esters or free fatty acids, represents some difficulties faced with oxidation, even in long time in association with antioxidant like tocopherol. We suggest by introducing in the structure of the molecule, unsaturated fatty acid compounds, an original way to stabilize such very fragile compounds. This new methodology seems to present an excellent way to vectorize some active compounds such as PUFA. Some further studies are still undergoing in order to validate these first results. Following up on this work, other applications linked to the maintainence of biological activity of active compounds in topic field need to be explored.

Acknowledgements

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