The conjugate of jasmonic acid and tetrapeptide as a novel promising biologically active compound†

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A novel biologically active compound, the conjugate of jasmonic acid and tetrapeptide, has been obtained. The newly synthesized conjugate was characterized using MS, NMR and FTIR spectroscopy. The purity and melting point of the conjugate were determined using a differential scanning calorimetry technique. The safety of topical application of the conjugate was theoretically estimated. The obtained compound may exhibit the activity of jasmonic acid as well as that of tetrapeptide, therefore it is expected to have a promising effect on the skin.

Topical applications of ointments, gels and emulsions containing therapeutics have become very popular as non-invasive methods for the delivery of active substances to the skin. The cooperation of dermatologists and pharmacists has allowed the development of a formulation from which the release of active substances is most efficient. However, the search for new active substances or modification of the already existing therapeutics that may exhibit even better effects on the skin is continuing. Jasmonic acid and its derivatives, called also jasmonates, have been widely used in both the pharmaceutical and cosmetic industries. Jasmonic acid (JA) is a plant hormone that regulates plant response to abiotic and biotic stresses as well as plant growth and development.1 Dalko disclosed a composition of (dihydro-) jasmonic acid derivatives suitable for treating excessive sebum secretion.2 Jasmonates have also been suggested as active ingredients of a pharmaceutical composition for stimulating epidermal renewal and smoothing skin texture.3 Jasmonates may also be used as depigmenting agents.4 It is believed that they may be used to stimulate the growth of human keratin fibres, to inhibit hair loss; therefore these compounds may be used, e.g., in therapy of different types of alopecia.5 Moreover, local administration of methyl jasmonate gave good results for the treatment of lichen planus and lentigomaligna of the face with mixed basal spinal cell carcinoma treatment.6 Jasmonates are also supposed to inhibit in vitro cell proliferation and to induce cell death in melanoma skin cancer.7 In vitro studies on the Episkin™ model proved that sodium tetrahydrojasmonate stimulates an increase in the amount of Ki67 (a nuclear protein that may be crucial for cellular proliferation processes) in the basal layer of the epidermis.8,9 Tran et al. have proposed the use of sodium tetrahydrojasmonate to exfoliate the skin and reduce visible signs of skin aging.10 In vivo studies have shown that LR2412 reversed steroid-induced atrophy of the skin and improved the deposition of fibrillin-rich microfibrils in the papillary dermis. According to the in vitro release studies of an LR2412 solution using a Franz diffusion cell on excised human skin, after 24 h of study, a total of circa 6% of the applied dose was released to the skin. This result has stimulated the search for a derivative of jasmonic acid that would show a higher percentage of release in in vitro release studies and hence show greater bioavailability than commercial sodium tetrahydrojasmonate.10 Other innovative active ingredients that have been widely used in pharmaceutical and skincare products are bioactive peptides such as Tyr-Pro-Phe-Phe-NH₂ (YPFF). This tetrapeptide is an agonist of opioid receptors and reduces neurogenic hyperreactivity associated with the release of the neuropeptide CGRP (calcitonin gene related peptide).11 As a commercial product, Skinasensyl® is a synthetic acetylated YPFF tetrapeptide (AcYPFF), which when applied to the skin weakens the stimulation of nerve endings. This results in a reduction in hypersensitivity of the skin.12-14 Jasmonic acid has been shown to occur in the conjugated form with a variety of amino acids (i.e. leucine, isoleucine and valine) in higher plants such as barley leaf tissue and arbuscular mycorrhizal barley roots.15 This finding prompted us to synthesize the conjugate of jasmonic acid and tetrapeptide. As mentioned above, both of these compounds provide beneficial effects on the skin, therefore we suggest that the newly obtained conjugate may be a promising active ingredient for topical applications. The aim of...
The synthesis of the JA–YPFF–NH₂ conjugate performed by LipoPharm.pl is presented in Fig. 1. The peptide YPFF–NH₂ was synthesized manually on the 0.3 millimole scale via a solid-phase method using AM RAM resin and the Fmoc/But procedure. The racemic jasmonic acid was purchased from TCI Europe N.V. and the amino-acid derivatives (Fmoc-L-Phe-OBt, Fmoc-L-Pro-OH, and Fmoc-L-Tyr(tBu)-OH) were purchased from Sigma-Aldrich. The precise amounts of amino acid derivatives and jasmonic acid used in the conjugate synthesis were as follows: 585.1 mg of Fmoc-L-Phe-OH, 506.1 mg of Fmoc-L-Pro-OH, and 371.1 mg of jasmonic acid to A was performed using DIC in DMF and the Fmoc deprotection was performed using a 20% solution of piperidine in DMF. After each deprotection of the amino group, a chloranil test was performed to detect the amino groups. The result of the test was positive; dark blue to green beads of the resin implied that there were free amino groups and the deprotection was performed successfully. After careful washing of the resin, the JA–YPFF–NH₂ conjugate was contemporarily cleaved from the resin, via acidic treatment using a mixture of TFA: H₂O : TIS (v/v, 92.5:2.5:5) for 1.5 hour at 25 °C. Fig. 1 presents the general synthesis scheme of the conjugate and does not include the stereoisomeric structures of jasmonic acid. However, there are two chiral centers at C-3 and C-4 and therefore four possible stereoisomeric forms of JA, (3S,4S)-JA or (+)JA, (3R,4R)-JA or (-)JA, (3S,4R)-JA, known as (−)-epi-JA, and (+)-3R,4S)-JA, known as (+)-epi-JA, that may be generated. It was investigated that commercially available jasmonic acid is a racemic mixture containing approximately 3–5% each of (+)-epi-JA and (−)-epi-JA, and 45–47% each of (+)-JA and (−)-JA. No pure JA stereoisomers have been commercially available so far. Therefore it can be concluded that the obtained product is in the majority a mixture of (+)-JA and (−)-JA conjugates. Both forms are biologically active, however (−)-JA is believed to be the more biologically active compound.

The desired product was purified using RP-HPLC. In this process a Phenomenex Luna column (5 μm, C18(2), 100 Å, 10 × 250 mm) was employed. As a mobile phase water and a mixture of acetonitrile and 0.1% of TFA were used in the gradient elution. The purity of the obtained conjugate was >97%. The conjugate of jasmonic acid and tetrapeptide YPFF-NH₂ is a white powder with poor solubility in water, but a high solubility in organic solvents such as methanol and ethanol.

To confirm the molecular weight of the conjugate, the mass spectra were recorded using a hybrid QTOF instrument (AB Sciex, model 5600+). Ions were generated using electrospray ionization (ESI) under the following conditions: a flow rate of 10 ml min⁻¹, a dry gas flow of 8 L min⁻¹, a nebulizer pressure of 1.5 bar, a spray voltage of 5500 V, and a drying gas temperature of 250 °C. Analyst 1.6TF software (AB Sciex) was used to process the raw spectra. The MS spectrum was obtained in the positive mode. The conjugate of JA and tetrapeptide was successfully identified using mass spectrometry. The positive ion mode presents a peak of a protonated molecular ion [M + H]+ at m/z 765.39. Additionally, a molecular ion with associated sodium [M + Na]+ at m/z 787.37 was detected (Fig. 2).

Fourier-transform infrared spectroscopy was carried out using a Nicolet FTIR 200 spectrophotometer (Thermo Fisher Scientific, Inc., USA). The spectrum was collected in the wave-length range of 500–4000 cm⁻¹. The typical chemical bonds of the synthesized compound were successfully characterized. FTIR measurements of JA–YPFF–NH₂ and the identified functional groups are presented in Fig. 3. The band at about 3302 cm⁻¹ corresponds to O–H stretching vibrations of the OH group. The band at about 3028 cm⁻¹ is assigned to —C–H
strectching vibrations. Moreover, at 2964 cm\(^{-1}\) the stretching vibrations of C–H were identified. The C=C–O group was manifested as a single band at about 1738 cm\(^{-1}\) region, whereas the C=C–H and C=C–O groups were manifested as a single band at about 1738 cm\(^{-1}\). Moreover, at 2964 cm\(^{-1}\), 1716 cm\(^{-1}\), 1676 cm\(^{-1}\), 1574 cm\(^{-1}\), 1508 cm\(^{-1}\), 1480 cm\(^{-1}\), 1408 cm\(^{-1}\), 1367 cm\(^{-1}\), 1340 cm\(^{-1}\), 1301 cm\(^{-1}\), 1291 cm\(^{-1}\), 1240 cm\(^{-1}\), 1191 cm\(^{-1}\), 1161 cm\(^{-1}\), 1110 cm\(^{-1}\), 1070 cm\(^{-1}\), 1023 cm\(^{-1}\), 984 cm\(^{-1}\), 934 cm\(^{-1}\), 873 cm\(^{-1}\), 853 cm\(^{-1}\), 793 cm\(^{-1}\), 784 cm\(^{-1}\), 743 cm\(^{-1}\), 700 cm\(^{-1}\), 682 cm\(^{-1}\), 654 cm\(^{-1}\), 635 cm\(^{-1}\), 604 cm\(^{-1}\), 562 cm\(^{-1}\), 513 cm\(^{-1}\), and 498 cm\(^{-1}\) vibrations of C–H were identified. The C=C–O vibrations of C–H were identified. The C=C–O vibrations of C–H were identified. The C=C–O vibrations of C–H were identified.

To confirm the structure of JA–YPFF–NH\(_2\), its nuclear magnetic resonance (NMR) spectra were recorded using a NMR spectrometer (Bruker Avance 600 MHz; Bruker, UK). The conjugate sample was dissolved in chloroform-d (Sigma-Aldrich). The number of the atoms in the structure scheme (Fig. 4) was produced in order to present the NMR spectroscopy results. The \(^1\)H NMR shift for CDCl\(_3\) is 7.24 ppm and its \(^13\)C NMR shift is 77.0 ppm.

**Stereoisomer 1**

\(^1\)H NMR (CDCl\(_3\), 600 MHz): \(\delta H 0.90 (3H, t, H-10), 1.29 (2H, m, H-5), 1.62 (2H, br, H-23), 1.70–1.78 (1H, m, H-3), 1.85 (2H, br, H-24), 1.98 (2H, quint, H-9), 2.01–2.06 (2H, m, H-11), 2.24 (2H, br, H-6), 2.27 (1H, br, H-4), 2.32 (2H, br, H-5), 2.47 (2H, br, H-1), 2.89 (2H, br, H-28), 2.96 (2H, br, H-37), 3.19 (2H, br, H-14), 3.67 (2H, br, H-22), 4.37 (1H, br, H-25), 4.70 (1H, br, H-27), 4.78 (1H, br, H-36), 4.88 (1H, br, H-13), 5.14–5.20 (1H, m, H-7), 5.33–5.48 (1H, m, H-8), 6.61–6.83 (2H, m, H-16, H-20), 6.94 (2H, br, H-17,H-19), 7.03–7.26 (10H, m, H-30-H-34 and H-39-H-43), 7.31 (1H, s, H-45), 7.53 (1H, s, H-48), 7.93 (1H, s, H-47).

\(^13\)C NMR (CDCl\(_3\), 600 MHz): \(\delta C 14.06 (C-10), 20.52 (C-9), 24.55 (C-23), 25.36 (C-6), 26.60 (C-5), 28.55 (C-24), 37.15 (C-14), 37.57 (C-11), 38.16 (C-37), 38.29 (C-28), 38.82 (C-4), 40.2 (C-11), 40.50 (C-1), 47.66 (C-22), 52.29 (C-13), 53.98 (C-36), 53.76 (C-3), 54.55 (C-27), 60.55 (C-25), 115.7 (C-17, C-19), 124.79 (C-7), 125.72–129.15 (C-30-C-34, C-39-C-43), 129.37 (C-15), 130.50 (C-16, C-20), 134.02 (C-8), 135.85 (C-29, C-37), 155.81 (C-18), 171.22 (C-26), 171.58 (C-35), 172.01 (C-21), 172.12 (C-44), 173.39 (C-12), 220.30 (C-2).

**Stereoisomer 2**

\(^1\)H NMR (CDCl\(_3\), 600 MHz): \(\delta H 0.86 (3H, t, H-10), 1.29 (2H, m, H-5), 1.62 (2H, br, H-23), 1.70–1.78 (1H, m, H-3), 1.85 (2H, br, H-24), 1.98 (2H, quint, H-9), 2.01–2.06 (2H, m, H-11), 2.24 (2H, br, H-6), 2.27 (1H, br, H-4), 2.32 (2H, br, H-5), 2.47 (2H, br, H-1), 2.89 (2H, br, H-28), 2.96 (2H, br, H-37), 3.19 (2H, br, H-14), 3.52 (2H, br, H-22), 4.37 (1H, br, H-25), 4.70 (1H, br, H-27), 4.78 (1H, br, H-36), 4.88 (1H, br, H-13), 5.23–5.30 (1H, m, H-7), 5.33–5.48 (1H, m, H-8), 6.61–6.83 (2H, m, H-16, H-20), 6.94 (2H, br, H-17,H-19), 7.03–7.26 (10H, m, H-30-H-34 and H-39-H-43), 7.31 (1H, s, H-45), 7.53 (1H, s, H-48), 7.93 (1H, s, H-47).

\(^13\)C NMR (CDCl\(_3\), 600 MHz): \(\delta C 14.13 (C-10), 20.52 (C-9), 24.57 (C-23), 25.39 (C-6), 26.76 (C-5), 28.55 (C-24), 37.26 (C-14), 37.66 (C-11), 38.16 (C-37), 38.29 (C-28), 38.82 (C-4), 40.2 (C-11), 40.60 (C-1), 46.83 (C-22), 52.34 (C-13), 54.08 (C-36), 53.66 (C-3), 54.60 (C-27), 60.55 (C-25), 115.95 (C-17, C-19), 124.94 (C-7), 125.72–129.15 (C-30-C-34, C-39-C-43), 129.41 (C-15), 130.38 (C-16, C-20), 134.09 (C-8), 136.18 (C-29, C-37), 156.28 (C-18), 171.22 (C-26), 171.58 (C-35), 172.01 (C-21), 172.12 (C-44), 173.52 (C-12), 220.1 (C-2).

The melting point of the conjugate crystals was determined using differential scanning calorimetry (DSC) using a differential scanning calorimeter (Q2000; TA Instruments, USA). The conjugate sample was weighted on an aluminium pan which was then hermetically closed and placed into a DSC analyser. The initial and final temperatures of the measurement were 0 and 100 °C, respectively, and the temperature ramp was 5 °C min\(^{-1}\). The results of the DSC study are plotted with the percentage heat flow on the Y-axis and temperature on the X-axis. The DSC device is designed to maintain the test sample and the reference sample at the same temperature when they are heated. The precise and high quality data obtained from DSC provide information on the thermal stability of the samples in process development and in the formulation of potential therapeutics. Moreover, the knowledge of the melting point of the active compounds is essential in order to select the temperature for...
In vivo skin irritation before performing it is important to investigate whether this substance may cause corrosive to the skin. Moreover, this estimation gave a negative response to both genotoxic and nongenotoxic carcinogenicity of the conjugate. Therefore, it can be concluded that JA–YPFF–NH₂ may be used in topical applications and should cause no damage to skin appearance, condition and health. Therefore, it may be a promising active ingredient to add to topical formulations.

Since the conjugate is not highly soluble in water, we suggest that ethanol could be used as a solvent for the topical use of the active compound. This solvent is widely used in topical applications in cosmetology, dermatology and pharmacy. The excessive use of ethanolic solutions on the skin may cause dryness. Therefore, we suggest using a small amount of ethanol to dissolve the conjugate (e.g., 200 μl of EtOH per 5 mg of JA–YPFF–NH₂) and to mix the obtained ethanolic solution of the conjugate with other components of pharmaceutical formulations. Moreover, ethanol is a well-known skin penetration enhancer. Furthermore, the conjugate could also be encapsulated into nanocarriers or mixed with an oily phase of the pharmaceutical formulation, as long as the temperature during the preparation process does not exceed 35–40 °C (a higher temperature may cause the risk of conjugate degradation). Characterization of the newly obtained conjugate JA–YPFF–NH₂ was carried out successfully. The theoretical mass of the obtained compound was confirmed using MS spectrometry. The structure of JA–YPFF–NH₂ was confirmed using NMR spectroscopy and the typical chemical bonds of the conjugate were structuring chemicals in order to make a TTC estimation is the most popular approach for structuring chemicals in order to make a TTC estimation is the Cramer classification tree. The Toxtree software was evolved to implement diverse rule-based estimation approaches and was commissioned by the European Chemicals Bureau (ECB).

Prior to in vitro and in vivo studies the potential risk of topical application of the conjugate was theoretically assessed. In order to estimate the potential toxic hazard of JA–YPFF–NH₂, the open source application Toxtree was used. The results of this estimation show that the conjugate of jasmonic acid and tetrapeptide does not include skin sensitization reactivity domains and is not corrosive to the skin. Moreover, this estimation gave a negative response to both genotoxic and nongenotoxic carcinogenicity of the conjugate. Therefore, it can be concluded that JA–YPFF–NH₂ may be used in topical applications and should cause no damage to skin appearance, condition and health. Therefore, it may be a promising active ingredient to add to topical formulations.

The potential toxic hazard of JA–YPFF–NH₂ was estimated using the application Toxtree. This application makes structure-based predictions for a number of toxicological endpoints. The conjugate is intended for topical applications. Therefore, it is important to investigate whether this substance may cause skin irritation before performing in vivo studies on volunteers. Toxtree analysis is based on the Threshold of Toxicological Concern (TTC) concept designed to establish a safety level of exposure to chemical compounds. The most popular approach for structuring chemicals in order to make a TTC estimation is the Cramer classification tree. The Toxtree software was evolved to implement diverse rule-based estimation approaches and was commissioned by the European Chemicals Bureau (ECB).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Financial support from the Polish Ministry of Science and Higher Education is acknowledged. The authors would like to express their gratitude to Dr. Marta Krysmann (University of Central Lancashire, UK) for the support and cooperation with DSC studies. The authors also would like to thank Dr. Tomasz Pędziński (Adam Mickiewicz University in Poznan, Poland) for the support with the MS studies.

References and notes

24 M. E. Lane, *Int. J. Pharm.*, 2013, **447**(1–2), 12.