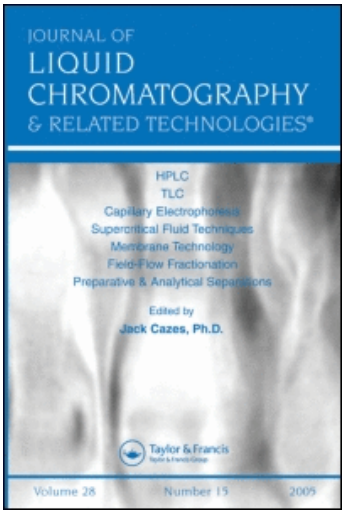

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Synthesis, Purification, and Activity of Salidroside

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Synthesis, Purification, and Activity of Salidroside

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Abstract: Our goal was to synthesise this compound by glucosylation of *p*-tyrosol. The 74% yield of the reaction was not satisfactory and instead of optimising the procedure, we preferred to purify the product. This was achieved by high performance centrifugal partition chromatography with an optimised biphasic system followed by a solid phase extraction on a graphitic stationary phase. The final product was found to be pure at 95%. The structure of salidroside was confirmed by both NMR and ESIMS^2. Each ^{13}C NMR, ^{1}H NMR, and ESIMS^2 signals were attributed to the salidroside structure. The last step of this work was to assess the biological activity of the synthetic salidroside. It was found to be more biologically active than ginsenoside Rb1 on the ATP production rate of keratinocytes.

Keywords: *Rhodiola*, Synthesis, Purification, Salidroside, HPCPC, Porous graphitic carbon

INTRODUCTION

For the last decades, the interest of consumers for natural products and particularly traditional medicine plants has been growing. Plant extracts have been

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studied more and more and included in cosmetics and pharmaceuticals, amongst them the *Rhodiola* plants that are of special interest.

The genus *Rhodiola* consists in nearly 200 species, such as *Rhodiola rosea* L. *Rhodiola* grows naturally in dry sandy soils, in mountainous regions of North and Middle Europe and Asia.

In various traditional medicines (India, Russia, China) *Rhodiola* is used to increase resistance to high altitude stress, physical endurance and sickness, promote longevity, and to treat fatigue, depression, anaemia, impotence, gastrointestinal ailments, infections, and nervous system disorders. It is also known as an adaptogen.

The phytochemistry investigation of *Rhodiola* root has revealed the presence of six distinct groups of chemical compounds: Phenylpropanoid derivaties such as rosavin, rosin, rosarin; Phenylethanoid derivatives such as salidroside (rhodioloside), tyrosol; Flavanoids such as rodiolin, rodionin, rodiosin, acetylrodalgin, tricin; Monoterpernes such as rosiridol, rosaridin; Triterpenes such as daucosterol, β-sitosterol; Phenolic acids such as chlorogenic and hydroxycinnamic, gallic acids.

Salidroside is considered as one of the compounds responsible for the pharmacological properties of *R. rosea*.[1–5] Therefore, the *R. rosea* extracts approved by the Russian Pharmacopoeia Committee were standardized to a minimum of 0.8% salidroside content.[6]

The aim of this work was to synthesise salidroside, to purify the synthesis product by counter current chromatography and identify it before the evaluation of its biological properties. The synthetic salidroside will then be used notably as a pure standard in biological tests and in analytical methods.

EXPERIMENTAL

General Methods

NMR spectra were recorded in $CDCl_3$ or CD_3OD solutions at 300°K using a Brüker Avance DRX 500 spectrometer equipped with a Brüker CryoPlatform and a 5 mm cryo TXI probe. The temperature of the probe and preamplifier was 30°K. Chemical shifts were referenced to CD_3OD: $\delta_H = 3.31$ ppm, $\delta_C = 49.0$ ppm. Resonance multiplicities for ^{13}C signals were established via the acquisition of DEPT spectra. For two-dimensional experiments, Brüker microprograms using gradient selection (gs) were applied. gs-COSY spectra were obtained with an F_2 spectral width of 10 ppm and 2 K data points and an F_1 spectral width of 256 t_1 increments with sine-bell windows in both dimensions. The gs-HMQC spectra resulted from 256 × 1024 data matrix size with 2–16 scans per t_1 depending on the sample concentration, an inter-pulse delay of 3.2 ms, and a 5:3:4 gradient combination. gs-HMBC spectra were measured using a pulse sequence optimized for 10 Hz (interpulse delay for the evolution of long-range couplings 50 ms) and the same gradient ratios.

Electrospray ionisation mass spectrometry (ESIMS) was used to obtain the mass spectra of the salidroside. The ESIMS system used was a Quatro Ultima triple quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) equipped with a pneumatically assisted electrospray ionisation source. Data acquisition and processing were performed using the MassLynx 4.0 software. The analyte (salidroside with 50 mM of ammonia for the direct ESIMS analysis) was introduced into the mass spectrometer via the ESI probe with a Harvard Apparatus pump 11 (Harvard Apparatus, Massachusetts, USA) with a flow rate of 5 μL min^{-1}. Desolvatation and source temperatures were 350 and 125°C, respectively. Full scan mode was used in negative ionisation mode. Capillary voltage was set to −3.5 kV. The mass scan range was 100–300 amu, for 1 min total scan time, with 500 ms scan time, and 0.1 s inter-scan time. All mass spectra were smoothed twice using Savitzky Golay algorithm with 0.75 Da peak width.

ESIMS^2 experiments were performed with the same mass spectrometer. Collision gas was nitrogen and collision energy was 15 eV. The mass spectrometer was programmed to allow the $[M\text{-}H]^-$ ions of salidroside at m/z 299 to pass through the first quadrupole (Q1) into the collision cell (Q2). Full scan was acquired in the third quadrupole (Q3) in the range 100–300 amu, for 1 min total scan time, with 500 ms scan time, and 0.1 s inter-scan time.

HPLC was performed using Agilent HP 1100 apparatus with a UV detector. The analytical column used was a porous graphitic carbon Hypercarb (100 × 4.6 mm, 5μm) supplied by Thermo Electron (Shandon, UK). Mobile phase was constituted with (A) water containing 0.01% (v/v) of trifluoroacetic acid (TFA), (B) methanol containing 0.01% of TFA, (C) acetonitrile containing 0.01% of TFA. Elution was in gradient mode starting with A and B (50:50) from A-C (50:50) in 20 min to 100% C until 30 min. The flow rate was 1 mL/min and detection was performed at 275 nm. Filtered and deionised water was obtained from a UHQ system (Elga, High Wycombe, UK).

Synthesis of Salidroside

Glucosylation of p-Tyrosol with Ag_2CO_3

A mixture of p-tyrosol (0.83 g, 6 mmole) and Ag_2CO_3 (2.07 g, 7.5 mmole) in dry benzene-ether (9:1, 10 mL) was stirred at room temperature under N_2 atmosphere for 1.5 h, then a solution of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (2.06 g, 5 mmole) and Ag_2CO_3 (1.66 g, 6 mmole) in dry benzene (5 mL) was added rapidly to the mixture and stirred for one night. The reaction mixture was treated with saturated aqueous solution of $NaHCO_3$ and extracted with CH_2Cl_2. The organic layers were washed with brine, and then dried over

$MgSO_4$. Evaporation of the solvent afforded a residue, which was purified by column chromatography on silica gel using hexane-ether (2:1) as the eluent to afford **3** (1.72 g, 74%) as a colourless oil.

^{1}H NMR ($CDCl_3$) δ 1.91 (3H, s, OAc), 1.98 (3H, s, OAc), 2.02 (3H, s, OAc), 2.08 (3H, s, OAc), 2.80 (2H, t, $J = 7.1$ Hz, CH_2), 3.65 (2H, t, $J = 7.1$ Hz, CH_2), 3.69 (1H, m, C_5H), 4.10 (1H, d, $J = 12.0$ Hz and $J = 2.0$ Hz, C_6H), 4.23 (1H, d, $J = 12.0$ Hz and $J = 4.0$ Hz, C_6H), 4.46 (1H, d, $J = 7.0$ Hz, C_1H), 4.80-5.21 (3H, m, $C_{2,3,4}$-H), 6.71 (2H, d, $J = 9.0$ Hz, CH_{arom}), 7.02 (2H, d, $J = 9.0$ Hz, CH_{arom}).

Alkaline Hydrolysis of the β-Glycoside

K_2CO_3 (2.27 g, 16.4 mmole) was added to a solution of β-glycoside **3** (0.77 g, 1.64 mmole) in MeOH (10 mL). After stirring overnight, the reaction mixture was filtered through celite. The filtrate was neutralized with Amberlite IRA-120 to pH 6, concentrated under a reduced pressure, and the residue was chromatographed on silica gel using MeOH-$CHCl_3$ (1:4) as eluent to afford salidroside **4** (0.49 g, 100%).

^{1}H NMR (CD_3OD) see Fig. 4 δ 2.82 (2H, td, $J = 7.9$ and $J = 2.9$ Hz, $(CH_2)_5$), 3.16 (1H, dd, $J = 9.0$ and $J = 7.8$ Hz, H_8), 3.24 (1H, td, $J = 9.1$ and $J = 2.2$ Hz, H_{11}), 3.27 (1H, t, $J = 9.4$ Hz, H_{10}), 3.33 (1H, t, $J = 8.9$ Hz, H_9), 3.65 (1H, td, $J = 11.9$ and $J = 5.3$ Hz, H_{12a}), 3.67 (1H, td, $J = 9.6$ and $J = 6.9$ Hz, H_{6b}), 3.85 (1H, dd, $J = 11.9$ Hz and $J = 2.0$ Hz, H_{12b}), 4.02 (1H, ddd, $J = 9.6$ Hz and $J = 8.2$ Hz and $J = 7.0$, H_{6a}), 4.28 (1H, d, $J = 7.8$ Hz, H_7), 6.68 (2H, dm, $J = 8.5$ Hz, $2H_{2arom}$), 7.05 (2H, dm, $J = 8.5$ Hz, $2H_{3arom}$).

Purification of Salidroside

HPCPC Purification of Salidroside

An LLB high performance centrifugal partition chromatography (HPCPC) system (Sanki, Kyoto, Japan) was used for the purification of salidroside. The total column volume was 230 mL. This HPCPC system was equipped with a Shimadzu VP series solvent delivery pump (Shimadzu, Tokyo, Japan). A UV detector (Shimadzu) was installed at the outlet of the HPCPC before the collector (Advantec SF-2120, Dublin, CA, USA). The crude sample was injected through a 3 mL loop.

Shake flask experiments were performed to determine the enrichment constants E_c of the salidroside and p-tyrosol between the two phases of the biphasic tested system.[7,8] Various two-phase systems were prepared by stirring, at room temperature, hexane, water, acetonitrile, methanol, ethyl acetate, and trichloromethane before separating into upper and lower phases. To measure the distribution constants, an aliquot (100 μL)

of the reaction mixture was evaporated to dryness under nitrogen stream and dissolved in 1 mL of the upper (or lower) phase. The solution was shaken with an equal volume of lower (or upper) phase for 5 min. The two phases were then separated by centrifugation for 10 min. The concentrations of the molecules in each phase were determined by HPLC. The aqueous phase was diluted in the same volume of water before injection and the organic phase was evaporated to dryness under nitrogen stream and dissolved in the mobile phase. The enrichment constant E_c was calculated as follows:

$$E_c = [C]_{TYR}/[C]_{SAL} = A_{TYR}/A_{SAL}$$

Where $[C]_{TYR}$ and $[C]_{SAL}$ are the concentration of *p*-tyrosol and the salidroside, respectively; A_{TYR} and A_{SAL} are the HPLC peak areas for *p*-tyrosol and salidroside, respectively.

To separate salidroside from *p*-tyrosol, a two-phase solvent system composed of ethyl acetate-acetonitrile-water with volume ratios of 50/12.5/37.5, respectively was selected.

The mobile phase (aqueous phase) and the stationary phase (organic phase) were prepared by mixing the solvents until a biphasism occurred.

The HPCPC column was first entirely filled with the stationary phase with a rotation of the column at 200 rpm. Then the apparatus was rotated at 1200 rpm while the mobile phase was pumped through the column in descending mode at 4 mL/min. After the mobile phase front emerged and equilibrium was established in the column (76% retention of the stationary phase), 3 mL of the sample solution were then injected with a flow rate of 4 mL/min. The effluent of the column was continuously monitored with UV at 275 nm and the collected fractions (8 mL) were analysed by HPLC. At the end of the run, the stationary phase was pushed out from the column with compressed air, and methanol was pumped through the column at 10 mL/min with a 200 rpm rotor speed to wash the column.

SPE Cleanup Procedure

Some residual impurities were present in the CPC fractions. The solvent in the fractions was evaporated under nitrogen steam at 35°C. The dry residue was reconstituted in 10 mL of water. The ENVI-Carb SPE cartridge (500 mg, Bellefonte, PA, USA) was conditioned with 12 mL of methanol, then 12 mL of water. The sample was loaded on the cartridge and salidroside was eluted with 5 mL of a mixture of water and acetonitrile (50/50:v/v) whereas the impurities were retained by the stationary phase. The eluate was then evaporated at 50°C under reduced pressure.

Biological Assays

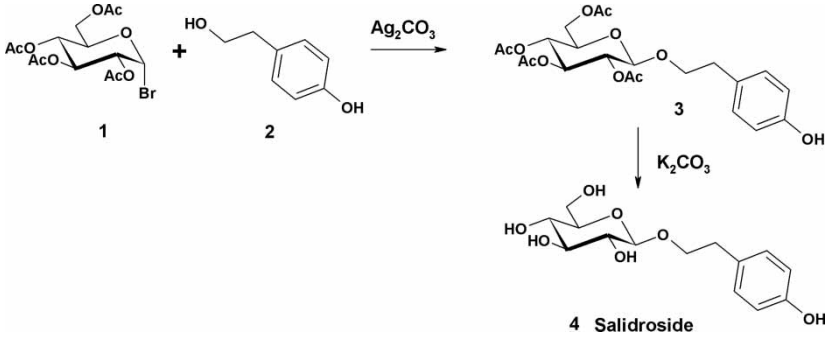
Resulting salidroside was tested to evaluate its biological activity on cultured cells. To perform growth experiments, cells were seeded (20,000 cells/well) in 96-well flat-bottomed plates (Cultureplates, Perkin-Elmer Life Science). After 24 h, the media were replaced, and after one wash those containing the tested products were added. Three independent experiments were performed in quadruplicate. After 72 h of culture in the presence of the tested compounds, the plates were harvested and the number of viable cells was estimated by dosing ATP using the ATPlite kit (Perkin-Elmer Life Science). The kit was employed following manufacturer's instructions.

RESULTS AND DISCUSSION

Synthesis and Purification

The synthesis of salidroside from *p*-tyrosol is described in Scheme 1. Salidroside (**4**) was obtained in 2 steps following the procedure described by Endo et al.[9] The condensation of acetobromo-α-D-glucose (**1**) and *p*-tyrosol (**2**) was performed via Koenigs-Knorr method in the presence of silver carbonate. The expected *p*-hydroxyphenylethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**3**) was generated in 74% yield. Subsequent deprotection using potassium carbonate in methanol led to salidroside (**4**) in quantitative yield.

By the HPLC technique, the injection of salidroside and *p*-tyrosol on a C_{18} column did not lead to enough selectivity to perform a reproducible chromatographic separation. The order of elution of *p*-tyrosol and salidroside could be exchanged according to the chromatographic pump used. This was due to a small difference of the retention factor in the highly aqueous content of the mobile phase.[10] The HPLC separation of salidroside and



Scheme 1. Synthesis of salidroside (4) by glucosylation of p-tyrosol (2).

p-tyrosol was, thus, performed on a porous graphitic carbon (PGC) Hypercarb stationary phase. On PGC, the retention factor increased by increasing the number of polar substituents and was shown to depend on both the field and the mutual resonance effects of the different subsituents on the aromatic ring,[11] thus, salidroside and *p*-tyrosol could be easily separated on PGC (Figure 1).

Purification of salidroside depends mainly on the separation of salidroside and *p*-tyrosol at the semi-preparative scale. High performance centrifugal partition chromatography purification (HPCPC) was easily implemented by the determination of the enrichment constants (Ec) (selectivity factor). These Ec's were determined for four ternary biphasic systems (ethyl acetate-acetonitrile-water, ethyl acetate-methanol-water, chloroform-methanol-water, hexane-methanol-water, each in 50-12.5-37.5 v/v/v proportions). The best phase separation between salidroside and *p*-tyrosol was obtained with the ethyl acetate-acetonitrile-water system. This separation system led to a better efficiency and a faster separation than the ethyl acetate-n-butanol-water, chloroform-methanol-isopropanol-water, or n-butanol-ethyl acetate-water systems used by Han *et al.*[12] and Li and Chen.[13]

Pure salidroside and *p*-tyrosol are both white powders. The product obtained after synthesis was brown. This colouring was due to polymerisation of traces of 5-hydroxymethylfurfural coming from glucose dehydration. The coloured impurity was highly retained on both C_{18} and PGC stationary phases and, thus, was not detected by chromatography. During the purification of HPCPC the coloured impurity was found to partially coelute with

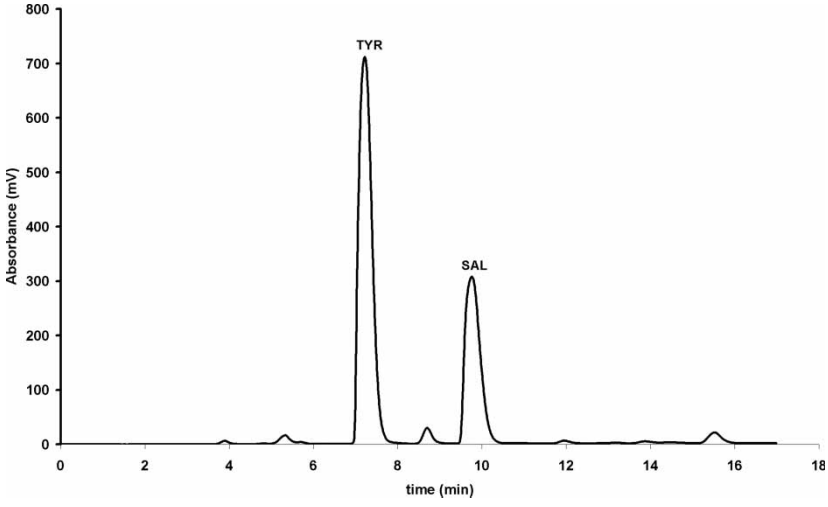


Figure 1. Separation of p-tyrosol (TYR) and salidroside (SAL) after synthesis on a PGC column (for experimental conditions see text).

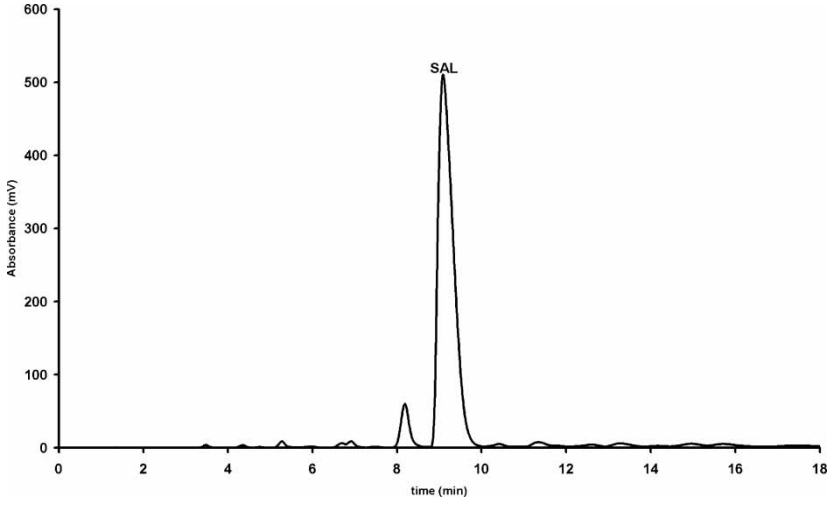


Figure 2. Chromatogram of the purified salidroside (SAL) on a PGC column (for experimental conditions see text).

salidroside. Because of the great affinity of the impurity with the PGC stationary phase, a EnviCarb SPE cartridge was used. This procedure easily removed the coloured impurity. The final purified product was found to be pure at 95% by HPLC (Figure 2).

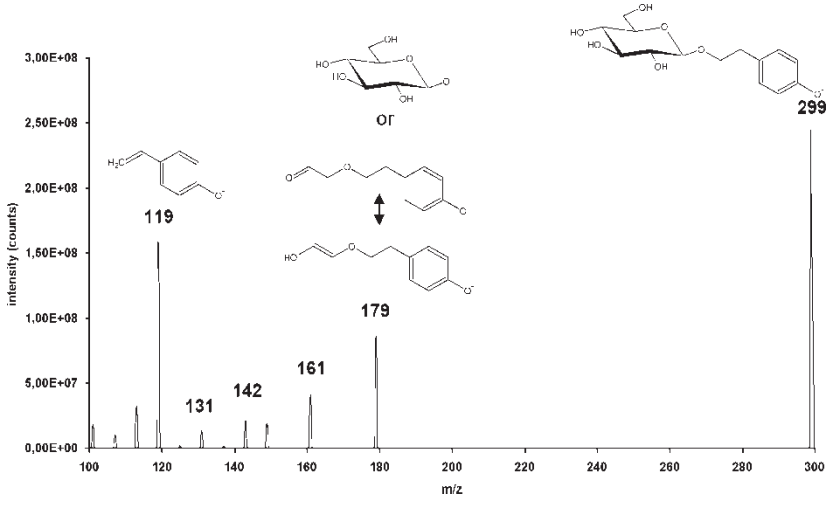


Figure 3. ESIMS^2 product scan of the ion 299. The structure of the main fragments is shown.

Structural Confirmation

The structure of the final purified compound was resolved by both MS and NMR.

Tandem Mass Spectrometry

Negative electrospray mass spectrum of the parent ion $m/z = 299$ was performed with an collision energy of 15 eV. The fragmentation pattern of the compound was found to be characteristic of salidroside m/z of [salidroside$-H^-$] = 299 (Figure 3).

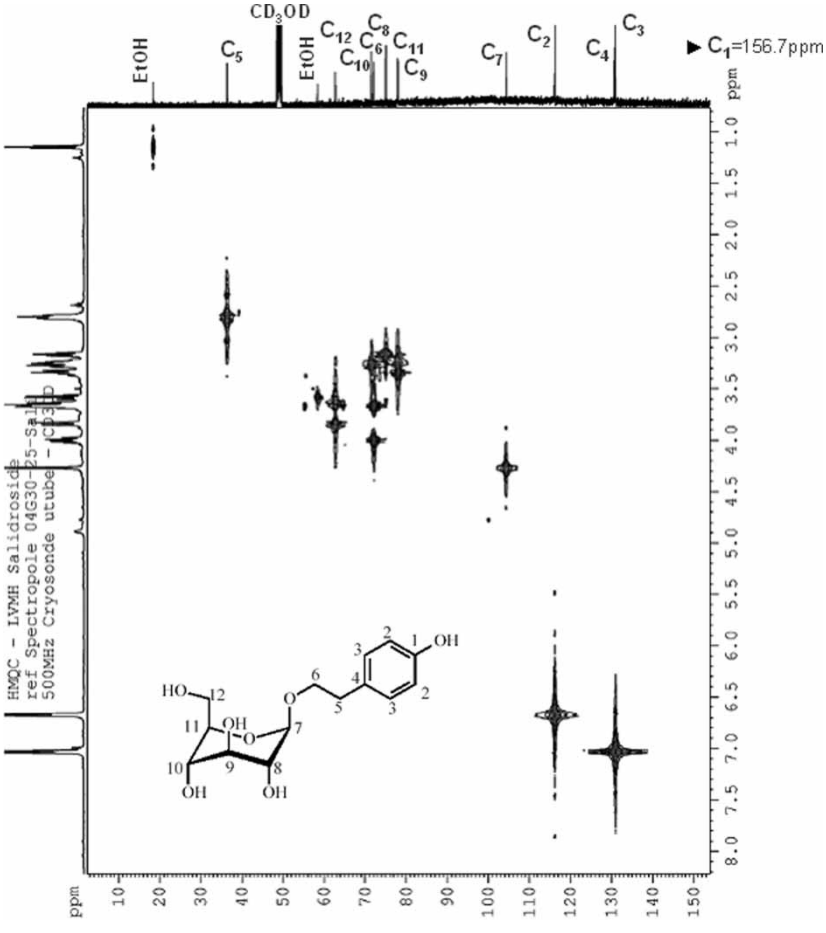


Figure 4. HMQC C-H correlation NMR analysis of the purified salidroside. The structure was confirmed and the purity of the product is established.

No fragment at the mass corresponding to $[p\text{-tyrosol}-H]^-$ ($m/z = 137$) was found in the mass spectrum. A fragment common with the mass spectrum of *p*-tyrosol was, however, present ($m/z = 119$) because of the same aglycone part of both molecules.

HMQC C-H Correlation NMR Spectroscopy

C-H correlation HMQC NMR spectrum confirmed the structure of salidroside. Each ^{13}C and ^{1}H signal can be attributed (Figure 4). Moreover NMR confirmed the absence of detectable impurities in the final purified product.

Both MS^2 and NMR confirmed the structure and the purity of the synthesised and purified salidroside.

Biological Activity

ATP is a marker of cell viability because it is present in all metabolically active cells, and the concentration stands for the energy level of cells. To access this activity, we chose to carry out the test using skin cells keratinocytes. After a 24 hour treatment of culture, the rate of ATP produced by keratinocyte was measured.

The ATPlite-M assay system is based on the production of light caused by reaction of ATP with luciferase and D-luciferin. The emitted light is proportional to the ATP concentration within certain limits. Ginsenoside Rb1 (Extrasynthèse, Genay, France) was used as positive control in our laboratory because of its well known activity.[14] Activity of the synthetic salidroside was found to be more important than the one of ginsenoside (Figure 5).

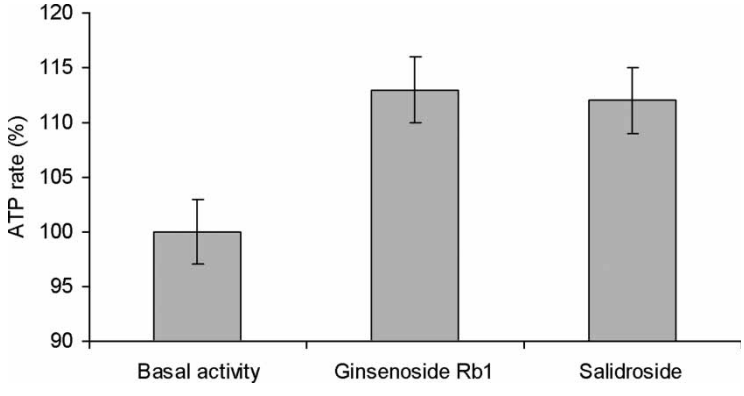


Figure 5. Biological activity assessment of purified salidroside based on the measurement of ATP production rate of keratinocytes for 3 substances. The all treatment concentration for each substance was 25 μg/mL. Ginsenoside Rb1 is used as positive control.

CONCLUDING REMARKS

Salidroside was synthesised, purified, identified, and biologically tested. This paper shows a global procedure for the synthesis of a pharmacologically and cosmetically interesting compound. We have preferred the purification of the product instead of optimizing the synthesis to reach a yield close to 100%. This choice leads to the preparation of salidroside with 95% purity following a rapid and easy procedure.

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