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Biotransformation of 6α-santonin and 1,2-dihydro-α-santonin by *Acremonium chrysogenum* PTCC 5271 and *Rhizomucor pusillus* PTCC 5134

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ABSTRACT

Biotransformation of 6α-santonin (**1**) and 1,2-dihyro-α-santonin (**2**) by two fungal strains *Acremonium chrysogenum* (*Cephalosporium chrysogenum*) and *Rhizomucor pusillus* has been investigated for the first time. After 8 days of incubation of **1** by *A. chrysogenum*, four known metabolites including 1,2-dihydro-α-santonin (**2**) (30%), 8α-hydroxyl-α-santonin (**3**) (22%), 15-hydroxy-α-santonin (**4**) (15%) and 4,5-dihydro-α-santonin (**5**) (10%) were obtained. Incubation of **1** by *R. pusillus* afforded two metabolites **2** (45%) and **3** (20%). Biotransformation of 1,2-dihyro-α-santonin by *A. chrysogenum* produced tetrahydro-α-santonin (**6**) with 52% yield and tetrahydroartemisin (**7**) with 33% yield. By *R. pusillus*, the yields of **6** and **7** were 32% and 21%, respectively. The structures of the products were identified on the basis of spectroscopic data.

1. Introduction

Eudesmanolide compounds are biogenetic and chemical precursors of a range of sesquiterpene lactones [1]. They possess a variety of biological and pharmaceutical activities such as cytotoxic, anti-cancer, anti-HIV and anti-tumor [2]. α-Methyl-γ-lactone and α,β-unsaturated ester side chain adjacent to α-methylene-γ-lactone are structure-activity requirement of the sesquiterpene lactones for high *in vivo* anti-tumor activity [3,4].

6α-Santonin (**1**), the cheap and abundant sesquiterpene lactone, is found in several species of genus *Artemisia* [3]. It has attracted a great deal of interest from chemists owing to its wide range of biological activities, such as cytotoxic, anti-tumor, immunosuppressive, insecticidal and anti-HIV activities [2,3,5,6]. 6α-Santonin is an important starting material on the account of many different functional groups to obtain several naturally bioactive terpenoid compounds bearing the guaiane or eudesmane skeleton [7–10].

Microorganisms catalyze various oxidative, reductive, conjugative and degradative reactions of many classes of natural products. They have been used as *in vitro* models in mammalian systems to mimic and predict the metabolic fate of pharmaceutical agents [11]. Since metabolites in mammalian systems are not large in quantities, they are difficult to identify. In contrast, by using microbial transformations in large-scale, metabolites can be produced in large quantities, which is easy to identify by spectroscopic methods.

Biotransformation introduced a regioselective and stereoselective alternatives for some of the enantiospecific synthesis of natural sesquiterpene lactones [12]. Bioconversion of sesquiterpenes is actually accepted as a preferable method, in combination with chemical reactions, for the semi-synthesis of products of interest with an enhanced bioactivity and low toxicity [9,13–15]. There have been number of reports on the biotransformation of 6α-santonin, which many different results such as hydration, epoxidation, reduction, hydroxylation or combination of these reactions have been concluded [1,12,16–21].

In this research, the ability of two fungi *Acremonium chrysogenum* (*Cephalosporium chrysogenum*) and *Rhizomucor pusillus* in the biotransformation of 6α-santonin and 1,2-dihydro-α-santonin was investigated.

Reduction of the double bonds in 6α-santonin by chemical synthesis methods needs metal catalyst and hydrogen gas. In order to reduce the production of harmful waste in chemical synthesis methods, biotransformation was chosen as an environmentally friendly alternative for reduction of the double bonds of 6α-santonin, but in addition to reduction of the double bonds in some metabolites, hydroxylation was observed. Since biotransformation

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of 1,2-dihydro-α-santonin has not been studied before by any fungal strains, this compound was selected for investigating the biotransformation ability of two mentioned strains.

In the previous reports on the biotransformation of 6α-santonin by fungal strains, the yield of the products was low. Despite various studies on bioconversion of 6α-santonin, trying to find new strains for its bioconversion and the production of pharmaceutical ingredients or precursors with good yield is still a great deal of interest.

2. Experimental

2.1. Materials

6α-Santonin was purchased from Sigma–Aldrich (Taufkirchen, Germany). Yeast extract and agar were purchased from Scharlau (Barcelona, Spain). Inorganic salts, analytical grade reagents, solvents and silica gel 60 GF_{254} fluorescent thin layer chromatographic plates (TLC) were obtained from Merck (Darmstadt, Germany).

2.2. Microorganisms and cultural conditions

The fungal strains *A. chrysogenum* PTCC 5271 and *R. pusillus* PTCC 5134 were obtained from the Persian type culture collection (Iranian Research Organization for Science and Technology).

R. pusillus was maintained on potato-dextrose agar plates (composition, g/L: glucose 20 g, diced potatoes 300 g, agar 15.0 g) and *A. chrysogenum* was maintained on yeast extract agar plates (yeast extract, 4.0 g; soluble starch, 15.0 g; K_2HPO_4, 1.0 g; $MgSO_4{\bullet}7H_2O$, 0.5 g; agar, 15.0 g, distilled water, 1000.0 ml) at 4 °C and freshly sub-cultured before using in the transformation experiment. All media were sterilized by autoclaving at 121 °C for 20 min. Ten 250-ml Erlenmeyer flasks, each containing 150 ml of liquid medium of potato-dextrose broth for *R. pusillus* and ten 250 ml Erlenmeyer flasks, each containing 150 ml of liquid medium of yeast extract broth for *A. chrysogenum*, were inoculated with freshly obtained spores from agar slope cultures and incubated 24 h at 26 °C in a rotary shaker (120 rpm).

2.3. Synthesis of 1,2-dihydro-α-santonin

A mixture of 1 g of 6α-santonin (4 mmol), 30 ml of dried CH_2Cl_2 and 150 mg of 2% Pd–C was stirred under 1 atm H_2 at room temperature for 10 h. Then the catalyst was removed and the filtrate was concentrated under reduced pressure on a rotary evaporator. The concentrated residue was re-dissolved in chloroform and loaded on preparative TLC with *n*-hexane/ethyl acetate (7:3 v/v) for further purification. Finally, 1,2-dihydro-α-santonin was separated and purified (60% yield) for using in biotransformation reaction.

2.4. General procedure for the biotransformation of 6α-santonin and 1,2-dihydro-α-santonin using whole cells

After preparation of fermentation media, 6α-santonin (1 g) was dissolved in acetone (10 ml); 1 ml of the solution was added to each 250-ml Erlenmeyer flask for each fungi. Higher concentrations of substrate resulted in lower conversion and a decrease in metabolites yields. The reaction was monitored by TLC. After 8 days of incubation under the same conditions, the highest conversion was achieved.

2.5. Extraction of 6α-santonin and 1,2-dihydro-6α-santonin transformation metabolites

At the end of incubation, all identical fermentation broths were combined and extracted three times with equal volume of ethyl acetate. The extract was evaporated under reduced pressure on a rotary evaporator. The concentrated residue was re-dissolved in chloroform and loaded on preparative TLC with *n*-hexane/ethyl acetate (4:6 v/v). All the purified metabolites were identified by spectral data. Further support was obtained by comparison with literature data.

2.6. Analytical methods

^{1}H NMR spectra were recorded using a Bruker Avance-300 MHz spectrometer with tetramethylsilane (TMS) as internal standard in $CDCl_3$. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Mass (MS) spectra were obtained on a Finnigan MAT TSQ-70 instrument by electron impact (EI) at 70 eV. Infrared (IR) spectra were recorded using KBr disks on a Magna-IR 550 Nicolet FTIR spectrometer. Thin layer chromatography (TLC) and preparative TLC was performed on 20 × 20 cm 0.25-mm thick layers of silica gel G (Silica gel 60 GF_{254}, Merck). Layers were prepared on glass plates and activated at 110 °C 1 h before use.

3. Result

3.1. Biotransformation of 6α-santonin by A. chrysogenum and R. pusillus

After 8 days of incubation of 6α-santonin (**1**) at 26 °C by *A. chrysogenum*, four metabolites **2**–**5** and by *R. Pusillus*, two metabolites **2** and **3** were obtained. On the basis of the spectral data including ^{1}H NMR, FT-IR and MS, the structures of the products were identified (Scheme 1). Their chemical and spectral data were in a good agreement with those reported in the literature. The observed bioconversion reactions were: C1–C2 double bond reduction in **2**, C-8 hydroxylation in **3**, 15-methyl hydroxylation in **4** and C4–C5 double bond reduction in **5**.

3.2. Biotransformation of 1,2-dihydro-α-santonin by A. chrysogenum and R. pusillus

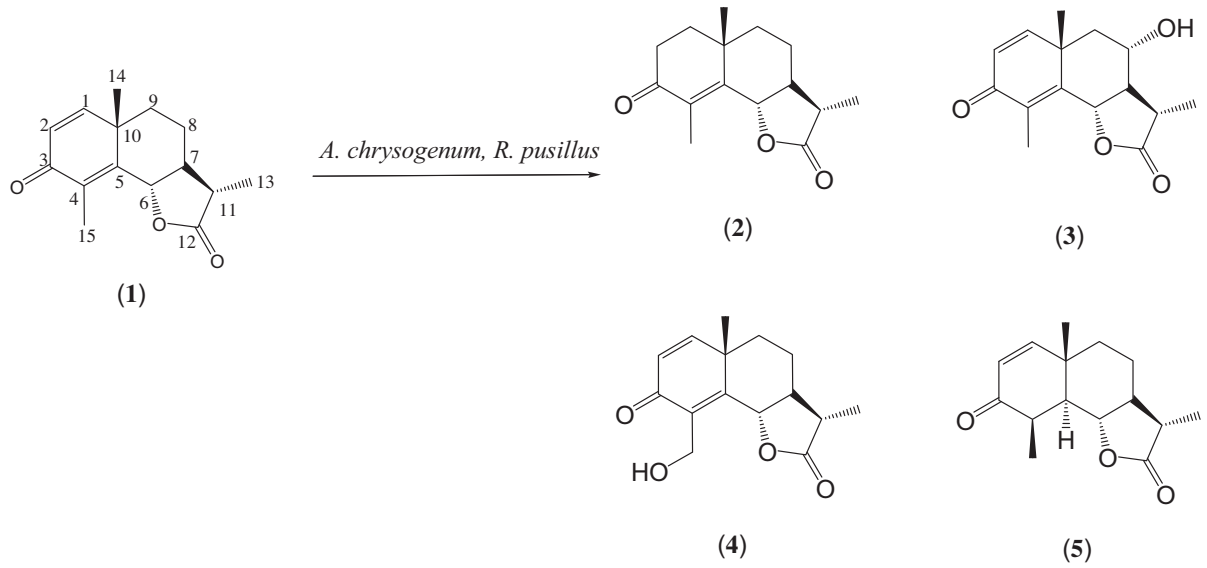
Biotransformation of 1,2-dihyro-α-santonin by *A. chrysogenum* and *R. pusillus* produced tetrahydro-α-santonin (**6**) and tetrahydroartemisin (**7**) by different yields (Scheme 2). The physical and spectral data were in good agreement with literature. The observed bioconversion reaction was reduction of C4–C5 double bond in metabolite **6** and reduction of C4–C5 double bond and C-8 hydroxylation in metabolite **7**.

The obtained yields of all the metabolites from the biotransformation of 6α-santonin and 1,2-dihyro-α-santonin by two fungi and a comparison of them with previously reported yields is shown in Table 1. Chemical shifts of all metabolites are shown in Table 2.

4. Discussion

Metabolite 2: The MS spectrum of metabolite **2** showed a molecular ion peak at *m*/*z* 248 and provided the molecular formula $C_{15}H_{20}O_3$, which was two units higher than 6α-santonin suggesting that double bond reduction was occurred. The optical rotation of this metabolite was measured; $[\alpha]_D{}^{25}$ +90° (c 6.1, $CHCl_3$) (lit. [14], $[\alpha]_D{}^{25}$ +89°). In ^{1}H NMR spectrum of compound **2**, elimination of signals at δ 6–6.7 confirmed reduction of C1–C2 double bond. Doublet at δ 4.67 was related to H-6. Addition of signals at δ 2.44 and δ 2.52 related to H-2a and 2b confirmed the structure. Singlet at δ 1.98 was related to Me-15. Chemical shift for this methyl group showed olefinic structure at C-4.

Previously, 1,2-dihydro-α-santonin (**2**) was obtained from the incubation of 6α-santonin with *Cunninghamella bainieri*,



Scheme 1. The biotransformation of 6α-santonin by *A. chrysogenum* (**2**–**5**) and *R. pusillus* (**2** and **3**).

Cunninghamella echinulata, *Mucor plumbeus* and *Rhizopus stolonifer* [14]. This metabolite was also obtained through biotransformations of 6α-santonin with *Aspergillus niger*, *Cunninghamella blaksleeana*, *Streptomyces aureofaciens*, *Bacillus subtilis*, *Bacillus cereus*, *C. echinulata*, *Rhizopus oryzae* and *Pseudomonas* sp. [12,14,18,22]. The reported yield of this metabolite after incubation by *Absidia officinalis* was 2.9% but in this study, the obtained yields were 45% and 30% by *R. pusillus* and *A. chrysogenum*, respectively [6].

Metabolite 3: The MS spectrum of metabolite **3** showed a molecular ion peak at m/z 262 consistent with the molecular formula of $C_{15}H_{18}O_4$, suggesting that hydroxyl group may be introduced. The ^{1}H NMR of **3** also showed the existence of a new oxymethine proton signal at δ 4.15. The optical rotation of this metabolite was measured, $[\alpha]_D^{20}$ −40.6° (c 0.51, $CHCl_3$) (lit. [6], $[\alpha]_D^{20}$ −41.1°), it was confirmed α-stereochemistry for OH by comparison with the literature. Accordingly, the structure of **3** was determined as 8α-hydroxy-α-santonin.

The fact of 8α-hydroxylation of 6α-santonin enables the formation of 8,12-eudesmanolide instead of 6,12-eudesmanolide and some other useful modification at C-8 position.

This metabolite was obtained by incubation of 6α-santonin with the cell cultures of *Abisidia coerulea* in a low yield (2%) but in this study by *R. pusillus* and *A. chrysogenum*, the yields were 20% and 22%, respectively [6].

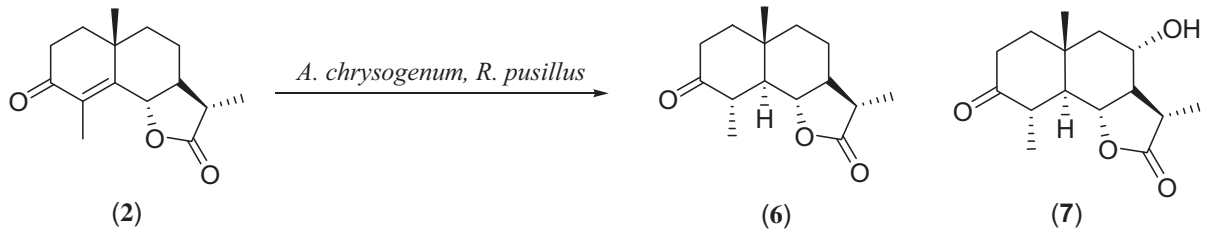
Metabolite 4: The MS spectrum of metabolite **4** showed a molecular ion peak at m/z 262, consistent with the molecular formula of $C_{15}H_{18}O_4$, suggesting that a hydroxyl group may be introduced. The ^{1}H NMR of **4** was similar to that of 6α-santonin except that the signal of H-15 at δ 2.07 was disappeared, however, one new oxy-methylene at δ 4.80 was appeared. The optical rotation of this metabolite was measured; $[\alpha]^{25}_D$ −90.2° (c 0.11, $CHCl_3$) (lit [6], $[\alpha]^{25}_D$ −92.0°). Therefore, the structure of **4** was identified to be 15-hydroxy-α-santonin.

This metabolite was reported in 2005 by biotransformation of 6α-santonin by cell suspension cultures of five plants (*Catharanthus roseus*, *Ginkgo biloba*, *Platycodon grandiflorum*, *Taxus cuspidate* and *Phytolacca asinosa*). Biotransformation of **1** by cell cultures of *Abisidia coerulea* afforded this metabolite with 1.0% yield but in our research, the yield was 15% by *A. chrysogenum* [6,15].

Metabolite 5: The MS spectrum of metabolite **5** showed a molecular ion peak at *m/z* 248 and provided the molecular formula $C_{15}H_{20}O_3$, which was two units higher than 6α-santonin suggesting that double bond reduction was occurred.

In comparison between ^{1}H NMR of **5** and 6α-santonin, a significant difference for the resonances of H-4, H-5 and Me-15 was observed. The C-15 methyl protons appeared as a doublet at δ 1.57 and it showed vicinal couplings with a C-4 methine proton (δ 2.60), which was confirmed the reduction of C4–C5 double bond. H-5 appeared at δ 2.31. Stereochemistry was established by comparison of optical rotation of this metabolite and published data in the literature, $[\alpha]_D^{25}$ +73.5° (c 9.7, $CHCl_3$) (lit. [14], $[\alpha]_D^{25}$ +75°).

Previously, metabolite **5** was obtained by *R. stolonifer* [14]. Biotransformation of 6α-santonin by cell suspension cultures of five plants (*Catharanthus roseus*, *Ginkgo biloba*, *Platycodon grandiflorum*, *Taxus cuspidate* and *Phytolacca asinosa*) were also produced this metabolite [15]. Incubation of 6α-santonin by *A. officinalis* produced this metabolite by yield of 5.8% but in our research by *A. chrysogenum*, the yield was increased to 10% [6].



Scheme 2. The biotransformation of 1,2-dihydro-α-santonin by *A. chrysogenum* and *R. pusillus*.

Table 1
Obtained yields from biotransformation of 6α-santonin and 1,2-dihydro-α-santonin by *A. chrysogenum* and *R. pusillus* and reported yields in literature.

Metabolite	Obtained yields by *A. chrysogenum*	Obtained yields by *R. pusillus*	Reported yields in literature
2	30%	45%	[a]Cell suspension of *Asparagus officinalis*: 2.9% [b]Cell suspension of *Phytolacca asinosa*: 62% [b]Cell suspension of *Taxus cuspidata*: 7% [b]Cell suspension of *Catharanthus roesus*: <2%
3	22%	20%	[a]Absidia coerulea IFO 4011: 2.0%
4	15%	–	[a]Cell suspension of *Asparagus officinalis*: 1.0% [b]Cell suspension of *Platycodon grandiflorum*: 26% [b]Cell suspension of *Phytolacca asinosa*: <2% [b]Cell suspension of *Catharanthus roesus*: <2%
5	10%	–	[a]Cell suspension of *Asparagus officinalis*: 5.8% [a]Cell suspension of *Catharanthus roesus*: <2%
6	52%	32%	–
7	33%	21%	–

[a] Ref. [16].
[b] Ref. [15].

Table 2
^{1}H NMR chemical shifts of metabolites **2**–**7**.

Carbon	**2** ^{1}H	**3** ^{1}H	**4** ^{1}H	**5** ^{1}H	**6** ^{1}H	**7** ^{1}H
1	1.55	6. 67	6.70	6.67	1.26	1.28
	1.76	–	–	–	1.46	1.46
2	2.44	6.27	6.27	5.36	1.82	1.82
	2.52	–	–	–	1.88	1.88
4	–	–	–	2.60	2.69	2.69
5	–	–	–	2.31	1.52	1.50
6	4.67	4.82	4.81	4.80	3.85	3.85
7	1.81	1.91	1.86	2.16	1.56	1.87
8	1.65	4.15	1.69	1.34	1.61	3.15
	1.89	–	2.05	2.05	2.01	–
9	1.70	1.45	1.56	1.34	1.17	1.53
	1.85	2.06	1.93	1.92	1.35	1.91
11	2.34	2.65	2.41	2.16	2.24	2.24
13	1.25	1.26	1.26	1.29	1.20	1.20
14	1.32	1.35	1.34	1.26	1.00	1.00
15	1.98	2.18	4.80	1.57	1.22	1.22

Metabolite 6: The MS spectrum of metabolite **6** showed a molecular ion peak at *m*/*z* 250 and provided the molecular formula $C_{15}H_{22}O_3$, which was two units higher than 1,2-dihydro-α-santonin suggesting that reduction of double bond was occurred. In ^{1}H NMR of **6**, elimination of signals at δ 6–6.7 confirmed reduction of C1–C2 double bond. The upfield chemical shift value of C-15 indicated that this methyl group was bonded to a sp^3 hybridized carbon atom. These data confirmed the reduction of double bond present between C4–C5. The stereochemistry of H-4 was determined to be β-configuration. The optical rotation of this metabolite was measured, $[\alpha]_D^{25}$ +30.7° (c 0.24, $CHCl_3$) (lit. [24], $[\alpha]_D^{25}$ +31°), and it was confirmed β-stereochemistry for H-4 by comparison with the literature.

This metabolite was obtained by incubation of 6α-santonin by *Phanerochaete chrysosporium* [1]. To the best of our knowledge, it is the first report of this compound by microbial method, previously **6** was synthesized from 6α-santonin by using hydrogen gas over 10% palladium on carbon [24,25].

Metabolite 7: The MS spectrum of metabolite **7** showed a molecular ion peak at *m*/*z* 266 and provided the molecular formula $C_{15}H_{22}O_4$, suggesting that a hydroxyl group may be introduced and also double bond reduction may be occurred. In ^{1}H NMR of **7**, elimination of signals at δ 6– 6.7 confirmed reduction of C1–C2 double bond. The upfield chemical shift value of C-15 indicated that this methyl group was bonded to a sp^3 hybridized carbon atom. These data confirmed the reduction of double bond present between C4–C5. The ^{1}H NMR of **7** also showed the existence of a new oxymethine proton signal at δ 3.15, which was related to H-8. The stereochemistry of H-4 was determined to be β-configuration by comparison with optical rotation of this metabolite, $[\alpha]_D^{14}$ +50.1° (c 2.6, $CHCl_3$) (lit. [24,26], $[\alpha]_D^{14}$ +50°). In our knowledge, there is no report on 8α-hydroxylation and double bonds reduction of 1,2-dihydro-α-santonin by enzymatic approaches. Previously, this compound was synthesized by chemical methods.

5. Conclusion

The ability of two fungal strains, *R. pusillus* and *A. chrysogenum*, in bioconversion of 6α-santonin (**1**) and 1,2-dihyro-α-santonin (**2**) has been studied for the first time. The observed bioconversion reactions were C1–C2, C4–C5 double bonds reduction, C-8 hydroxylation and 15-methyl hydroxylation in different metabolites of these two substrates. In this research, in comparison with the previous reports on biotransformation of 6α-santonin, yields of all metabolites were improved. It is the first report of biotransformation of 1,2-dihydro-α-santonin and the production of metabolite **7** and **8** by enzymatic approaches.

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