Microbial Transformations of α-Santonin

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Fungal biotransformations of α-santonin (**1**) were conducted with *Mucor plumbeus* (ATCC 4740), *Cunninghamella bainieri* (ATCC 9244), *Cunninghamella echinulata* (ATCC 9245), *Curvularia lunata* (ATCC 12017) and *Rhizopus stolonifer* (ATCC 10404). *Rhizopus stolonifer* (ATCC 10404) metabolized compound **1** to afford 3,4-epoxy-α-santonin (**2**) and 4,5-dihydro-α-santonin (**3**) while *Cunninghamella bainieri* (ATCC 9244), *Cunninghamella echinulata* (ATCC 9245) and *Mucor plumbeus* (ATCC 4740) were capable of metabolizing compound **1** to give a reported metabolite, 1,2-dihydro-α-santonin (**4**). The structures of these transformed metabolites were established with the aid of extensive spectroscopic studies. These fungi regiospecifically reduced the carbon-carbon double bond in ring A of α-santonin.

Key words: 1,2-Dihydro-α-santonin, 4,5-Dihydro-α-santonin, 3,4-Epoxy-α-santonin

Introduction

Microorganisms are known for their ability to catalyze a large number of oxidative, reductive, conjugative and degradative reactions of many classes of natural products in a xenobiotic fashion (Charney and Herzog, 1967). Most interestingly, common microorganisms can metabolize xenobiotics in a manner remarkably similar to that shown by mammals. This similarity in metabolic profile is largely explained by the presence of the enzyme cytochrome P-450 monooxygenase in these organisms (Shocken *et al.*, 1997). Microorganisms have been used successfully as *in vitro* models to mimic and predict the metabolic fate of pharmaceutical agents in mammalian systems (Smith and Rosazza, 1982). In the mammalian system, metabolites are not produced in large quantities and are difficult to identify, whereas through the use of microbial transformations, metabolic products can be produced in large quantities by using large-scale fermentation, and the metabolites can then be identified by spectroscopic methods. The metabolites obtained through this method are often very closely parallel those obtained from human biotransformations (Orabi *et al.*, 1999). In organic synthesis, microbial reactions may be applied to improve the regioselectivity and stereoselectivity of some chemical reactions during the enantiospecific synthesis of natural sesquiterpene lactones (Barrero *et al.*, 1999). Bioconversion of bioactive natural products produces metabolites with an enhanced bioactivity and low toxicity (El Sayed *et al.*, 1998, 1999).

α-Santonin (**1**) is a sesquiterpene lactone and it is found in several species of genus *Artemisia* (Marshall and Wuts, 1978; El-Feraly *et al.*, 1983). It had been used in the treatment of nervous complaints and as an anthelmintic (Sareen *et al.*, 1961). This compound has shown a number of biological activities such as anti-inflammatory and antipyretic activities (Alharbi *et al.*, 1994). We are interested to do microbial transformations of bioactive natural products isolated in our lab in order to study their structure-activity relationships. We used α-santonin as a model compound to perform microbial transformations. In this paper, we report the fungal biocatalysis of α-santonin (**1**) and structure elucidation of transformed products, 3,4-epoxy-α-santonin (**2**), 4,5-dihydro-α-santonin (**3**) and 1,2-dihydro-α-santonin (**4**). Spectroscopic methods were used to establish the structures of compounds **2**–**4**.

Results and Discussion

The first biotransformed metabolite, 3,4-epoxy-α-santonin (**2**), was obtained as a colorless gum after incubation with *R. stolonifer* (ATCC 10404). Its UV spectrum showed a maximum absorption at 229 nm indicating the lack of any conjugated π bond. The IR spectrum displayed intense absorption bands at 1795 (γ-lactone), 1588 (C=C) and 1100 (C-O) cm^{-1}. The high-resolution electron-im-

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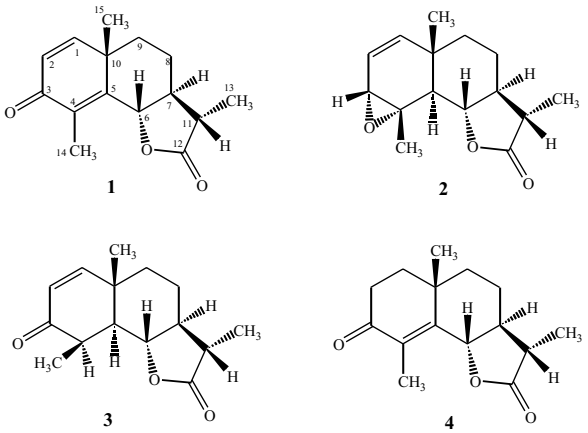

Fig. 1. Structures of α-santonin (**1**), 3,4-epoxy-α-santonin (**2**), 4,5-dihydro-α-santonin (**3**), and 1,2-dihydro-α-santonin (**4**).

pact mass spectrum (HREIMS) of compound **2** showed a molecular ion peak at *m/z* 248.1416 (calcd. 248.1412) which provided the molecular formula $C_{15}H_{20}O_3$.

The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of compound **2** showed two signals at δ 1.19 and 1.67, integrating for three protons each, due to the C-15 and C-14 methyl protons, respectively. A three-hydrogen doublet at δ 1.26 (J = 6.9 Hz) was assigned to the C-13 secondary methyl protons. A signal for the C-6 methine proton at δ 4.66 (J_1 = 10.0 Hz, J_2 = 1.49 Hz) was also observed in the ^{1}H NMR spectrum. The COSY-45° spectrum was recorded to assign the ^{1}H NMR chemical shift assignments of compound **2** and it revealed the presence of two isolated spin systems in the molecule. The first spin system (C-1H ~ C-3H) started from the olefinic C-1 methine proton (δ 5.63), which showed cross-peaks with the C-2 methine proton (δ 5.30), which in turn exhibited cross-peaks with the C-3 methine proton (δ 3.49). The second fragment (C-5H ~ C-13H_3) was traced from the C-6 methine proton (δ 4.66), which showed ^{1}H-^{1}H spin correlations with C-5 (δ 2.45) and C-7 (δ 1.96) methine protons. The latter exhibited cross-peaks with C-11 methine (δ 2.15) and C-8 methylene (δ 1.42 and 2.12) protons. The vicinal couplings of H-11 with the C-13 methyl protons (δ 1.26) were also observed in the COSY-45° spectrum. H_2-8 showed ^{1}H-^{1}H spin shift correlations with the C-9 methylene protons (δ 1.37 and 2.00).

The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **2** showed the resonance of all fifteen carbon atoms in the molecule. The DEPT experiment was also performed to establish the multiplicity of each signal in the ^{13}C NMR broadband spectrum. It revealed the presence of three CH_3, two CH_2 and seven CH carbon signals in compound **2**. The subtraction of DEPT spectrum from the broadband ^{13}C NMR spectrum indicated the presence of three quaternary carbon atoms in this compound. The C-6 signal, geminal to the lactone moiety, resonated at δ 80.1, while two olefinic signals at δ 136.2 and 129.8 were assigned to the C-1 and C-2 carbon atoms, respectively. The C-12 signal appeared at δ 175.1. The additional methine signal at δ 63.9 in the DEPT spectrum was due to C-3 and in the HMQC spectrum showed ^{1}H/^{13}C one-bond shift correlation with H-3 (δ 3.49). The downfield ^{1}H and ^{13}C NMR chemical shift values suggested the presence of a geminal oxygen functionality. The ^{1}H NMR spectrum of compound **2** was also recorded in pyridine-d_5 to confirm the epoxide nature of oxygen between C-3 and C-4. The C-3 methine proton showed a paramagnetic shift from δ 3.49 to 3.52. It has been reported in the literature that a pronounced shift of ≈ 0.2 ppm was observed in case of protons adjacent to hydroxyl group, when ^{1}H NMR spectrum was recorded in pyridine-d_5 (Atta-ur-Rahman *et al.*, 1992). These spectral observations indicated the presence of an oxirane ring at C-3 and C-4. The presence of an epoxide in compound **2** was also evident from the mass spectrum, which provided the molecular formula $C_{15}H_{20}O_3$ and six degrees of unsaturation in compound **2**. Two oxygen atoms were due to a five-member lactone ring, while the third one was involved in the epoxidation at C-3/C-4. Five degrees of unsaturation were accounted for three rings, a double bond in the ring A and a lactone carbonyl moiety. The presence of an oxirane ring in this compound also satisfied the sixth degree of unsaturation for this new metabolite. Complete ^{13}C NMR chemical shift assignments and ^{1}H/^{13}C one-bond shift correlations of all hydrogen-bearing carbon atoms, as determined from HMQC spectrum of compound **2**, are shown in Table I.

The HMBC spectrum of compound **2** was used to assign the ^{13}C NMR chemical shift assignments of all quaternary carbon atoms of compound **2** and to establish the gross-structure of compound **2** from the sub-structures obtained form COSY-45°

	2		3		4	
Carbon	^{1}H	^{13}C†	^{1}H	^{13}C†	^{1}H	^{13}C†
1	5.63	136.2 (d)	6.67	156.7 (d)	1.57	33.6 (t)
	–	–	–	–	1.71	–
2	5.30	129.8 (d)	5.39	130.3 (d)	2.45	38.32 (t)
	–	–	–	–	2.50	–
3	3.49	63.9 (d)	–	198.6 (s)	–	198.8 (s)
4	3.27	61.2 (s)	2.77	40.4 (d)	–	128.6 (s)
5	2.45	55.4 (d)	2.42	55.6 (d)	–	152.5 (s)
6	4.66	80.1 (d)	4.65	80.2 (d)	4.67	81.9 (d)
7	1.96	53.2 (d)	2.12	51.4 (d)	1.80	52.9 (d)
8	1.42	40.3 (t)	1.39	39.7 (t)	1.65	41.7 (t)
	2.12	–	2.00	–	1.90	–
9	1.37	24.9 (t)	1.34	25.4 (t)	1.65	24.61 (t)
	2.00	–	1.88	–	1.60	–
10	–	40.2 (s)	–	39.4 (s)	–	38.3 (s)
11	2.15	41.3 (d)	2.10	41.3 (d)	2.35	41.3 (d)
12	–	175.1 (s)	–	176.7 (s)	–	177.7
13	1.26	14.9 (q)	1.27	15.3 (q)	1.27	12.4 (q)
14	1.67	21.0 (q)	1.44	11.5 (q)	2.00	24.3 (q)
15	1.19	12.0 (q)	1.21	12.3 (q)	1.33	11.24 (q)

Table I. ^{1}H and ^{13}C NMR chemical shift assignments and ^{1}H/^{13}C one-bond shift correlations determined from HSQC spectra of compounds **2**–**4**.

† Multiplicity was determined by DEPT expriment.

spectrum. Important HMBC interactions of compound **2** are shown around structure **2** in Fig. 2.

The NOESY spectrum of **2** was used to establish the relative stereochemistry at various chiral centers. H-3 (δ 3.49) showed a NOE with C-14 methyl protons (δ 1.67), which in turn showed cross-peaks with the C-15 methyl protons (δ 1.19). The latter showed cross-peaks with the C-6 methine proton (δ 4.66). H-6 methine and C-15 methyl protons have β-orientations in α-santonin, thus suggesting the β-orientations of H-3 and H_3-14 in compound **2**. These observations derived from the NOESY spectrum suggested a α-stereochemistry for an epoxide functionality at C-3/C-4. The H-5 (δ 2.45) showed NOESY interactions with H-7 (δ 1.96). The H-7 had an α-orientation in α-santonin, thus indicating an α-orientation for H-5 in compound **2**. The probable conformation of rings A, B, C and D obtained from the NOESY spectrum in compound **2** and important NOE interactions are shown in Fig. 2. Based on these spectroscopic studies, structure **2** was assigned to this new transformed product.

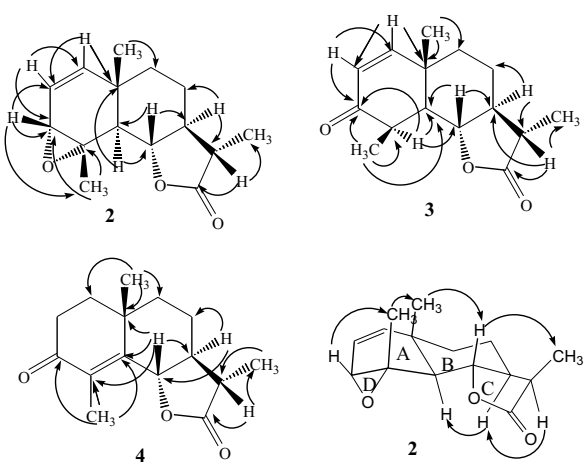

Fig. 2. Important HMBC interactions of compounds **2**–**4** and probable conformations of rings A, B, C and D in compound **2** obtained from NOESY spectrum.

The second transformed compound, 4,5-dihydro-α-santonin (**3**), was also isolated as a colorless gum from the fermentation flask of *R. stolonifer* (ATCC 10404). The UV spectrum showed a maximum absorption at 246 nm indicating the presence of an α,β-unsaturated carbonyl chromophore in the molecule. Its IR spectrum displayed intense absorption bands at 2909 (CH), 1785 (γ-lactone), 1680 (C=O) and 1599 (C=C) cm^{-1}. The HREIMS of compound **3** showed a molecular ion peak at *m/z* 248.1402 (calcd. 248.1412) and provided the molecular formula $C_{15}H_{20}O_3$.

The ^{1}H NMR spectrum of compound **3** was distinctly similar to that of compound **1**. However the former spectrum exhibited a significant difference for the resonances of H-4, H-5, H_3-14. The C-14 methyl protons appeared as a doublet at δ 1.44 (J = 6.8 Hz) and it showed vicinal couplings with a C-4 methine proton (δ 2.77), which in turn ex-

hibited ^{1}H-^{1}H spin correlations with H-5 (δ 2.42) in the COSY-45° spectrum. The latter showed COSY-45° interactions with H-6 (δ 4.65). The remaining observations in the COSY-45° spectrum for compound **3** were the same, as were observed in the COSY-45° spectrum of compound **1**. The ^{13}C NMR spectrum of compound **3** was similar to that of compound **1** except that it showed differences in the resonances of C-4 (δ 40.4), C-5 (δ 55.6) and C-14 (δ 11.5) signals. The upfield chemical shift value of C-15 indicated that this methyl group was bonded to an sp^3 hybridized carbon atom. The DEPT and HMQC spectra of compound **3** indicated that C-4 and C-5 were methine carbon atoms. The ^{13}C NMR chemical shift values also indicated that C-4 and C-5 were sp^3 hybridized carbon atoms. This ^{13}C NMR data suggested that a double bond present between C-4/C-5 had been reduced by *R. stolonifer* (ATCC 10404). Complete ^{1}H/^{13}C one-bond shift correlations of all hydrogen-bearing carbon atoms of compound **3**, as determined from HMQC spectrum, are shown in Table I. Important HMBC interactions of compound **3** are shown around structure **3** in Fig. 2.

The NOESY spectrum was also recorded to establish the relative stereochemistry at various chiral centers in compound **3**. H-4 (δ 2.77) and H-5 (δ 2.42) showed NOE cross-peaks with C-7 methine (δ 2.12) and C-13 methyl (δ 1.27) protons. α-Santonin has α-orientations for H-7 and the C-13 methyl group and this NOE data helped us to assign the α-stereochemistry for H-4 and H-5. H-6 (δ 4.65) also showed cross-peaks with C-14 (δ 1.44) and C-15 (δ 1.21) methyl protons. H-6 and H_3-15 have β-orientations in α-santonin, thus suggesting a β-orientation of the C-14 methyl group. Based on these spectral studies, structure **3** was proposed for this transformed metabolite.

Our third metabolite, 1,2-dihydro-α-santonin (**4**), was obtained from the incubation of α-santonin with *C. bainieri* (ATCC 9244), *C. echinulata* (ATCC 9245), *M. plumbeus* (ATCC 4740). Previously, this metabolite was obtained through biotransformations of α-santonin with *Aspergillus niger*, *Cunninghamella blaksleeana*, *Streptomyces aureofaciens*, *Bacillus subtilis*, *B. cereus* (UI1477) and *Pseudomonas* sp. (ATCC 43388) (Atta-ur-Rahman *et al.*, 1999; Calaco *et al.*, 1993; El-Sharkawy *et al.*, 1996). The UV, IR, ^{1}H, ^{13}C NMR and mass spectra (see Experimental Section and Table I) of compound **4** were identical to those of 1,2-dihydro-α-santonin reported in the literature (Atta-ur-Rahman *et al.*, 1999; Calaco *et al.*, 1993; El-Sharkawy *et al.*, 1996).

In summary, we have identified two different types of fungi, which are capable of reducing the carbon-carbon double bond in α-santonin. Additionally, we also got an epoxy derivative of α-santonin.

Experimental

General

Mass spectrometric measurements were conducted on a Varian MAT 312 double focussing mass spectrometer connected to a DEC PDP 11/34 computer system. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Varian Inova and an AM 500 Bruker NMR spectrometer at 200 and 500 MHz, while the ^{13}C NMR spectra were recorded on an AM 500 Bruker NMR spectrometer at 125 MHz with TMS as an internal standard. The IR spectra were recorded on a Jasco-IRA1 IR spectrophotometer. Hewlett Packard GC-MS was used to monitor the microbial reactions. The UV spectra were recorded on a Shimadzu UV 240 instrument. The optical rotations were measured on a Polatronic D polarimeter (Hitachi) and the purities of the samples were checked on TLC (silica gel, GF 254 precoated plates purchased from Merck).

Microorganisms

Fungi, *Mucor plumbeus* (ATCC 4740), *Cunninghamella bainieri* (ATCC 9244), *Cunninghamella echinulata* (ATCC 9245), *Curvularia lunata* (ATCC 12017) and *Rhizopus stolonifer* (ATCC 10404), were purchased from ATCC and maintained on potato dextrose agar and stored in a refrigerator at 4 °C.

Preparation of media

The medium for *M. plumbeus* (ATCC 4740), *C. bainieri* (ATCC 9244), *C. echinulata* (ATCC 9245), *C. lunata* (ATCC 12017) was prepared by mixing the following ingredients in 1 l of distilled water: Dextrose (20 g), yeast (5 g), sodium chloride (5 g), potassium hydrogen phosphate (5 g) and soy flour (5 g). The pH was adjusted to 7.0 before autoclaving.

The medium of *R. stolonifer* (ATCC 10404) was prepared by mixing tartaric acid (3.5%), sucrose (5%), KH_2PO_4 (2%), $MgSO_4 \cdot 2H_2O$ (1%),

NH_4NO_3 (2%), $Zn(OAc)_2$ (0.03%) in 1 l of distilled water. The pH was adjusted to 8.0 with the aid of 0.04 N NaOH before autoclaving.

Incubation experiment

First of all, biotransformation experiments were performed on analytical scale to screen abovementioned five fungal strains. During our initial screening, we found that *C. echinulata* (ATCC 9245), *C. bainieri* (ATCC 9244), *M. plumbeus* (ATCC 4740) and *R. stolonifer* (ATCC 10404) were capable of metabolizing α-santonin (**1**) into compounds **2**, **3** and **4**. Biotransformation experiments with these fungi were carried out on a preparative scale. Both analytical and preparative fermentation experiments were conducted by using standard two-stage fermentation experiments (Atta-ur-Rahman *et al.*, 1994; Herath *et al.*, 2003). For the preparation of stage I liquid cultures, ten 500 ml flasks (each containing 150 ml of liquid culture media) were inoculated with microorganisms to be screened and incubated on a shaker for 24 h. Stage II cultures were prepared by adding α-santonin (**1**) (500 mg) as a solution in ethanol having a concentration of 0.33 mg/ml to each flask having four different microorganisms. These fermentation experiments were also monitored by including two controls, a "culture control" and a "substrate control" to eliminate the possibility that metabolic products were not microbial secondary metabolites and that the culture media has not done any chemical transformation on the substrate. Culture control contained only fermentation blanks in which the microorganisms were grown under identical conditions but without substrates, while the substrate control consisted of the substrate in sterile liquid media. All these flasks were again placed on a shaker at room temperature. After 10 d, these culture media were extracted with ethyl acetate and the crude extracts concentrated under reduced pressure and analyzed by TLC and GC-MS. The crude extracts were subjected to silica gel column chromatography using gradient elution with hexane/ethyl acetate (0–100%) and ethyl acetate/methanol (0–100%) to purify compounds **2** (21.2 mg), **3** (65 mg) and **4** (25 mg).

α-Santonin (**1**): $[\alpha]_D^{25}$ = + 121° (*c* 9.7, $CHCl_3$). – UV(MeOH): λ_{max} = 258 nm. – IR($CHCl_3$): ν_{max} = 2910 (CH), 1780 (γ-lactone), 1590 cm^{-1} (C=C). – ^1H NMR (200 MHz, $CDCl_3$): δ = 1.22 (3H, d, *J* = 6.5 Hz, H_3-13), 1.31 (3H, s, H_3-14), 2.16 (3H, d, *J* = 1.5 Hz, H_3-15), 4.77 (1H, dd, J_1 = 10.6 Hz, J_2 = 1.5 Hz, H-6), 6.22 (1H, d, *J* = 10.0 Hz, H-2), 6.65 (1H, d, *J* = 10.0 Hz, H-1). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 10.9 (q, C-14), 12.5 (q, C-13), 23.1 (q, C-15), 25.1 (t, C-9), 37.8 (t, C-8), 41.0 (d, C-11), 41.3 (s, C-10), 53.5 (d, C-7), 81.4 (d, C-6), 125.9 (d, C-2), 128.8 (s, C-4), 150.8 (s, C-5), 154.8 (d, C-1), 177.5 (s, C-12) 186.3 (s, C-3). – EIMS: *m/z* (rel. int.) = 246 ($C_{15}H_{18}O_3$, 10%), 231 ($C_{14}H_{15}O_3$, 8), 91 (80), 41 (100).

3,4-Epoxy-α-santonin (**2**): $[\alpha]_D^{25}$ = + 112° (*c* 10.3, $CHCl_3$). – UV(MeOH): λ_{max} = 229 nm. – IR ($CHCl_3$): ν_{max} = 2904 (CH), 1795 (γ-lactone), 1588 (C=C), 1100 cm^{-1} (C-O). – ^1H NMR (500 MHz, $CDCl_3$): see Table I. – ^{13}C NMR (125 MHz, $CDCl_3$): see Table I. – HREIMS: *m/z* (rel. int.) = 248.1416 ($C_{15}H_{20}O_3$, calcd. 248.1412, 7), 233.1250 ($C_{14}H_{17}O_3$, calcd. 233.1255, 14).

4,5-Dihydro-α-santonin (**3**): $[\alpha]_D^{25}$ = + 75° (*c* 9.7, $CHCl_3$). – UV (MeOH): λ_{max} = 246 nm. – IR($CHCl_3$): ν_{max} = 2909 (CH), 1785 (γ-lactone), 1680 (C=O), 1599 cm^{-1} (C=C). – ^1H NMR (500 MHz, $CDCl_3$): see Table I. – ^{13}C NMR (125 MHz, $CDCl_3$): see Table I. – HREIMS: *m/z* (rel. int.) = 248.1402 ($C_{15}H_{20}O_3$, calcd. 248.1412. 35), 233.1259 ($C_{14}H_{17}O_3$, calcd. 233.1255, 29).

1,2-Dihydro-α-santonin (**4**): $[\alpha]_D^{25}$ = + 89° (*c* 6.1, $CHCl_3$). – UV(MeOH): λ_{max} = 245 nm. – IR($CHCl_3$): ν_{max} = 2914 (CH), 1789 (γ-lactone), 1600 cm^{-1} (C=C). – ^1H NMR (500 MHz, $CDCl_3$): see Table I. – ^{13}C NMR (125 MHz, $CDCl_3$): see Table I. – HREIMS: *m/z* (rel. int.) = 248.1399 ($C_{15}H_{20}O_3$, calcd. 248.1412, 15), 233.1246 ($C_{14}H_{17}O_3$, calcd. 233.1255, 10).

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