Full Length Research Paper

Biotransformation of *ß*-estradiol isolated from *Sonchus eruca*

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The biotransformation of *ß*-estradiol (1,3,5-estratriene-3,17,*ß*-diols) by *Bacillus subtillus* resulted in four metabolites which were (1) 3,5-estratriene-3-ol–17–one, (2) 1,3,5-estratriene-3,16,17-triol, (3) 1,3,5,7-estratetra-ene-3-ol-17-one and (4) 1,3,5,6, estratetra-ene-3-ol-17-one. The structures were elucidated on the basis of spectroscopic techniques. Compound 3 showed good inhibitory activity against lipoxygenase enzyme.

Key words: Microbial transformation, *ß*-estradiol (1,3,5-estratriene-3,17,ß-diols), *Bacillus subtillus, Aspergillus niger,* lipoxygenase.

INTRODUCTION

Biotransformation is increasingly exploited as a useful and often unique reaction step in the semi synthesis of pharmaceuticals or for structural modification of complex natural compounds (Jovetic et al., 1998). In a chemical synthesis, a variety of catalysts and protecting group are needed for well defined chemical transformation. An alternative way of structural modification of substrate is the use of some bio-cataylst (isolated enzymes, cell extract, plants and animals, whole cell of bacteria, yeast, micro algae and fungi) under mild conditions collectively called biotransformation. The transformation of substrate that occurred by this pathway are regio-and stereo-selective or sometimes novel (Javid et al., 2010). *ß*-Estradiol was isolated from chloroform soluble fraction of *Sonchus eruca* belonging to the family of asteraceae. The asteraceae or sunflower family (formerly composite, also known as the Aster family) is a family of dicoty-ledonous flowering plants. It is the largest family of angiosperms and comprises of over 1535 genera and 23000 species, distributed in three sub-families and 17 tribes. It is also the largest plant family in Pakistan, repre-sented by over 650 species distributed in 15 tribes (Carvalho et al., 2006). *ß*-estradiol is the most potent and naturally occurring estrogen in human beings followed by estrone and estriol (Bulent and Inci, 2002).

Estradiol is one of the most abundant and important estrogens occurring in animals and man (Jan et al., 2002). Estrogen plays a central role in the development and maintenance of the female reproductive organs, mammary glands and other sexual characteristics. The decreased production of estrogen has been linked to a series of postmenopausal pathologies, such as mood alterations, sleeplessness, hot flushes, sweats and sexual dysfunction (Muhammad et al., 2005).

In continuation of the work with microbial transfor-mation of steroid, incubation of compound **1** with *Bacillus subtilus* and *Aspergillus niger* resulted in the formation of four metabolites (Scheme 1) which were reported for the first time via *B. substillus.* Metabolite **2** underwent reduction at C-19, metabolite **3** underwent hydroxylation at C-18, metabolite **4** underwent reduction at C-19 and also exhibited dehy-drogenation at C-7 and C-8, and metabolite **5** underwent reduction at C-19 and also exhibited

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ehydrogenation at C-6 and C-7.

MATERIALS AND METHODS

Plant materials

The plant *Sonchus eruca* was collected at Parachinar Kurram agency, N.W.F.P Pakistan, in July 2008 and was identified by plants taxonomist. The voucher specimen has been deposited in the herbarium of the Botany Department of Kohat University of Science and Technology Kohat (KUST). The whole plant was air-dried for 10 days and milled into powder with an electrical grinder and finally, was stored in airtight bottles before analysis.

General experimental procedure

The electron ionization mass spectra (EI-MS) were measured on a Varian MAT 311 A mass spectrometer. The HR-EI-MS spectra were recorded on Joel JMS-600H mass spectrometer. The ^{1}H NMR spectra (δ in ppm, J in Hz) were obtained in deuterated solvent (CDCl$_3$) at 400 MHz on Bruker Avance-400 NMR spectrometer, while ^{13}C NMR spectra were recorded in CDCl$_3$ on the same instrument at 100 MHz. The FTIR were recorded on Shimadzu FTIR-8900 spectrophotometer. UV spectra were recorded on advance UV spectrophotometer. Optical rotation measurement was performed on Jasco DIP-360 digital polarimeter. Melting point was taken on Buchi 535 apparatus. The metabolites were purified on column chromatography on silica gel. The purity of sample was checked on precoated TLC plates (Si 60, PF 254, 20 × 20, 0.25 mm, E. Merck).

Preparation of media

The medium for *B. subtillus* (NRRL-68751) was prepared by mixing the following reagents and distilled water (2 L): glucose (10 g), peptone (2 g), yeast extract (10 g) and sucrose (4 g). The media for *A. niger* (NRRL-68751) was prepared by mixing glucose (20 g), peptone (10 g), KH$_2$PO4 (10 g), yeast extract (10 g), glycerol (10 ml) and NaCl (10 g) in distilled water (2 L) and the pH was maintained at 5.6.

Culture and fermentation procedure

The following procedure was used for both *B. subtllus* and *A. niger*. Two days old spores of the bacteria were aseptically transferred into the broth medium flask containing 100 ml of freshly prepared and auto claved medium. The seed flask thus obtained was incubated on a shaker table at 30°C for two days. The two days old broth culture from the 100 ml seed flask was inoculated into 20 media flask (250 ml) containing 100 ml of the medium and fermentation was continued for further 24 h. Compound **1** (700 mg) was diluted with acetone (10 ml) and the resulting solution was evenly distributed among 20 conical flask having the shake culture and the fermentation was continued for 10 days for *B. subtillus* and for 12 days for *A. niger*. The mycelia were filtered, washed with ethyl acetate and the broth thus obtained was extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate and was concentrated in vacuum to obtain gums. These were adsorbed on equal quantity of Silicon (Si) gel and were eluted with various solvents, gradients of petroleum ether, EtOAc and MeOH. The obtained compounds **2**, **3**, **4** and **5** had 14.7, 6.2, 20.2 and 10.2% yield, respectively. The remaining compound (compound **1**) was recovered unchanged. The yield of compounds **2** and **3** from *A. niger* was 3.2 and 5.4%, respectively.

Assay of lipoxygenase inhibition

Lipoxygenase inhibiting activity was conveniently measured by slightly modifying the spectrometric method developed by Tappel (Tappel, 1962). Lipoxygenase (1.13.11.12) type I-band linoleic acid was purchased from Sigma (St. Louis, MO, USA). 160 μl of 0.1 mM sodium phosphate buffer (pH 7.0), 10 μl of the test-compound solution and 20 μl of lipoxygenase solution were mixed and incubated at 25°C for 5 min. The reaction was then initiated with the addition of 10 μl of linoleic acid (substrate) solution, and resulted in the formation of (9Z, 11E)-(13S)-13-hydroperoxyoctadeca-9, 11-dienoate, the change in absorbance was read after 10 min at a wavelength of 234 nm. The test compound and control were dissolved in 50% EtOH. All reactions were performed in triplicate. The IC$_{50}$ values were then calculated using the EZ-fit enzyme kinetics program (Perrella Scientific Inc., Amherst, USA).

RESULTS AND DISCUSSION

The fermentation of compound **1** with *B. subtillus* for 10 days yielded the reductive metabolite **2**. The molecular formula of compound **2** determined from HR-EI-MS was found to be C$_{18}$H$_{22}$O$_2$ and showed M$^+$ at m/z 270.3706. The IR spectrum of compound **2** showed kenotic and hydroxyl absorption at 1720 and 3335 cm^{-1}, respectively. The ^{13}C NMR data of compound **2** was the same to the authentic sample except for the absorption at C-17. The unusual ^{13}C NMR spectrum (100MHz, C$_5$D$_5$N) has absorption at δ 219.6 for C-17 instead of absorption at δ 81.22 for the authentic sample (Table 2), due to the presence of five member ring that caused angle strain. ^{1}H NMR further confirmed this by the disappearance of absorption at δ 3.92 (J = 8 Hz, H-17) in compound **2** as absent in the authentic sample (Table1).

The molecular formula of compound **3** determined from HR-EI-MS was found to be C$_{18}$H$_{22}$O$_3$, and showed M$^+$ at m/z 288.3858. The NMR data of compound **3** was also the same to the authentic sample but only was different in ^{1}H NMR absorption at H-17 and H-16 in ^{1}H NMR spectrum and C-17 and C-16 in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum of compound **3** had an overlapped peak at 4.68 (H-17) instead of a peak at 3.92 (J = 8 Hz, H-17) in the authentic sample (Table 1). This assumption was further confirmed with ^{13}C NMR spectrum having absorption at δ 82.30 for C-16 of methyne, instead of absorption at δ 30.90 for C-16 methylene (Table 2). Furthermore, no interaction was observed in NOE between H-16 and H-17, which confirmed that H-16 and H-17 had different stereochemistry; H-16 was ß while H-17 was α. In NOE spectrum, the position of 16-OH was further confirmed through COSY interaction of H-16 with H-17 (Horeau and Kagan, 1964), and was used to assign the stereochemistry of the newly formed hydroxyl group at position-17 of the compound **3**.

The molecular formula of compound **4** determined from HR-EI-MS was found to be C$_{18}$H$_{20}$O$_2$, and showed M$^+$ at m/z 268.3548. The IR spectrum of compound **4** showed olefinic, ketonic and hydroxyl absorption at 1604, 1665 and 3335 cm^{-1}, respectively. The ^{1}H NMR spectrum of

Table 1. ^{1}H NMR assignments of compound (**1**) and its metabolites **2** to **5**.

Proton	1	2	3	4	5
H-1	7.09 d (12.6)	7.06 d (12.6)	7.27 d (12.6)	7.11 d (12.6)	7.04 d (12.6)
H-2	7.00 dd (10.4, 6.6)	6.99 dd (10.4, 6.6)	6.58 dd (10.4, 6.6)	7.00 dd (10.4, 6.6)	6.78 dd (10.4, 6.6)
3-OH	8.70s	8.71s	8.70 s	8.69 s	8.69 s
H-4	6.99 dd (16.2, 4.3)	7.00 dd (16.2, 4. 3)	6.98 dd (16.2, 4. 3)	7.01 dd (16.2, 4. 3)	6.72 dd (16.2, 4. 3)
H-5	-	-	-	-	-
H-6	2.81t (2.5, 6.2)	2.82 t (2.5, 6.2)	2.85 t (2.5, 6.2)	3.42 t (2.5, 6.2)	5.56 d
H-7	1.26, 1.78	1.27, 1.79m	1.40, 1.66 m	5.41 m	5.48 dd
H-8	1.29 m	1.30 m	1.43 m	-	3.38 m
H-9	2.09 m	2.08 m	2.08 m	3.43 t	2.10 m
H-10	-	-	-	-	
H-11	2.17, 1.29 m	2.29, 1.37m	2.24, 1.30 m	2.21, 1.63 m	2.23, 1.28 m
H-12	1.12, 1.73d (11.7)	1.29, 1.80 d (11.7)	1.46, 1.66 d (11.7)	1.10, 1.64 d (11.7)	1.16, 1.80 d (11.7)
H-13	-	-	-	-	-
H-14	1.79 m	1.44 m	1.50 m	2.43 m	1.52 m
H-15	1.79 m	1.79 m	1.66, 1.08 m	1.64, 1.08 m	1.58, 1.28 m
H-16	1.78, 1.32 m	1.93, 1.43t (2.2, 25)	4.03 m	1.94,173 t	1.84, 1.30 t (2.2, 25)
16-OH	-	-	4.66 s	-	-
H-17	3.89 t (17.1, 8.5)	-	4.05 d (20.08)	-	-
17-OH	3.92 s	-	4.68 s	-	-
H-18	0.98	0.80	1.06	0.70	0.86

The signal appeared in ^{1}H-NMR spectrum recorded in C_5H_5N.

Table 2. ^{13}C NMR chemical shifts and multiplicities of metabolites **1** to **5**.

Carban	1	2	3	4	5
C-1	126.8 (CH)	126.9 (CH)	126.8 (CH)	128.6 (CH)	128.4 (CH)
C-2	113.8 (CH)	113.9 (CH)	113.9 (CH)	115.0 (CH)	115.0 (CH)
C-3	156.6 (Q)	156.8(Q)	156.7 (Q)	157.0 (Q)	157.3 (Q)
C-4	116.2 (CH)	116.3 (CH)	116.3 (CH)	116.0 (CH)	116.0 (CH)
C-5	138.1 (Q)	138.0 (Q)	138.1 (Q)	136.5 (Q)	135.9 (Q)
C-6	29.9 (CH_2)	29.8 (CH_2)	29.7 (CH_2)	30.1 (CH_2)	126.9 (CH)
C-7	26.8 (CH_2)	26.3 (CH_2)	26.3 (CH_2)	122.9 (CH)	116.3 (CH)
C-8	39.4 (CH)	38.7(CH)	38.8 (CH)	123.2 (Q)	40.4 (CH)
C-9	43.7 (CH)	44.3 (CH)	45.0 (CH)	49.5 (CH)	49.5 (CH)
C-10	131.4 (Q)	130.8 (Q)	130.9 (Q)	129.3 (Q)	129.2 (Q)
C-11	27.6 (CH_2)	26.8 (CH_2)	26.9 (CH_2)	30.1 (CH_2)	30.1 (CH_2)
C-12	37.4 (CH_2)	35.9 (CH_2)	36.9 (CH_2)	35.5 (CH_2)	36.7 (CH_2)
C-13	43.7 (Q)	44.3 (Q)	44.5 (Q)	49.5 (Q)	49.6 (Q)
C-14	50.2 (CH)	50.3 (CH)	50.4 (CH)	50.7(CH)	50.7 (CH)
C-15	23.4 (CH_2)	21.6 (CH_2)	22.6 (CH_2)	19.9 (CH_2)	20.9 (CH_2)
C-16	30.9 (CH_2)	32.1 (CH_2)	82.3 (CH)	32.7 (CH_2)	32.7 (CH_2)
C-17	81.2 (CH)	219.0 (C=O)	82.2 (CH)	218.1 (C=O)	220.2 (C=O)
C-18	11.6 (CH_3)	13.8 (CH_3)	12.2 (CH_3)	13.7 (CH_3)	13.6 (CH_3)

Multiplicities were determined by DEPT experiment.

compound **4** was similar to that of compound **2**; the only difference was the olefinic group at C-7 and C-8 position. The ^{1}H NMR data of compound **4** had only difference in absorption at δ 5.41(m) H-7 for H-7 methine instead of at δ1.27 for H-7 methyne (Table 1). This assumption was further confirmed through ^{13}C NMR spectrum with a
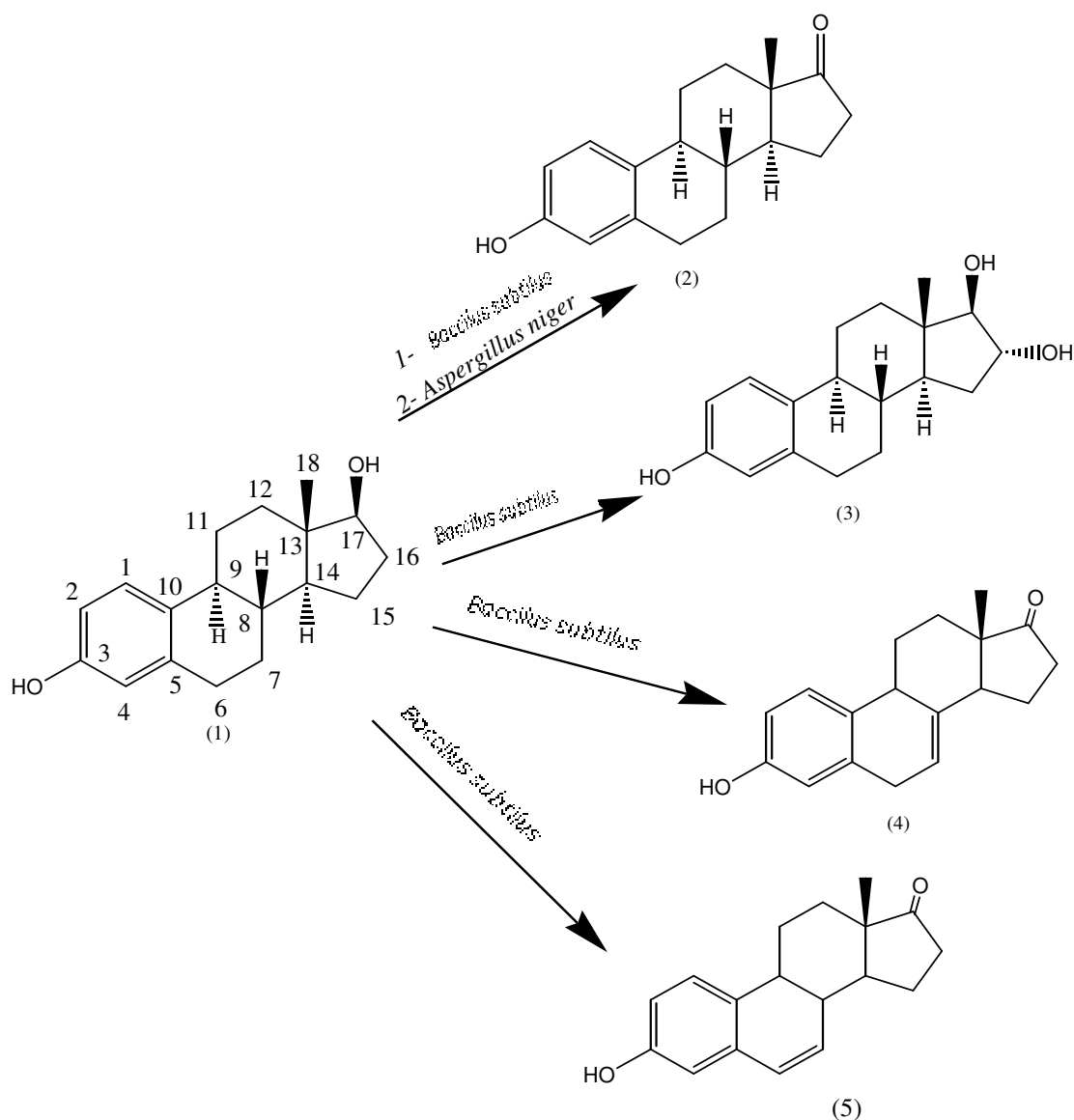
Scheme 1: Microbial transformation of compound **1**.

signal for C-7 at δ 122.95 (CH) instead of a signal at δ 26.88(CH$_2$), while C-8 was at δ 123.25(q) instead of at δ 39.40(CH) (Table 2). Further, the position of the double bond was confirmed by the HMBC interaction of the signal of H-6 at δ 5.41 (overlap), with C-8, C-9 and C-14, respectively.

The molecular formula of compound **5** determined from HR-EI-MS was found to be C$_{18}$H$_{20}$O$_2$, and showed M$^+$ at m/z 268.3467. The IR spectrum of compound **5** showed olefinic, ketonic and hydroxyl absorption at 1607, 1667 and 3338 cm^{-1}, respectively. The ^{1}H NMR spectrum of compound **5** was similar to that of compound **4**. The only difference was for the olefinic group which shifted from C-7 and C-8 in compound **4** to C-6 and C-7 positions in

compound **5**. The ^{1}H NMR spectrum of compound **5** showed absorption peak for H-6 at δ 5.56(CH) instead of at δ 3.42(CH$_2$) (Table 1). The assumption was further confirmed through ^{13}C-NMR spectrum with a signal for C-6 at δ 126.93 (CH), instead of at δ 30.17(CH2) in compound **4** (Table 2). Further, the position of the double bond between C-6 and C-7 was confirmed by the HMBC interaction experiment.

Compounds **1** to **5** were screened for the inhibition of lipoxygenase enzyme. Lipoxygenase constitute a family of non-haem iron containing dioxygenases that are widely distributed in animals and plants. In mammalian cells, these are key enzymes in the biosynthesis of variety of bioregulatory compounds such as hydroxyeicosa- tetraenoic

Table 3. Inhibitory activity (IC_{50} (µM)) of compounds **1** to **5** against lipoxygenase enzyme.

Compound	Lipoxygenase IC_{50}
1	374.4±5.2
2	Inactive
3	168.4±3.2
4	197.4±4.1
5	195.4±1.3
Baicalcin (control)	22.4±1.3

acids (HETEs), leukotrienes, lipoxins and hepaxylines.

Lipoxygenases are therefore, regarded as an attractive target for the rational drug design and discovery of mechanism-based inhibitors of treatment of variety of disorders such as bronchial asthma, inflammation, cancer and autoimmune diseases (Lili et al., 2004). The IC_{50} values of metabolites **1** to **5** are shown in Table 3, along with the positive control of lipoxygenase (Baicalcin). The biological screenings revealed that, metabolite **3** had more inhibitory potential against the lipoxygenase enzyme than *β*-estradiol, while metabolites **4** and **5** also had moderate inhibitory activity against the lipoxygenase enzyme (Table 3). Similarly, compound **1** showed non-significant potential followed by compound **2** which was inactive.

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