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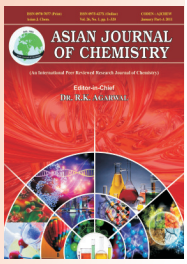

Microbial Transformation of Topical Corticosteroid: Prednicarbate

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In the present research, the steroidal antiinflammatory topically used drug prednicarbate (**1**) was subjected to microbial biotransformation by *Cunninghamella elegans*. Prednicarbate (**1**) was transformed into various metabolites. One new and two known metabolites were purified named as prednisolone 17-ethylcarbonate (**2**) Prednisolone (**3**) and 4-(4-hydroxybenzyl)-2-(3-methylbut-2-enyl)phenyl methyl carbonate (**4**). The compound (**4**) was separated as a new compound and was not reported in literature. Its structure did not show resemblance with prednicarbate so possibly derived from fungal mass. Their structures were elucidated by using modern spectroscopic techniques *e.g.* ^{13}C NMR, ^{1}H NMR, HMQC, HMQC, COSY, NOESY and mass spectrometry *e.g.* EI-MS.

Keywords: Biotransformation, *Cunninghamella elegans*, Prednicarbate, Topical.

INTRODUCTION

Now a day's microbial biotransformation is flourished in order to find the new modified drug molecules. In microbes, bacteria and fungi are used to modify existing drug molecules with better the rapeutic profile, efficacy and least resistant[1]. The factors that govern the microbiological hydroxylation of steroids involve a combination of effects based on the site, stereochemistry and nature of the existing functional groups in the substrate[2]. The importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when the Upjohn Company patented the process of 11α-hydroxylation of progesterone by Rhizopus species[3]. A variety of steroids are widely used as antiinflammatory, diuretic, anabolic, contractive, antiandrogenic, progesteronal and anticancer agents as well as in other applications[4]. Using microorganisms in the biotransformation procedures has been investigated for decades with the advantages in production of the molecules with regio- and stereospecificity[5,6]. One of the major biotechnological aspects in this area is the application of a wide range of the microorganisms including bacteria, fungi and micro algae in converting steroid substances into the pharmacologically active compounds or other useful intermediates[7-9]. Prednicarbate is quite a new topically applied corticosteroid drug. Its potency is like hydrocortisone. No microbial biotransformation has been referenced in the literature until now. It prescribed for the treatment of inflammatory skin diseases, such as atopic dermatitis[10]. It is a non-halogenated corticosteroid, double ester derivative of prednisolone.

EXPERIMENTAL

Prednicarbate (**1**) was obtained from Stiefel Laboratories Pakistan (Pvt) Ltd. Thin layer chromatography was carried out on precoated plates (Silica gel, Merck, PF254). Column chromatography (CC) was performed by using silica gel (E. Merck, Germany). ^{1}H- and ^{13}C NMR spectra were recorded in $CDCl_3$ on Bruker Avance-NMR spectrometers. The chemical shifts (δ values) are presented in ppm and the coupling constants (J values) are in Hertz. JEOL (Japan) JMS-600H mass spectrometer was used for recording EI-MS in *m/z*.

Microbial culture: Culture of *Cunninghamella elegans* was purchased from FCBP (764), grown on Saboraud dextrose agar (SDA) England. The culture medium for *C. elegans* was prepared by dissolving glucose (40 g), yeast extract (20 g), peptone (20 g), NaCl (20 g), KH_2PO_4 (20 g) and glycerol (40 mL) in distilled water (4 L).

Fermentation of prednicarbate with *Cunninghamella elegans* and purification of metabolites: In experiment forty 250 mL conical flasks for the distribution of 4000 mL media such that each flask containing 100 mL media. 1 g of prednicarbate was dissolved in 20 mL acetone and was equally divided among all the flasks such that (25 mg/0.5 mL/flask), which were then placed on shaker at 25-28 °C for 14 days. After completion of fermentation, the fungal mass was filtered and the filtrate was extracted with dichloromethane. The extract (organic phase) was dehydrated over anhydrous Na_2SO_4, evaporated under high vacuum in rotary evaporator to get brown gummy crude extract which was put to analysis through

different chromatographic techniques. Slurry of this crude extract has been prepared in a petri dish. A wide width column has been prepared and packed with silica gel and solvent petroleum ether. Slurry was loaded on to the column while maintaining its surface and put a cotton onto the slurry for assuring its surface balance during the experiment. Now this slurry was eluted with gradient ethyl acetate/petroleum ether solvent system. Flow rate of the column was quite fast in this experiment. Fractions obtained were dried in vacuum hood and subjected to thin layer chromatography and similar fractions were compared and compile. Five fractions were obtained from the main column after compilation. Fraction 3 was run on normal phase recycling HPLC.

RESULTS AND DISCUSSION

Fermentation of prednicarbate (**1**) with *Cunninghamella elegans* for 14 days yielded three known compounds (**2,3**) which were identified as prednisolone 17-ethylcarbonate (**2**), prednisolone (**3**) and 4-(4-hydroxybenzyl)-2-(3-methylbut-2-enyl)phenyl methyl carbonate (**4**).

Products resulting from the biotransformation of prednicarbate are presented in Fig. 1.

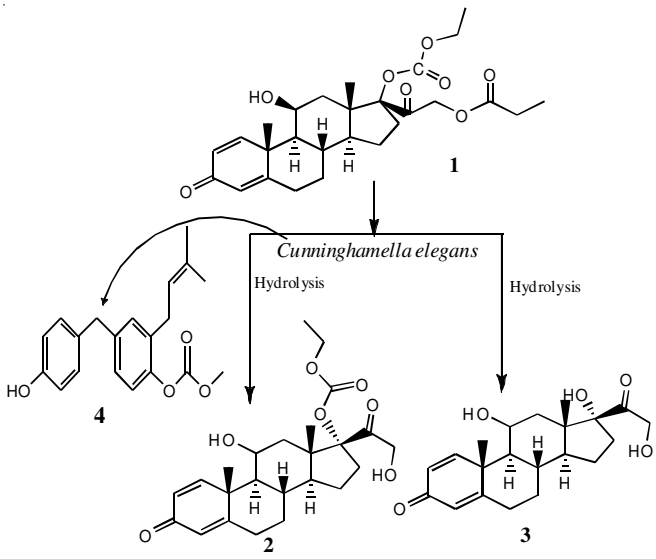

Fig .1. *Drug Prednicarbate (**1**) is transformed into three metabolites prednisolone 17-ethylcarbonate (**2**) and prednisolone (**3**) by means of *Cunninghamella elegans* and one new metabolite not reported in literataure derived from *Cunninghamella elegans* as 4-(4-hydroxybenzyl)-2-(3-methylbut-2-enyl)phenyl methyl carbonate

Prednisolone 17-ethylcarbonate (**2**) was purified on normal phase HPLC as a colour less solid. R_f = 0.40 (petroleum ether/acetone = 7:3), m.f.: $C_{24}H_{32}O_7$, m.w.: 432.506680 g/mol, HREI-MS:*m/z* (m.f., calcd value): 432.2143 ($C_{24}H_{32}O_7$,432.2148).

Prednisolone (**3**) was purified on normal phase HPLC as a white colour crystalline solid. R_f = 0.25 (petroleum. ether/acetone = 6:4), m.p.: 235 °C, m.f.: $C_{21}H_{28}O_5$, m.w.: 360.444020 g/mol, HREI-MS:*m/z* (m.f., calcd value): 360.1923 ($C_{21}H_{28}O_5$, 360.1937).

4-(4-Hydroxybenzyl)-2-(3-methylbut-2-enyl)phenyl methyl carbonate (**4**) was isolated as colourless amorphous powder was soluble in methanol by column chromatography technique. The molecular formula of the compound was found to be $C_{20}H_{22}O_4$ which was established by EI-MS. Molecular weight of the compound was 310 and melting point was 390 °C.

Prednisolone 17-ethylcarbonate (**2**) had an elemental composition $C_{24}H_{32}O_7$, deduced through HREI-MS (M^+ = *m/z* 432.2143, calcd 432.2148) and molecular weight is 432.5067. The compound showed fluorescence under UV light. This suggested that the compound has unsaturated carbonyl system. The UV analysis showed absorbance at (238 nm) for a conjugated ketone. The ^{13}C and ^1H NMR spectra signaled the presence of the double bond between C-1/C-2 and C-4/C-5. The ^1H and ^{13}C NMR data for metabolite (**2**) is given in Table-1. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) (including BB, dept 90 and dept135) of (**2**) signaled the resonances for three methyl, seven methylene, seven methine and seven quaternary carbons. Mass spectra indicated that the mass of this compound is 432.2143 Da which is 56 mass unit less than the substrate (488.241) which showed the removal of CH_2CH_2CO (56 mass units). So metabolite (**2**) was thus identified as prednisolone 17-ethylcarbonate[11].

TABLE-1
^1H- (400 MHz, $CDCl_3$) AND ^{13}C NMR (100 MHz, $CDCl_3$) CHEMICAL SHIFT ASSIGNMENTS OF **2**

Carbon No.	δ_C	Multiplicity	δ_H (*J* = Hz)
1	156.0	CH	7.23, d (10)
2	127.9	CH	6.25, dd (1.6,10)
3	186.5	C	-
4	122.5	CH	5.99, s
5	169.8	C	-
6	31.9	CH_2	2.3, dd; 2.56, td (3.2,13.2)(4.8,13.6)
7	34.0	CH_2	1.09, m; 2.10, m
8	31.2	CH	2.15, m
9	55.3	CH	1.06, m
10	39.8	C	-
11	70.1	CH	4.48, br s
12	39.8	CH_2	1.65, m; 2.05, m
13	47.9	C	-
14	51.4	CH	1.62, m
15	23.8	CH_2	1.48, m; 1.81, m
16	34.8	CH_2	1.45, m; 2.75, m
17	89.6	C	-
18	17.0	CH_3	0.98, s
19	21.1	CH_3	1.44, s
20	204.4	C	-
21	70.2	CH_2	4.82, d; 5.02, d (17.6,17,6)
22	155.0	C	-
23	64.6	CH_2	4.21, q (7.2)
24	14.2	CH_3	1.32, t (7.2)

Prednisolone (**3**) had an elemental composition $C_{21}H_{28}O_5$, deduced through HREI-MS (M^+ = *m/z* 360.1923, calculated 360.1937) molecular weight is 360.444. The compound showed fluorescence under UV light. This suggested that the compound has unsaturated carbonyl system. The UV analysis showed absorbance at (238 nm) for a conjugated ketone. The ^{13}C and ^1H NMR spectra signaled the presence of the double bond between C-1/C-2 and C-4/C-5. The ^1H and ^{13}C NMR data for metabolite 3 is given in Table-2. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) (including BB, dept 90 and dept135) of data showed the resonances for two methyl, six methylene, seven methine and six quaternary carbons. Mass spectra displayed that the mass of this compound is 360.1923

Da which is 128 mass unit less than the substrate (488.241) which showed the removal of CH_2CH_2CO (56 mass units) and removal of CH_2CH_2OCO (72 mass units). This metabolite was thus characterized as prednisolone (**3**)[12].

TABLE-2
^1H (400 MHz, $CDCl_3$) AND ^{13}C NMR (100 MHz, $CDCl_3$) CHEMICAL SHIFT ASSIGNMENTS OF COMPOUND **3**

Carbon No.	δ_C	Multiplicity	δ_H (J = Hz)
1	155.8	CH	7.22, d (10)
2	128.0	CH	6.25, dd (2,10)
3	186.4	C	-
4	122.6	CH	6.00, s
5	169.6	C	-
6	31.9	CH_2	2.32, m; 2.56, td (4.4, 13.2)
7	33.9	CH_2	1.16, m; 2.08, m
8	31.3	CH	2.12, m
9	55.2	CH	1.08, m
10	43.9	C	-
11	70.2	CH	4.48, br, t
12	39.8	CH_2	1.46, m; 2.02, dd (3.6,14.4)
13	48.1	C	-
14	51.2	CH	1.66, m
15	23.9	CH_2	1.48, m; 1.85, m
16	34.2	CH_2	1.54, m; 2.70, m
17	88.6	C	-
18	17.5	CH_3	0.96, s
19	21.1	CH_3	1.43, s
20	212.0	C	-
21	67.5	CH_2	4.27, dd; 4.62, dd (4.8,19.6)

4-(4-Hydroxybenzyl)-2-(3-methylbut-2-enyl)phenyl methyl carbonate (**4**) was isolated as colourless amorphous powder. The molecular formula of the compound was found to be $C_{20}H_{22}O_4$ which was established by EI-MS. Molecular weight of the compound was 310 and melting point was 390 °C.

^1H and ^{13}C NMR data for metabolite **4** is given in Table-3. The ^1H NMR shows the peak at 7.605 (d) with J-9 and another peak at 6.846 (d) with J = 9 of benzene ring so it is again a *para* substituted ring. It also showed doublet of doublet at 6.54 (d,d) with J = 8.4, 2 and 6.47 (d) J = 8 so it's *ortho* in position and 6.41 (d) with J = 2 so it's *meta* substituted and thus benzene ring is tri substituted. It also showed a triplet methine at 5.05 which attribute double bond present in molecule. It also shows two methylene group at 3.40 (s) and 3.06 (d) and two methyl at 1.65 and 1.56 which are singlet so attached to quaternary center. By using the HMBC correlation and COSY correlation structure was established and important correlation was given in Table-3 to established structure. The ^{13}C-NMR shows the signals at 130.0 (CH), 116.4 (CH), 129.7 (CH), 115 (CH), 132.4 (CH), of benzene ring and 123.6 (CH) of double bond. A signal at 53.6 shows Methoxy and two methylene signals at 39.6 and 28.7. It also shows two signals of methyl present at 25.9 and 17.7.

This was potential route of synthesis of these compounds. Chemical reactions required variety of chemicals and conditions to accomplish the reactions while in biotransformation a number of reactions can be accomplished in one container and reactions under mild conditions was required even at room temperature. As there was no hazardous substances used for the production of these metabolites so it could be denominated as green chemistry while chemical synthesis involved hazardous chemicals lead to the environmental pollution. *Cunninghamella elegans* had an ability to hydrolyze the compounds due to the presence of hydrolase enzymes in its enzyme system[13]. The reaction involved in the synthesis of metabolites was ester hydrolysis shown in Fig. 1. It is expected that the new metabolites formed may have more antiinflammatory activity, better therapeutic profile, safety, efficacy and least resistance and can be employed in the treatment of asthma as that of the parent compound.

TABLE-3
^1H- (400 MHz, $CDCl_3$) CHEMICAL SHIFT ASSIGNMENTS OF METABOLITE PRODUCED (**4**)

H NMR	δ_C	Multiplicity	δ_H (J = Hz)
7.605 (d) J = 9	130.0	CH	158.7 (Q), 130.0 (CH), 39.6 (CH_2), 128 (Q)
6.846 (d) J = 9	116.4	CH	128 (Q), 158 (Q), 116.4
6.54 (dd) J = 8.4 J = 2.0	129.7	CH	115.02 (Q), 132.4 (CH), 128.4 (Q), 39.6 (CH_2)
6.47 (d) J = 8	115.0	CH	155.02 (Q), 125.3 (Q), 128.4 (Q)
6.41 (d) J = 2	132.4	CH	129.7 (CH), 155.02 (Q), 39.6
5.05 (t)	123.6	CH	28.7 (CH_2), 132.9 (Q)
3.75(s)	53.6	MEO	172.1 (Q)
3.40 (s)	39.6	CH_2	128.4 (Q)
3.06 (d)	28.7	CH_2	128.3 (Q), 123.6 (CH)
1.65 (s)	25.9	CH_3	123.6 (CH), 132.9 (Q), 17.7 (CH_3)
1.56 (s)	17.7	CH_3	25.9 (CH_3), 123.6 (CH), 132.9 (Q)

It is expected that the new metabolites formed may have more antiinflammatory activity related to skin, better therapeutic profile, safety, efficacy and least resistance and can be employed in the treatment of inflammatory skin diseases *i.e.* dermatitis, eczema, *etc.* as that of the parent compound. In the present study we could produce these compounds outside the body which showed the definite potential of the emerging route of metabolism of drug as well as new drug discovery from the existing one.

Conclusion

Microbial biotransformation of prednicarbate by *Cunninghamella elegans* resulted first time from this fungus into two known and one new metabolite which illustrates the enzymatic potential of the fungus. The new metabolite was not reported in literature and its structure did not showed structural resemblance with substrate so it was assumed to be a fungal metabolite.

The two known compounds included prednisolone and prednisolone 17-ethylcarbonate and one new metabolite as 4-(4-hydroxybenzyl)-2-(3-methylbut-2-enyl) phenyl methyl carbonate. It is expected that new derivative may have more anti-inflammatory activity than the parent compound. Their structures of these compounds were elucidated by using modern spectroscopic techniques *e.g.*,^{13}C NMR, ^1H NMR, HMQC, HMQC, COSY, NOESY and mass spectrometry *e.g.* EI-MS.

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