Microbial Metabolism. Part 11.[1] Metabolites of Flutamide

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The yeast culture, *Rhodotorula mucilaginosa* (ATCC 20129) transformed flutamide (1) to three metabolites: 4-nitro-3-(trifluoromethyl)aniline (2), 2-methyl-*N*-[4-amino-3-(trifloromethyl)phenyl]propanamide (3) and *N*-[4-amino-3-(trifluoromethyl)phenyl]acetamide (4). The structures were established by spectroscopic methods.

Key words flutamide; microbial metabolism; *Rhodotorula mucilaginosa*

Flutamide, a nonsteroidal antiandrogen is a commonly used drug to treat advanced prostate cancer,[2] which is one of the leading causes of death in men in the United States.[3] It is absorbed rapidly from the gastrointestinal track of humans and rats after oral administration and undergoes extensive metabolism in the liver[3—5] through hydrolysis, hydroxylation, *N*-acetylation and nitroreduction to yield several metabolites.[6] The major metabolites detected in plasma are 2-hydroxyflutamide (OH-FLU) and 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1)[7] with concentrations higher than that of flutamide.[8] It is suggested that the antiandrogen activity of flutamide is largely associated with its main metabolite, 2-hydroxyflutamide.[6] Its activity is based on the ability to attach itself to the receptors of the cancer cells preventing the attachment of testosterone, the male hormone which is essential for the growth of the prostate cancer cells. Flutamide with the structure similar to that of testosterone[9] however, is a weak antiandrogen.[10] FLU-1, which is devoid of any antiandrogenic activity[7] is formed by carboxyesterase-catalysed hydrolysis.[6] It is a product of a clearance pathway. Thus, the main metabolite identified in urine is 2-amino-5-nitro-4-(trifluoromethyl)phenol (FLU-3).[3] However, urinary excretion is not a major pathway of flutamide elimination in humans.[7]

The use of flutamide as therapeutic agent against prostrate cancers is eclipsed by rare incidences of idiosyncratic liver injury.[6] Although it is suggested that flutamide and its toxic metabolites could be responsible for such hepatic injury the mechanism of toxicity remains presently unknown.[6] It is observed that the serum concentration of FLU-1 is higher[3,7] and that of OH-flutamide is lower in patients with liver dysfunction than in those with normal liver function.[11] The formation of OH-flutamide from the parent compound is catalyzed by CYP1A2. Thus, a lower concentration of OH-flutamide is due to reduced activity of the enzyme.[11] Therefore, it is suggested that since CYP1A2 seems to be involved in the initiation of flutamide-induced liver injury, attention should be paid to patients with low CYP1A2 activity before administrating flutamide.[11] Genetic mutations and factors such as smoking, food and drugs are shown to influence the activity of the enzyme.[11] Smoking induces CYP1A2 activity and reduces the risk of liver toxicity due to flutamide.[3]

In the present investigation we used microbial models retrospectively in an attempt to obtain mammalian metabolites of **1**. No more than three metabolites were detected in the culture media of the forty microorganisms used. Among them the most prominent compound was FLU-1, detected in almost all the cultures used. *Rhodotorula mucilaginosa* culture which gave all three metabolites in good yields was selected for scale up studies. The structures of the metabolites (**2**—**4**) were elucidated by detailed study of their high resolution spectroscopic data.

Results and Discussion

Of the forty microorganisms screened using the standard two stage method, *R. mucilaginosa* was selected for scale-up studies.[12] The metabolites obtained were 4-nitro-3-(trifluoromethyl)aniline (**2**), 2-methyl-*N*-[4-amino-3-(trifloromethyl)phenyl]propanamide (**3**) and *N*-[4-amino-3-(trifluoromethyl)phenyl]acetamide (**4**) (Chart 1).

The molecular formulae of all the metabolites were determined by HR-ESI-MS.

Metabolite **2** (Flu-1) (50 mg, 11.1%) was isolated as a yellow solid with a molecular formula, $C_7H_5F_3N_2O_2$. It was less polar than **1**. The NMR data were similar to those of flutamide except for the absence of signals due to isobutanoyl group. NMR correlation data along with the published data (^{1}H-NMR) were used to characterize the compound as 4-nitro-3-(trifluoromethyl)analine.[2,13]

The light yellow solid **3** (15 mg, 3.1%) gave a molecular ion peak at *m*/*z* 247.1123 $[M-H]^+$ in its HR-ESI-MS corresponding to the elemental formula, $C_{11}H_{13}F_3N_2O$. The ^{1}H-NMR data showed close resemblance to those of **1** except for the presence of a two proton singlet due to an amino group at δ 5.30. It was identified as 2-methyl-*N*-[4-amino-3-(trifloromethyl)phenyl]propanamide (**3**) by correlation spectra

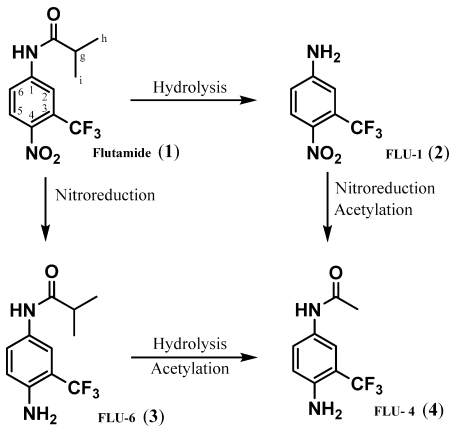

Chart 1. Possible Metabolic Pathway of Flutamide in *R. mucilagenosa*

The short names as per Aizawa *et al.*[7] and Takashima *et al.*[18] publications.

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compounds **2**—**4** (500/125 MHz)

Position	**2** (Flu-1)			**3** (Flu-6)			**4** (Flu-4)		
	δ ^{1}H ppm mult. (*J*, Hz)		δ ^{13}C ppm	δ ^{1}H ppm mult. (*J*, Hz)		δ ^{13}C ppm	δ ^{1}H ppm mult. (*J*, Hz)		δ ^{13}C ppm
1	—		154.8	—		142.5	—		142.6
2	7.06	d (2.5)	112.2	7.73	d (2.0)	117.5	7.68	d (2.0)	117.4
3	—		125.4	—		125.6	—		125.5
4	—		134.2	—		142.5	—		142.6
5	8.00	d (9.0)	114.9	6.79	d (9.0)	117.6	6.80	d (9.0)	117.7
6	6.81	dd (9.0, 2.5)	130.1	7.43	d (9.0, 2.0)	125.6	7.41	d (9.0, 2.0)	125.5
g	—		—	2.53	m	35.2	1.99	s	24.0
h	—		—	1.08	d (7.0)	20.0	—		—
i	—		—	1.08	d (7.0)	20.0	—		—
NH_2	—		—	5.30	s	—	5.30	s	—
NH	6.97	s	—	9.62	s	—	9.73	s	—
CF_3	—		124.1	—		128.9	—		128.8
C=O	—		—	—		175.1	—		168.6

together with published data.[6,8)]

Compound **4** (14 mg, 3.0%) was isolated as an off-white solid with a molecular formula $C_9H_9F_3N_2O$. The ^{1}H-NMR data of **4** differed from those of **3** in the absence of signals due to the isopropyl group and the presence of a methyl group instead. It was characterized as *N*-[4-amino-3-(trifluoromethyl)phenyl]acetamide (**4**) by correlation spectra.

Due to the limited availability of published data on **2**—**4**, their NMR spectral information is included in Table 1.

Conclusion

Many of the forty microbial cultures screened were able to transform **1** to a maximum of three metabolites with all showing the presence of FLU-1 (**2**) as the major product. Among the cultures, *Rhodotorula mucilaginosa* (ATCC 20129), *Beauveria bassiana* (ATCC 7159) and *Nocardia* sp. (NRRL 5646) which were capable of transforming **1** to all three metabolites. *R. mucilaginosa* was selected for large scale experiments as it showed a higher biotransformational efficiency. The three metabolites isolated and characterized were detected as products *in vitro* and *in vivo* experiments as well.[3,4,6—8)] The major routes of flutamide metabolism in humans include P450 mediated oxidation to 2-hydroxyflutamide and cleavage of the amide bond to FLU-1.[3)] In addition to 2-hydroxyflutamide mono-, di- and trihydroxylated flutamides have been identified.[8)] The absence of the hydroxylated metabolites and the predominant presence of FLU-1 along with FLU-4 and FLU-6 in culture media suggest that the fungal metabolism of flutamide takes place exclusively *via* amide bond cleavage pathway (Chart 1).[3)] Flutamide and its major microbial transformation product, FLU-1 are nitroaromatic compounds which undergo nitroreductive metabolism to yield FLU-6 and FLU-4 (detected in human urine).[6)] Nitroaromatic compounds undergo reductive and/or oxidative metabolism in mammalian cells and in microorganisms.[14)] The common pathway involves several reduction steps to yield the corresponding aniline which gets converted the corresponding amide by *N*-acetylation.[14)] The nitroso, *N*-hydroxy and the nitroanion radical intermediates formed during the reduction are suspected to be responsible for toxicity of nitroaromatic compounds.[6)] This investigation led to the isolation and characterization of important metabolites of flutamide. It shows that the microbial transformation studies carried out prospectively could give valuable information about the activity and possible toxicity of a drug before designing more expensive and elaborate experiments. The ability to mimic mammalian metabolism and to obtain sufficient quantities of metabolites for structure elucidation and further pharmacological studies are the advantages of using microbes as mammalian models of drug metabolism.[15)]

Experimental

General Experimental Procedures Unless otherwise stated the ^{1}H- and ^{13}C-NMR were obtained in $CDCl_3$ and DMSO-d_6 on a Bruker DRX-500 spectrometer. UV spectra were obtained using a Hewlett Packard 8452A diode array spectrometer. IR spectra were measured in $CHCl_3$ on an ATI Mattson Genesis series FTIR spectrophotometer. HR-ESI-MS data were obtained using a Bruker GioApex 3.0.

Substrate Flutamide (**1**) was purchased from Sigma Aldrich Chemical Co. (Milwaukee, Wisconsin) and its authenticity was confirmed by NMR data.

Organisms and Metabolism A two stage procedure[16)] was adapted to screen forty microbes from collection of The National Center for Natural Products Research of The University of Mississippi to identify organisms capable of successfully transforming flutamide (**1**) to its metabolites. Transformations were carried out under mild reaction conditions of normal aeration at ambient temperature and physiological pH to avoid decomposition of unstable metabolites that may be formed during the conversion. The progress of reaction was monitored at 7-d intervals using precoated Si gel 60 F_{254} TLC plates (E. Merck) with *p*-anisaldehyde as the spray reagent. *R. mucilagenosa* which gave all metabolites in comparatively good yields was selected for scale up studies. Preparative scale fermentation of **1** was carried out by administrating the substrate in dimethylformamide using six 1 l flasks, each containing 75 mg of substrate dispersed in 500 ml of medium-α[12)] by *R. mucilagenosa*. After 14 d of fermentation the incubation mixtures were filtered and the filtrates were extracted with EtOAc (500 ml×3). The combined extracts were evaporated *in vacuo* at 40 °C to yield a brownish residue (1.1 g). It was chromatographed on a Si gel column using CH_2Cl_2, gradually enriched with MeOH. The fractions containing the transformed compounds were combined and subjected to further column chromatography (CH_2Cl_2/MeOH) followed by preparative thin layer (Silica gel 60 F_{254}) chromatography (CH_2Cl_2/MeOH) to purify the metabolites. Substrate and culture controls were run along with the above experiments.[17)]

Microbial Transformation of Flutamide (1) by *Rhodotorula mucilaginosa* (ATCC 20129) Column chromatographic separation (Si gel 230—400 mesh: E. Merck, 30 g, column diameter: 20 mm) of the EtOAc extract of the combined fermentation broth was carried out using CH_2Cl_2 enriched with MeOH. The fractions obtained were further purified by repeated column and preparative layer chromatography (CH_2Cl_2–MeOH, 24 : 1) to obtain three compounds, **2**—**4** which were identified by spectroscopic along with published literature data.

4-Nitro-3-(trifluoromethyl)aniline (**2**) was isolated as a yellow solid (50 mg, 11.1%). *Rf* 0.87 [MeOH–CH_2Cl_2 (1 : 24)]; UV λ_{max} (MeOH) nm

(log ε): 367.9 (3.81), 313.4 sh (3.47), 232.0 (3.68) 203.0 (3.86); IR ν_{max} ($CHCl_3$) cm^{-1}: 1623, 1496, 1453, 1332, 1265, 1188, 1139, 1037, 880, 836; ^{1}H-NMR 400 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Table 1: HR-ESI-MS $[M+Na]^+$: (m/z) 205.0282 (Calcd for $C_7H_5F_3N_2O_2-H^+$: 205.0224).

2-Methyl-*N*-[4-amino-3-(triflоromethyl)phenyl]propanamide (**3**) (15 mg, 3.3%), was obtained as a light yellow solid with a *Rf* 0.51 [MeOH–CH_2Cl_2 (1 : 24)]; UV λ_{max} (MeOH) nm (log ε): 307.1 (3.38), 261.0 (4.06), 206.0 (3.99); IR ν_{max} ($CHCl_3$) cm^{-1}: 3301, 2925, 2854, 1662, 1550, 1513, 1302, 1140, 1110, 893, 829; ^{1}H-NMR 400 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Table 1: HR-ESI-MS $[M-H]^+$: (m/z) 247.1123 (Calcd for $C_{11}H_{13}F_3N_2O+H^+$: 247.1059).

N-[4-Amino-3-(trifluoromethyl)phenyl]acetamide (**4**) (14 mg, 3.0%), was a off-white solid with a *Rf* 0.22 [MeOH–CH_2Cl_2 (1 : 24)]; UV λ_{max} (MeOH) nm (log ε): 306.8 (2.60), 253.0 (3.95), 201.9 (3.68); IR ν_{max} ($CHCl_3$) cm^{-1}: 3263, 2925, 2854, 1662, 1566, 11549, 1498, 1465, 1412, 11382, 1330, 796, 722; ^{1}H-NMR 400 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Table 1: HR-ESI-MS $[M-H]^+$: (m/z) 219.0824 (Calcd for $C_9H_9F_3N_2O+H^+$: 219.0746).

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References and Notes

1) Part 10: Herath W., Mikell J. R., Khan I. A., *Nat. Prod. Res.*, **23**, 1231—1239 (2009).
2) Sortino S., Gluffrida S., De Guidi G., Chillemi R., Petralia S., Marconi G., Condorelli G., Scluto S., *Photochem. Photobiol.*, **73**, 6—13 (2001).
3) Goda R., Nagai D., Akiyama Y., Nishikawa K., Ikemoto I., Aizawa Y., Nagata K., Yamazoe Y., *Drug Metab. Dispos.*, **34**, 828—835 (2006).
4) Tevell A., Lennernäs H., Jönsson M., Norlin M., Lennernäs B., Bondesson U., Hedeland M., *Drug Metab. Dispos.*, **34**, 984—992 (2006).
5) Kang P., Dalvie D., Smith E., Zhou S., Deese A., *Drug Metab. Dispos.*, **35**, 1081—1088 (2007).
6) Wen B., Coe K. J., Rademacher P., Fitch W. L., Monshouwer M., Nelson S. D., *Chem. Res. Toxicol.*, **21**, 2393—2406 (2008).
7) Aizawa Y., Ikemoto I., Kishimoto K., Wada T., Yamazaki H., Ohishi Y., Kiyota H., Furuta N., Suzuki H., Ueda M., *Mol. Cell. Biochem.*, **252**, 149—156 (2003).
8) Kang P., Dalvie D., Smith E., Zhou S., Deese A., Nieman J. A., *Drug Metab. Dispos.*, **36**, 1425—1437 (2008).
9) Tzanavaras P. D., Themelis D. G., *J. Pharm. Biomed. Anal.*, **43**, 1820—1824 (2007).
10) Marsall K., *Altern. Med. Rev.*, **6**, 272—292 (2001).
11) Ozono S., Yamaguchi A., Mochizuki H., Kawakami T., Fujimoto K., Otani T., Yoshida K., Ichinei M., Yamashita T., Hirao Y., *Prostate Cancer Prostatic Dis.*, **5**, 128—131 (2002).
12) Herath W., Mikell J. R., Ferreira D., Khan I. A., *Chem. Pharm. Bull.*, **51**, 646—648 (2003).
13) Stabile R. G., Dicks A. P., *J. Chem. Educ.*, **80**, 1439—1443 (2003).
14) Boelsterli U. A., Ho H. K., Zhou S., Leow K. Y., *Curr. Drug Metab.*, **7**, 715—727 (2006).
15) Abourashed E. A., Khan I. A., *Chem. Pharm. Bull.*, **48**, 1996—1998 (2000).
16) Elmarakby S. A., Clark A. M., Baker J. K., Hufford C. D., *J. Pharm. Sci.*, **75**, 614—618 (1986).
17) Orabi K. Y., Clark A. M., Hufford C. D., *J. Nat. Prod.*, **63**, 396—398 (2000).
18) Takashima E., Iguchi K., Usui S., Yamamoto H., Hirano K., *Biol. Pharm. Bull.*, **26**, 1455—1460 (2003).