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THE C-1(2)-DEHYDTROGENATION OF 6-METHYLENE ANDROSTENEDIONE TO EXEMESTANE, AN AROMATASE INHIBITOR USED FOR THE TREATMENT OF BREAST CANCER

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ABSTRACT: Exemestane is an aromatase inhibitor widely used for the treatment of postmenopausal breast cancer patients. Several precursors are used for its synthesis; however the final step is C-1(2) dehydrogenation of the resultant steroid to yield exemestane. A sequence of hazardous chemical reactions is required for the final step of dehydrogenation. The present work was carried out to replace the chemical reaction with microbial catalysis. Microorganisms reported in the literature to catalyze C-1(2) dehydrogenation of various steroids were screened for dehydrogenation of 6-methylene androstenedione, a commonly used precursor for the synthesis of exemestane. The bacterial strains *Delftia acidovorans* MTCC 3364, *Arthrobacter simplex* IAM 1660 and *Arthrobacter simplex* NCIM 2449 have shown promising results and may be used for enzymatic catalysis and to develop eco-friendly route for the synthesis of this exemestane**.**

Key words: C-1(2)-Dehydrogenation, exemestane, 3-oxosteroid 1-dehydrogenase, breast cancer, *Delftia acidovorans* MTCC 3364.

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INTRODUCTION

Exemestane is widely used drug for the treatment of breast cancer in postmenopausal patients (Goss et al., 2011) and presently under phase III trial valuation in combination with bevacizumab (Trédan et al., 2016). A number of chemical synthetic routes have been developed for the synthesis of exemestane from various steroid precursors including androstenedione (Agarwal et al., 2012; Buzzetti et al., 1989) boldenone (Longo and Lombardi 1989), androstadienedione (Kunnen et al., 2012), 6-methylene androstenedione [6-MeAD] (Agarwal et al., 2009) and testosterone (Marcos-Escribano et al., 2009). All these routes involve multiple chemical steps and use of hazardous chemicals during the final step of C-1(2)-dehydrogenation, making them undesirable for the synthesis of exemestane.

An eco-friendly microbial route has also been developed using a bacterium *Nocardioides simplex* VKM Ac-2033D as a source of an enzyme 3-oxosteroid 1-dehydrogenase (EC 1.3.99.4) to catalyze C-1(2)-dehydrogenation of 6-MeAD, a commonly available precursor for the synthesis of exemestane (Figure 1) by Sukhodolskaya et al., (2010) and the process is patented (Savinova et al., 2011).

This enzyme is of great commercial importance due to its capacity to dehydrogenate various steroid substrates to their respective 3-oxo-delta1-steroids required for the synthesis of a variety of steroid hormones and drugs. 3-oxosteroid 1-dehydrogenase is widely distributed in microorganisms, particularly in the genera *Mycobacterium* (Zhang et al., 2013), *Arthrobacter (*Fokina et al., 2003; Li et al., 2013; Sukhodolskaya et al., 2010; 2017; *Pseudomonas* (Jethwa et al., 2011); R*hodococcus* (de las Heras et al., 2012).

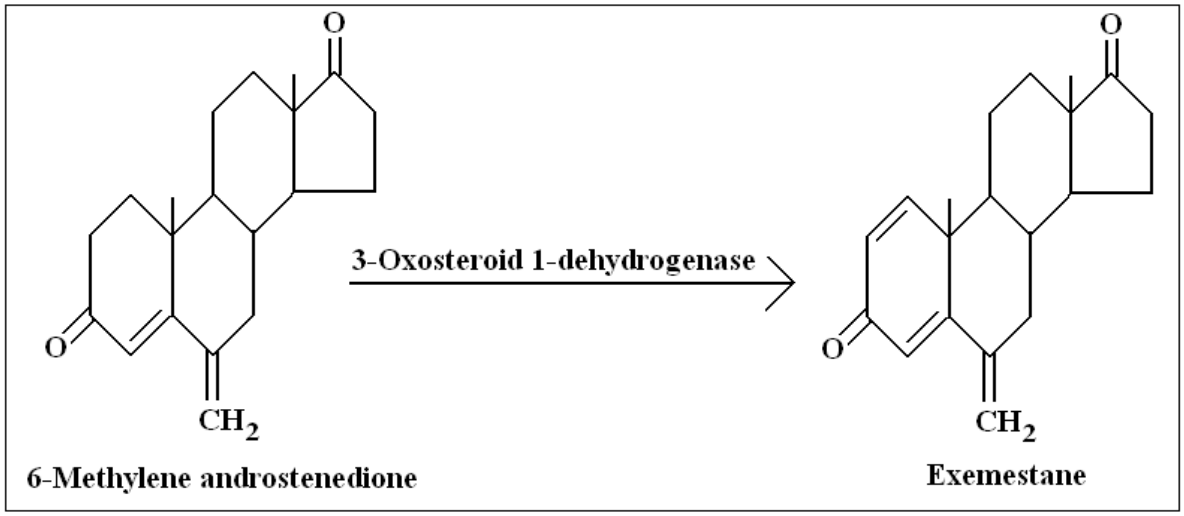

Figure 1. Enzymatic C-1(2)-dehydrogenation of 6-MeAD to exemestane

Comamonas (Horinouchi et al., 2003), *Delftia acidovorans* (Banerjee et al., 2014) and *Nocardia* (Kominek et al., 1985). In spite of wide spread 1(2)-dehydrogenation activity of various genera of bacteria, only *Arthrobacter simplex* and its variants including *Arthrobacter simplex* ATCC 6946 (Li et al., 2013), *Nocardioides simplex* VKM Ac-2033D (Sukhodolskaya et al., 2010, 2017) have so far been reported to catalyze C-1(2)-dehydrogenation of 6-MeAD to exemestane. The present work was undertaken to evaluate the feasibility of other strains of bacteria known to catalyze 1(2)-dehydrogenation of various steroids, so that efficient organism(s) for 1(2)-dehydrogenation of 6-MeAD to exemestane can be selected.

MATERIALS AND METHODS

Organisms

Bacterial strains used in the present study were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, India; National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, India; Institute of Applied Microbiology (IAM), University of Tokyo, Japan and Northern Regional Research laboratory (NRRL), Agricultural Research Service, USA. All organisms were grown on nutrient agar slants and preserved at 4^{o}C.

Bioconversion of 6-MeAD

Fifty ml nutrient broth (pH 7.2) was dispensed in 250 ml capacity Erlenmeyer flask and 50 mg 6-MeAD dissolved in 0.5 ml ethyl alcohol was added to it with continuous stirring on a magnetic stirrer. After sterilization at 121 ^{o}C for 15 minutes, the flasks were inoculated aseptically with 1 ml actively growing cultures of bacterial strains grown in nutrient broth and incubated at 32 $\pm$ 1 ^{o}C on a gyratory incubator shaker (180 rpm, 2.5 mm eccentric throw).

Thin layer chromatography

At regular intervals of 24 h, the samples of the incubation medium were drawn and analyzed by TLC for detection of residual 6-MeAD and exemestane accumulated in the medium. To 1 ml sample of medium in an eppendroff tube, 0.5 ml chloroform was added and thoroughly mixed on a cyclomixer. After centrifugation at 10,000 rpm for 5 minutes, lower chloroform phase was transferred to another tube and dried over anhydrous sodium sulphate. Ten microliter chloroform extract was spotted on pre-coated TLC plates (silica gel 60 F254, Merck, Darmstadt) along with authentic samples of exemestane obtained from Cipla Ltd., Mumbai, India and 6-MeAD synthesized from androstenedione (AD) procured from Jagsonpal Pharmaceuticals, New Delhi following the method described earlier (Patil et al., 2017).

The plates were developed in benzene-ethyl acetate (5:2) and the spots were visualized by spraying the plates with 50 % sulphuric acid followed by heating at 110 °C for 5 minutes in oven. Identification of exemestane spot on TLC plate was done by Rf value and color of the spot with that of authentic compound. Quantification of exemestane formed in the incubation medium was carried out by visual comparison of size and color density of spots on TLC plates.

RESULTS AND DISCUSSION

The relative concentrations of residual substrate left and exemestane formed in the incubation medium after different incubation periods by some bacterial strains are presented in Table 1. A spectrum of C-1(2)-dehydrogenase activity ranging between no activity to complete conversion of 6-MeAD to exemestane was shown by bacterial strains used in the present study. Based on these observations, the bacterial strains may be classified into following groups:

Group A: Organisms completely degrading 6-MeAD without accumulation of exemestane: *Pseudomonas* sp. MTCC 3365, *Pseudomonas* sp. MTCC 3366, *Corynebacterium equi* IAM 1038, *Rhodococcus equi* MTCC 2558, *Brevundimonas diminuta* MTCC 3361, *Delftia acidovorans* MTCC 3362 and *Delftia acidovorans* MTCC 3363.

Group B: Organisms partially degrading 6-MeAD and accumulating exemestane: *Rhodococcus rhodochorus* MTCC 291, *Arthrobacter protophormiae* MTCC 2682 and *Pseudomonas putida* MTCC 1194.

Table 1. Relative concentrations of residual 6-MeAD detected and exemestane formed in the incubation medium

Organism	Residual 6-MeAD detected after incubation period (h)					Exemestane formed after incubation period (h)				
	24	48	72	98	120	24	48	72	98	120
Pseudomonas putida MTCC 1194	+++	+++	+++	+++	++	ND	ND	+++	++	++
Pseudomonas sp. MTCC 3365	+	+	+	ND	ND	ND	ND	ND	ND	ND
Pseudomonas sp. MTCC 3366	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Corynebacterium equi IAM 1038	++	+	ND	ND	ND	ND	ND	ND	ND	ND
Rhodococcus equi MTCC 2558	+++	++	ND	ND	ND	ND	ND	ND	ND	ND
Rhodococcus rhodochorus MTCC 291	+++	+++	++	++	+	ND	++	++	++	+
Rhodococcus erythropolis MTCC 1548	+++	+++	+++	+++	+++	ND	ND	ND	ND	ND
Arthrobacter simplex IAM 1660	+	ND	ND	ND	ND	++	+++	+++	+++	+++
Arthrobacter simplex NCIM 2449	+	+	ND	ND	ND	+	++	+++	+++	+++
Arthrobacter protophormiae MTCC 2682	+++	+++	++	++	++	+	+	+	+	+
Comamonas testosteroni NRRL B-2611	++	+	+	ND	ND	ND	+	+	ND	ND
Brevundimonas diminuta MTCC 3361	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Delftia acidovorans MTCC 3362	+	+	+	ND	ND	ND	ND	ND	ND	ND
Delftia acidovorans MTCC 3363	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Delftia acidovorans MTCC 3364	+	+	ND	ND	ND	+	++	+++	++++	++++
Nocardia sp. MTCC 1534	++	++	+	ND	ND	ND	+	+	ND	ND

6-MeAD – 6-methylene androstenedione; + Relative spot density on TLC plates; ND- not detected

Group C: Organisms without any activity against 6-MeAD: *Rhodococcus erythropolis* MTCC 1548.

Group D: Organisms degrading the substrate completely with the formation of maximum exemestane: *Delftia acidovorans MTCC 3364, Arthrobacter simplex* IAM 1660 and *Arthrobacter simplex* NCIM 2449.

Although, the strains of bacterial genera selected for the present investigation are known for their 1(2)-dehydrogenation activity of a variety of steroid substrates, only six strains accumulated exemestane. Dodson and Muir (1961) and Sih *et al.* (1965a, b) have elucidated a general pathway of degradation of steroid nucleus by bacteria as early as 1970s. In a sequence of degradation pathway of steroid nucleus, C-1(2)-dehydrogenation and 9α-hydroxylation are identified as essential steps (Martin, 1977). It was evident from the present observation that both these enzymes are active in bacterial strains classified as group A, degrading 6-MeAD completely without the accumulation of exemestane.

The presence of additional functional group or the side chain may alter the activity of enzymes involved in steroid bioconversions. Arinbasarova *et al.* (1995) have shown that the presence of methyl group at 6α-position of hydrocortisone changes the biochemical characteristics of C-1(2)-dehydrogenation activity of *Arthrobacter globiformis* 193. Also dependence of steroid-1(2)-dehydrogenation on the nature of side chain attached to C-17 carbon atom has been reported (Srivastava and Patil,1995). It appears that the presence of additional methylene group at C-6 position of 6-MeAD altered the C-1(2)-dehydrogenation activity or inhibited it completely resulting into partial or complete loss of activity in organisms classified as group B and C respectively. Strains of bacteria included in group D are most suitable for C-1(2) dehydrogenation step involved in the synthesis of exemestane.

CONCLUSION

As a result of present work, it is concluded that *Delftia acidovorans* MTCC 3364, *Arthrobacter simplex* IAM 1660 and *Arthrobacter simplex* NCIM 2449 are suitable for scaling up an eco-friendly process for the production of exemestane from commonly available substrate, 6-MeAD.

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