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# Studies on *Bacillus stearothermophilus* Part IV. Influence of enhancers on biotransformation of testosterone

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**Abstract**

The impact of chemical enhancers on the biotransformation of testosterone has been exploited. Application of crude cell concentrates to produce *Bacillus stearothermophilus*-mediated bioconversion of testosterone at 65 °C for 72 h has been examined. After incubation, the xenobiotic substrate was added to the concentrated whole cell suspensions. The enhancer molecules were included in the whole cell suspension. The resultant products, after extraction into an organic solvent, were purified by thin layer chromatography and identification was carried out through spectroscopic data. Five steroid metabolites 9,10-seco-4-androstene-3,9,17-trione, 5α-androstan-3,6,17-trione, 17β-hydroxy-5α-androstan-3,6-dione, 3β,17β-dihydroxyandrost-4-ene-6-one and 17β-hydroxyandrost-4,6-diene-3-one were identified as biotransformation products of testosterone. A possible biosynthetic route for these bioconversion products is postulated.


*Keywords:* Enhancers; Biotransformation; *Bacillus stearothermophilus*; Cytochrome P-450

## 1. Introduction

Metabolism of steroids in healthy and unhealthy human organs, particularly in the liver, is the subject of extensive clinical and biomedical investigations. For this kind of work, it is essential that the authentic reference samples of pregnane and androstane based steroids, stereoselectively hydroxylated at the ring and bridge positions, become available. This is not feasible through synthesis. The potential use of microorganisms as a biomimetic model of xenobiotic metabolism in mammalian organisms has been used as an inexpensive and convenient method for the study of drug metabolism [1]. Microbial assisted hydroxylation entails an introduction of a hydroxyl group involving activation of chemically unreactive centers in steroid molecules. Holland has highlighted the industrial importance of microbial oxidation of steroids [2], and stereoselective hydroxylation of steroid skeletal positions has been reported [3,4].

Hydroxysteroids are important due to their physiological role in many situations. For instance, 6β-, 14α-hydroxyandrost-4-ene-3,17-dione and 14α-hydroxyandrost-4-ene-3,6,17-trione both possess androgenic activity and are useful inhibitors of breast cancer [5]. Similarly, allopregnanalone and allotetrahydrodeoxy-corticosterone are reported to be neuroactive steroids [6].

An important factor in the bioconversion capability of microorganisms is the induction of steroid hydroxylases. Steroid hydroxylases and cytochrome P-450 are known to be inducible [7,8]. In particular, animal liver microsomes, cytochrome P-450 IA and IIIA forms are highly inducible [9]. Thus, P-450 genes have been induced in laboratory animals and human liver by a number of chemically unrelated compounds [10]. In this context, the influence of cycloheximide on the induction of progesterone 11α-hydroxylating system has been reported [7]. Rifampicin is a potent CYP3A inducer in rabbit and human hepatocytes [11,12]. Salicylic acid can also act as a stimulator/enhancer for inducing hydroxylation in many biotransformation processes [13]. Enhanced biotransformation in enzymatic or microbial media supple-

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mented with cyclodextrin has also been investigated [14]. Microorganisms, besides hydroxylation, are also capable of carrying out a wide variety of other steroid transformation reactions [15].

It is recognized that thermostable enzymes of thermophiles are resistant to chemical denaturation [16]. However, little work has been reported about microbial transformation of steroids using thermophiles [17], and to our knowledge, 17β,5α-androstane derivatives, 6-dehydro derivatives and B-seco-androstene compounds have not been explored using thermophiles. Thermophile-mediated transformation of progesterone [18,19] and testosterone, without enhancers, has been previously reported from our laboratory [20]. These results encouraged us to further explore the potential of *B. stearothermophilus* to metabolize testosterone **1** in the presence of a variety of hydroxylase enhancers. In this endeavor, metabolism of **1** induced by salicylic acid, cycloheximide, rifampicin, chloramphenicol, cyclodextrin, dexamethasone, betanaphthoflavone, phenobarbital, riboflavin and the biotransformation products of **2** are reported in this study.

## 2. Experimental

### 2.1. Materials

Culture media were purchased from Difco Becton Dickinson and company (Sparks, USA) and Scharlau (Barcelona, Spain). All chemicals used were of analytical grade and were obtained from Fluka Riedel-dehaën, Merck (Darmstadt, Germany) and Scharlau. Kieselgel-60 $F_{254}$ fluorescent thin layer chromatographic plates (TLC) were obtained from Merck (Darmstadt, Germany). Steroid testosterone **1** and enhancers, cycloheximide, cyclodextrin, dexamethasone, β-naphthoflavone, chloramphenicol, riboflavin and rifampicin were obtained from Sigma-Aldrich Co. UK. Androstene-3,17-dione **2** was purchased from Steraloids Inc. (Newport, RI, USA). Salicylic acid was from BDH Laboratories (Poole, England) and phenobarbitone was from Courtin and Warner Ltd. (Lewes, East Sussex, England).

### 2.2. Organism

The details of *B. stearothermophilus* such as its maintenance and growth conditions have been described previously [20].

### 2.3. Influence of enhancers on the biotransformation of testosterone

Starter cultures (50 ml) of *B. stearothermophilus* were grown overnight on tryptone yeast extract (TYE) medium at 65 °C in a shaker incubator. The culture was transferred to 500 ml of fresh TYE media in a 2-L flask and kept under the same conditions until the end of log phase growth (4–4.5 h). The cells were collected by centrifugation and washed with 0.05 M sodium phosphate buffer pH 7. The cells were mixed with 10 ml of the same buffer and transferred to 500 ml of the phosphate buffer containing 10% Castenholtz mineral salt solution [21] in a 2-L flask. An ethanolic solution of testosterone and salicylic acid, made to final concentrations of 50 and 12.5 μg/ml, respectively, was added and the cultures were re-incubated at 65 °C for 72 h. Similarly, a final concentration of 50 μM rifampicin, 100 μg/ml of cycloheximide, 50 μg/ml chloramphenicol, 2 moles per one mole of substrate cyclodextrin, 2.5 μM dexamethasone, 50 μM betanaphthoflavone, 0.75 mM phenobarbitone, 1 mg/50 ml riboflavin and 50 μM rifampicin were also tested as enhancers for the biotransformation of testosterone. The transformation experiments of testosterone with enhancers were carried out in 0.05 M phosphate buffer pH 7, containing 10% Castenholtz mineral salt solution. Cycloheximide enhancer was used for biotransformation in 0.05 M phosphate buffer pH 7, containing 1% glucose. The inoculated flasks were incubated on a rotary shaker at 65 °C and 100 rev/min for 72 h.

### 2.4. Extraction, isolation and purification of metabolites

The fermentation mixture was harvested and exhaustively extracted three times with an equal volume of chloroform. The pooled extract was dried over anhydrous $Na_2SO_4$ and evaporated to dryness using a rotary evaporator to get a semi-solid residue. TLC separation of the residue on silica plates (Merck 60 $F_{254}$) with benzene: methanol: aceticacid (20:2:1 by volume) followed by second development in diethylether: toluene: ethylacetate (15:25:10 by volume) revealed the formation of a variety of metabolites. Since 5α-androstanes were undetected under UV light at 254 nm, the TLC chromatograms were stained with anisaldehyde reagent to get a spectrum of colors before separation and purification. The induced transformation of testosterone resulted in the formation of five compounds that were identified from their spectral data. These metabolites were identified as 9,10-seco-4-androstene 3,9,17-trione **3**, 5α-androstan-3,6,17-trione **4**, 17β-hydroxy-5α-androstan-3,6-dione **5**, 3β,17β-hydroxyandrost-4-ene-6-one **6** and 17β-hydroxyandrost-4,6-diene-3-one **7**.

### 2.5. Transformation of androstene-3,17-dione *2* by B. stearothermophilus

The starter culture and cells were grown and collected as described above, and the suspension was added to 500 ml sodium phosphate buffer (0.05 M and pH 7) containing 10% Castenholz mineral salt solution. An ethanolic solution of androstene-3,17-dione was added to achieve a final concentration of 50 μg/ml, and the culture was re-incubated for 72 h. Collection, separation, purification and identification of metabolites were carried out as described above for testosterone.

### 2.6. Quantification of the compounds

Relative percentage yield of each metabolite was measured from its TLC spot density using Shimadzu Dual Wavelength Flying Spot Scanner model CS-9000, interfaced with Quantascan 2D analysis program P/N 206-18597.

### 2.7. Analytical methods

Details of the analytical methods have been described previously [20].

## 3. Results and discussion

We have previously reported the biotransformation of testosterone **1** by *B. stearothermophilus*, and the major metabolite androst-4-ene-3,17-dione **2** was found in 90.2% relative yield along with four other monohydroxy metabolites [20]. However, the addition of hydroxylase enhancers like salicylic acid, chloramphenicol, cyclodextrin, cycloheximide, dexamethasone, betanaphthoflavone, phenobarbitone, riboflavin and rifampicin in *B. stearothermophilus* cultures resulted in the formation of additional metabolites. Enhanced yields of 9,10-seco-4-androstene-3,9,17-trione **3**, 5α-androstane-3,6,17-trione **4**, 17β-hydroxy-5α-androstan-3,6-dione **5** and other monohydroxy derivatives [20] were obtained using these enhancers (Table 1). Addition of cycloheximide in the presence of glucose resulted in the formation of 3β,17β-hydroxyandrost-4-ene-6-one **6** as a major metabolite. This compound was produced as a minor metabolite with other enhancers, such as salicylic acid, chloramphenicol, cyclodextrin, cycloheximide, dexamethasone and β-naphthoflavone. The metabolite 17β-hydroxyandrost-4,6-diene-3-one **7** was formed only with the addition of rifampicin. The molecular structures of these metabolites are shown in Fig. 1, and the influence of different enhancers on the biotransformation of **1** is shown in Table 1.

An extensive use of $^{13}$C NMR and $^{1}$H NMR spectroscopy assisted identification of these metabolites. The metabolite 9,10-seco-4-androstene-3,9,17-trione **3** was identified by comparison of its mass spectral fragmentation and $^{1}$H NMR, $^{13}$C NMR and IR spectral data with those reported in the literature [18,22,23]. The spectral details of this compound are shown in Tables 2–5. In the $^{1}$H NMR spectrum of the B-Seco compound **3**, the fundamental signals for testosterone were missing, confirming that the metabolite did not have the androstene skeleton. For instance, the 4-H at δ6.24 ppm was too high for the 4-H that normally appears at δ5.74 ppm in **1** and at δ5.76 ppm in androstenedione **2**. The $C_{19}$ methyl protons showed a split triplet centered at δ1.22 ppm due to coupling with the methylene protons at $C_1$ and the methyne proton at $C_{10}$. This confirmed the bond cleavage between $C_9$ and $C_{10}$ of androstene with a concurrent oxygenation at $C_9$ to give the B-seco compound **3** [18]. However, the $C_{18}$–$CH_3$ group showed a characteristic singlet at the expected position δ0.93 ppm.

Table 1
Testosterone transformation products using enhancers

| Enhancers | Relative percentages of metabolites | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
| Testosterone | 90.2 ± 0.71 | – | – | – | – | – | 1.1 ± 0.12 | 0. 9 ± 0.09 | 3. 9 ± 0.43 | 3. 9 ± 0.36 |
| Androst-4-ene-3,17-dione | 62.08 ± 0.66 | 1.98 ± 0.11 | 2.96 ± 0.20 | – | – | – | 20.88 ± 0.75 | 12.1 ± 0.92 | – | – |
| β-Naphthoflavone | 72.78 ± 0.39 | 1.38 ± 0.03 | 2.08 ± 0.09 | 2.12 ± 0.35 | 1.50 ± 0.24 | – | 4.77 ± 0.92 | 2.06 ± 0.10 | 8.50 ± 0.16 | 4.80 ± 0.29 |
| Dexamethasone | 85.35 ± 0.78 | 0.57 ± 0.01 | 1.00 ± 0.04 | 0.39 ± 0.04 | 0.21 ± 0.01 | – | 4.99 ± 0.39 | 2.61 ± 0.14 | 2.55 ± 0.18 | 2.32 ± 0.10 |
| Rifampicin | 92.29 ± 0.48 | 2.40 ± 0.03 | 1.87 ± 0.26 | – | – | 2.01± 0.22 | 0.80 ± 0.04 | 0.43 ± 0.04 | 0.11 ± 0.01 | 0.09 ± 0.01 |
| Riboflavin | 74.47 ± 1.15 | 2.03 ± 0.16 | 2.43 ± 0.24 | 0.48 ± 0.07 | – | – | 7.48 ± 0.21 | 6.86 ± 0.16 | 3.56 ± 0.39 | 2.70 ± 0.36 |
| Phenobarbital | 83.99 ± 0.09 | 0.98 ± 0.04 | 1.55 ± 0.03 | – | – | – | 6.74 ± 0.21 | 3.16 ± 0.03 | 2.27 ± 0.11 | 1.32 ± 0.09 |
| Cycloheximide | 78.50 ± 0.60 | 1.77 ± 0.08 | 1.58 ± 0.05 | 2.73 ± 0.10 | 2.86 ± 0.03 | – | 5.49 ± 0.82 | 3.54 ± 0.08 | 2.07 ± 0.18 | 1.46 ± 0.17 |
| Chloramphenicol | 86.00 ± 0.24 | 2.57 ± 0.04 | 1.61 ± 0.07 | 0.54 ± 0.01 | 0.64 ± 0.05 | – | 4.73 ± 0.22 | 2.96 ± 0.12 | 0.61 ± 0.05 | 0.34 ± 0.02 |
| Cyclodextrin | 88.07 ± 0.51 | 0.51 ± 0.09 | 0.74 ± 0.05 | 0.18 ± 0.01 | 0.15 ± 0.01 | – | 2.28 ± 0.14 | 2.04 ± 0.10 | 3.07 ± 0.18 | 2.96 ± 0.13 |
| Salicylic acid | 79.92 ± 0.72 | 0.83 ± 0.05 | 0.86 ± 0.19 | 0.75 ± 0.07 | 0.81 ± 0.05 | – | 7.28 ± 0.41 | 3.34 ± 0.26 | 3.40 ± 0.16 | 2.80 ± 0.22 |

Androst-4-en-3,17-dione **2**; 9,10-Seco-4-Androstene-3,9,17-trione **3**; 5α-Androstane-3,6,17-trione **4**; 17β-Hydroxy 5α-androstane-3,6-dione **5**; 3β,17β-Hydroxyandrost-4-ene-6-one **6**; 17β-Hydroxyandrost 4,6-diene-3-one **7**; 6β-hydroxy androst-4-ene-3,17-dione **8**; 6α-hydroxy androst-4-ene-3-one **9**; 6β,17β-hydroxy androst-4-ene-3-one **10**; 6α,17β-hydroxy androst-4-ene-3-one **11**. Standard deviation was calculated from three independent measurements.

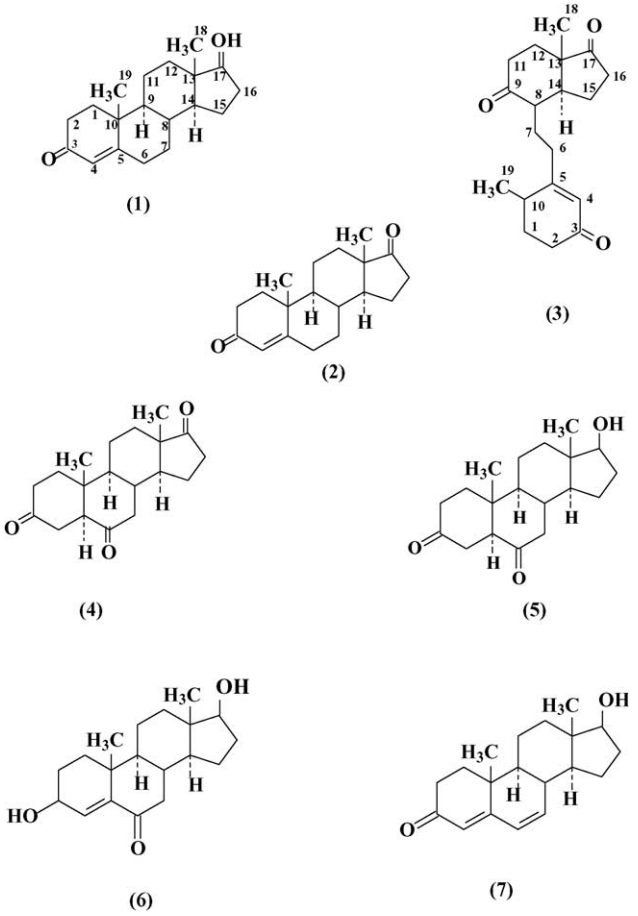


Fig. 1. Structures of testosterone metabolites. Testosterone (**1**) Androst-4-en-3,17-dione (**2**) 9,10-Seco-4-Androstene-3,9,17-trione (**3**) 5α-Androstane-3,6,17-trione (**4**) 17β-Hydroxy 5α-androstane-3,6-dione (**5**) 3β,17β-hydroxyandrost-4-ene-6-one (**6**) 17β-Hydroxyandrost 4,6-diene-3-one (**7**).

The three carbonyl groups at $C_3$, $C_9$ and $C_{17}$ were also apparent at 1680.09, 1698.43 and 1738.11 $cm^{-1}$ respectively in the FTIR spectrum. Other IR absorption frequencies included 2924.01 $cm^{-1}$ for C–H stretching and 2851.41 $cm^{-1}$ for CH–$CH_3$ stretching for bridge methyl groups (Table 5).

$^{13}$C NMR of compound **3** was very distinctive when compared to the parent compound **1**. Thus, signals at 126.5 ($C_4$), 163.9 ($C_5$), 40.0 ($C_6$), 30.3 ($C_7$), 46.0 ($C_8$), 208.2 ($C_9$) and 51.5 ($C_{10}$) ppm were all different compared to **1**. This cluster of signals was the main evidence for the molecular structure of compound **3**. Except for the signal at 30.3 ppm for $C_7$, all other signals for carbon positions 4-, 5-, 6-, 8-, 9- and 10- had shifted upfield. The additional $^{13}$C signals, including the $C_{17}$ carbonyl carbon at 220.1 ppm instead of 181.3 ppm in testosterone **1**, were all comparable to those of androstenedione **2** and the values are given in Table 3. Mass spectral fragmentation of the B-seco compound also supported the proposed molecular structure with a parent ion at *m*/*z* 302 and a base peak at *m*/*z* 149 (Table 4).

The metabolite **4** was identified as 5α-androstane-3,6,17-trione. All spectroscopic details of the compound corresponded to its molecular structure. Its $^1$H NMR spectral data showed that the fundamental signals of the androstene ring A had an upfield shift confirming the loss of the androstene ring structure resulting in 5α-androstane as shown in Table 2. The compound was undetectable at 254 nm on TLC chromatograms but showed a bright yellow color with anisaldehyde stain that was used for its detection on TLC chromatograms. The $^{13}$C NMR spectral data (Table 3) when compared with the reported values [24–26], confirmed the structure **4** for this metabolite. Mass spectral fragmentation, as reported earlier [24], was also in full agreement with the proposed structure. The FTIR absorption spectrum also sup-

Table 3
$^{13}$C NMR of testosterone and its metabolites in $CDCl_3$

| Carbon atom | Chemical shifts (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **(1)** | **(2)** | **(3)** | **(4)** | **(5)** | **(6)** | **(7)** |
| 1 | 36.1 | 36.0 | 36.07 | 38.0 | 38.6 | 34.5 | 34.5 |
| 2 | 34.1 | 34.3 | 34.48 | 37.5 | 37.9 | 30.9 | 34.5 |
| 3 | 198.0 | 199.8 | 199.6 | 211.3 | 210.2 | 67.4 | 200.2 |
| 4 | 124.2 | 124.5 | 126.5 | 41.7 | 41.8 | 126.2 | 128.6 |
| 5 | 170.4 | 170.8 | 163.9 | 58.1 | 58.1 | 161.30 | 164.3 |
| 6 | 32.8 | 32.9 | 40.0 | 208.7 | 208.6 | 202.5 | 124.3 |
| 7 | 32.2 | 31.1 | 30.3 | 45.9 | 46.6 | 46.9 | 140.9 |
| 8 | 36.1 | 35.5 | 46.0 | 37.9 | 36.8 | 34.6 | 38.2 |
| 9 | 54.6 | 54.1 | 208.2 | 54.1 | 54.1 | 51.6 | 48.8 |
| 10 | 39.0 | 39.0 | 51.5 | 38.6 | 38.7 | 36.5 | 36.9 |
| 11 | 21.2 | 20.7 | 20.8 | 21.2 | 21.9 | 21.1 | 20.9 |
| 12 | 37.1 | 36.1 | 31.5 | 31.6 | 37.5 | 36.1 | 30.9 |
| 13 | 43.2 | 47.9 | 47.5 | 48.6 | 44.0 | 39.7 | 44.4 |
| 14 | 51.1 | 51.2 | 52.8 | 52.1 | 51.8 | 51.8 | 51.4 |
| 15 | 23.8 | 22.1 | 22.2 | 22.2 | 23.8 | 23.7 | 23.5 |
| 16 | 30.7 | 31.6 | 36.0 | 36.2 | 30.9 | 30.9 | 30.2 |
| 17 | 81.3 | 220.9 | 218.5 | 220.1 | 82.0 | 81.9 | 81.9 |
| 18 | 11.3 | 14.1 | 14.2 | 14.2 | 11.7 | 11.6 | 11.6 |
| 19 | 17.3 | 17.7 | 18.2 | 13.2 | 13.2 | 18.1 | 16.9 |

Table 2
$^1$H NMR data for testosterone metabolites produced by the addition of enhancers

| Compounds | 4-*H* | 17α-O*H* | 18-*CH*$_3$ | 19-*CH*$_3$ | C*H*OH | Other significant signals |
|---|---|---|---|---|---|---|
| **1** | 5.74 | 3.67 | 0.81 | 1.21 | – | – |
| **2** | 5.76 | – | 0.93 | 1.22 | – | – |
| **3** | 6.24 | – | 0.92 | 1.22 | – | |
| **4** | 2.11 (4α-H), 2.38 (4β-H) | – | 0.88 | 1.01 | – | 1.59 (5α-H) |
| **5** | 2.11 (4α-H), 2.39 (4β-H) | 3.64 | 0.80 | 0.99 | 3.64 (17α-*H*) | 1.63 (5α-H) |
| **6** | 6.20 | 3.66 | 0.82 | 1.20 | 4.07 (3.66) | |
| **7** | 5.69 | 3.69 | 0.86 | 1.14 | 3.69 (17α-*H*) | 6.12 (6-H), 6.12 (7-H) |

Table 4
Mass spectral data of testosterone metabolites. Values in brackets represent relative abundance

| Compound | Significant ions and their intensities |
|---|---|
| **1** | 288:$M^+$ (85); 273:$M^+$ −$CH_3$ (16); 270:$M^+$ −$H_2O$ (15); 246:$M^+$ −CO−$CH_2$ (78); 228:$M^+$ −CO−$CH_2$−$H_2O$ (29); 213:$M^+$ −CO−$CH_2$−$H_2O$−$CH_3$ (19); 147: ring cleavage between B and C (64) |
| **2** | 286:$M^+$ (100); 271:$M^+$ −$CH_3$ (10); 258:$M^+$ −CO (9); 244:$M^+$ −$C_2H_2O$ (70); 201:$M^+$ −$C_2H_2O$−CO−$CH_3$ (25); 148: ring cleavage between B and C |
| **3** | 302:$M^+$ (10); 300:$M^+$ −$H_2$ (18); 288:$M^+$ −$CH_2$ (28); 273:$M^+$ −$CH_2$−$CH_3$ (16); 260:$M^+$ −CO−$CH_2$ (20); 231:$M^+$ −CHO−CO−$CH_2$ (19); 219:$M^+$ −CO−$CH_2$−$C_3H_5$ (35); 203:$M^+$ −CO−$CH_2$−$C_3H_5O$ (21.2); 161:$M^+$−CO−$CH_2$−$C_3H_5O$−CO−$CH_2$ (21); 149: ring cleavage between B and C (35) |
| **4** | 302:$M^+$ (65); 287:$M^+$ −$CH_3$ (58); 273:$M^+$ −CHO (82); 231:$M^+$ −CO−$CH_3$−CO (49); 231:$M^+$ −CO−$CH_3$−CO−CO (49); 203:$M^+$ −CO−$CH_3$−CO−CO (25); 175:$M^+$ −CO−$CH_3$−$CH_2$−$CH_2$−CO−CO (17);145: ring cleavage between B and C |
| **5** | 304:$M^+$ (100); 289:$M^+$ −$CH_3$ (18); 286:$M^+$ −$H_2O$ (11); 275:$M^+$ −$CH_3$−$CH_2$ (45); 261:$M^+$ −$CH_3$−$CH_2$−$CH_2$ (12); 245:$M^+$ −$CH_3$−$CH_2$−$CH_2O$ (38); 231:$M^+$ −$CH_3$−$CH_2$−$CH_2$−$CH_2O$ (17); 215:$M^+$ −$CH_3$−$CH_2$−$CH_2O$−$CH_2O$ (6); 147:$M^+$ −$CH_3$−$CH_2$−$CH_2O$−$CH_2O$−$C_3H_4$−CO (6) |
| **6** | 302:$M^+$ (46); 287:$M^+$ −$CH_3$ (38); 284:$M^+$ −$H_2O$ (34); 274:$M^+$ −CO (76); 260:$M^+$ −CO−$CH_2$ (22); 243:$M^+$ −CO−$CH_3O$ (35); 147: ring cleavage between B and C (37) |
| **7** | 286:$M^+$ (100); 268:$M^+$ −$H_2O$ (17); 253:$M^+$ −$H_2O$−$CH_3$ (12); 227:$M^+$ −$H_2O$−$CH_3$−$M^+$−$CH_3$−$C_2H_2$ (14); 214:$M^+$ −$H_2O$−$C_3H_2O$ (8); 185:$M^+$ −$H_2O$−$C_3H_2O$−CHO (9); 171:$M^+$ −$H_2O$−$C_3H_2O$−CHO−$CH_2$ (8); 151: ring cleavage between B and C (26) |

Table 5
Infrared spectra of testosterone and its metabolites

| Compounds | Group frequencies | | | |
|---|---|---|---|---|
| | −CO | −OH | >CH | >CH−$CH_3$ |
| **1** | 1656.62 | 3383.06 | 2943.92 | 2846.75 |
| **2** | 1736.08 | – | 2939.77 | 2856.26 |
| | 1670.95 | | | |
| **3** | 1738.11 | – | 2924.01 | 2851.41 |
| | 1698.43 | | | |
| | 1680.09 | | | |
| **4** | 1735.38 | – | 2926.27 | 2855.68 |
| | 1713.11 | | | |
| **5** | 1733.33 | 3418.03 | 2917.51 | 2849.19 |
| | 1713.00 | | | |
| **6** | 1684.34 | 3648.44 | 2930.31 | 2854.67 |
| | | 3596.88 | | |
| **7** | 1729.55 | 3466.92 | 2941.95 | 2841.73 |

ported 5α-pregnane structure through absorption frequencies at 1713.00 and 1735.38 $cm^{-1}$ for the overlapped carbonyl functions at $C_3$, $C_6$ and $C_{17}$, respectively (Table 5).

The metabolite 17β-hydroxy-5α-androstan-3,6-dione **5** was identified by comparing its spectral data with those of compounds reported in the literature [27,28]. The presence of the 17β-hydroxyl group was apparent from its $^{13}C$ NMR and $^1H$ NMR and mass spectrum of **5**. The other signals for 5α-androstane and the 3,6-keto groups were obvious from the $^1H$ NMR, $^{13}C$ NMR and FTIR spectra when compared to the metabolite **4** (Tables 2–5).

The spectral data of metabolite, 3β,17β-hydroxyandrost-4-ene-6-one **6** was compared with the reported data of similar compounds [28,29]. In the $^1H$ NMR spectra, the 3β-hydroxylation and the carbonyl function at $C_6$ resulted in a downfield shift for the 4-H signal to δ6.20 ppm. The 3β- and 17β-hydroxyl groups were evident from the signals at δ4.07 ppm and δ3.66 ppm, respectively (Table 2). In its $^{13}C$ NMR spectrum, values for the $C_6$ carbonyl function (202.5 ppm) and the 3β- and 17β-hydroxyl signals (67.4 and 81.9 ppm, respectively) confirmed the proposed molecular structure. A shift for $C_5$ (161.3 ppm) and $C_7$ signals (46.9 ppm) also supported the carbonyl group at $C_6$ (Table 3). The mass spectral analysis for 6 showed a parent ion at *m*/*z* $302^+$ that confirmed addition of an oxygen atom to the original molecule after its transformation. The additional oxygen appeared as a carbonyl group at 1684.34 $cm^{-1}$ in the FTIR spectrum.

From the spectroscopic details, metabolite **7** was identified as 17β-hydroxyandrost-4,6-diene-3-one (Tables 2–5). The $^1H$ NMR features of the 4,6-dienone systems were in full agreement with the 6-dehydrotestosterone. The reported $^{13}C$ NMR, $^1H$ NMR spectral data [30–32] and the infrared absorption frequencies at 1604.17 and 1646.75 $cm^{-1}$ were all in agreement with the conjugated dienone system. The presence of a 17β-hydroxyl group was supported from the position of the 17α-H in its $^1H$ NMR spectrum (Tables 2 and 3). The mass spectral data of compound **7** showed the parent ion at

*m*/*z* 286$^+$ with loss of a water molecule from the CH(OH) group at $C_{17}$with a fragment ion at *m*/*z* 268$^+$. The carbonyl group at $C_3$ was confirmed from its FTIR and $^{13}$C NMR spectrum.

The biotransformation of the androst-4-ene-3,17-dione **2** resulted in five compounds, that were identified from their spectral data by comparison with testosterone biotransformation products and also with values reported in the literature. These compounds were identified as 9,10-seco-4-androstene-3,9,17-trione, 5α-androstane-3,6,17-trione, testosterone, 6β-hydroxyandrost-4-en-3,17-dione and 6α-hydroxyandrost-4-en-3,17-dione.

We have previously reported 6-dehydro- and 5α-pregnane derivatives of progesterone transformation products with increased incubation period [19]. From the transformation products, it is clear that the compound testosterone is transformed into androstenedione **2**, which is then transformed into **3**, and **4**. At the same time, testosterone is transformed into compounds **5** and **6**. All these metabolites were produced in very small amounts along with the previously identified monohydroxy metabolites [20]. With increased incubation periods, the yield of these metabolites increased many folds after the addition of enhancers indicating the influence of these enhancers on the hydroxylation of these steroids. 17β-Hydroxyandrost-4,6-diene-3-one **7** probably also arises from testosterone **1**, through a process of dehydrogenation. Formation of these compounds from a thermophilic bacillus has not been reported before.

With the increasing knowledge of microbial transformation, particularly with respect to P-450 hydroxylases, and an ongoing interest in thermostable enzymes, this work may initiate a natural progression away from the microbial biotransformation by whole cell cultures. With the progress made in this study, we are confident that our new isolate can lead to new approaches for the efficient transformation of potential biologically active steroids.

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