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The generation of a steroid library using filamentous fungi immobilized in calcium alginate

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Dedicated to the memory of Professor Sir John W. Cornforth, University of Sussex (1917–2013).

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ABSTRACT

Four fungi, namely, *Rhizopus oryzae* ATCC 11145, *Mucor plumbeus* ATCC 4740, *Cunninghamella echinulata* var. *elegans* ATCC 8688a, and *Whetzelinia sclerotiorum* ATCC 18687, were subjected to entrapment in calcium alginate, and the beads derived were used in the biotransformation of the steroids 3β,17β-dihydroxyandrost-5-ene (**1**) and 17β-hydroxyandrost-4-en-3-one (**2**). Incubations performed utilized beads from two different encapsulated fungi to explore their potential for the production of metabolites other than those derived from the individual fungi. The investigation showed that steroids from both single and crossover transformations were typically produced, some of which were hitherto unreported. The results indicated that this general technique can be exploited for the production of small libraries of compounds.

1. Introduction

The concept of using a number of microorganisms in a single fermentation vessel for the bioconversion of a single substrate to multiple products is not new. Unfortunately, such an environment would lead to inhibitory competition, where either one microorganism prevails whilst the other dies, or the growth of both suffers. Presumably even if the fermentation were successful, the conditions would not be highly reproducible. Additionally, as each microorganism has different nutritional requirements, it could be difficult to find a suitable medium to perform the fermentation [1]. Employing immobilized mycelial cells could avert these problems. With each microorganism trapped in its own sphere, the possibility of negative competition would be reduced or eliminated.

Most of the applications of cell immobilization have been concentrated on monoculture bioconversions. It is, therefore, of immense interest to explore the potential of mixed cultures. There have been previous reports of co-immobilized biocatalysts [1]. Typically, the microorganisms employed for such an objective tend to be symbiotic. For example, one might be oxygen consuming while the other is oxygen producing, as in the case of *Cephalosporium acremonium* (an oxygen consuming fungus) and *Chlorella pyrenoidosa* (an oxygen producing alga). Both were co-immobilized and used for the production of cephalosporin C [2]. A similar mixed culture system was previously used for the production of β-lactam antibiotics [3]. Another study, with entrapped cells of *Chlorella vulgaris* and *Provedencia* sp., as well as *Aspergillus nidulans* and *Provedencia* sp., were used produce α-keto acids from amino acids [4]. A mixed culture-entrapped preparation of *Chlorella pyrenoidosa* and *Glucanobacter oxydans* was employed in the bioconversion of glycerol to dihydroxyacetone, in an effort to study the effects that co-immobilization with oxygen-producing algae had on the transformation yields [5].

It has been shown that immobilized cell bioconversions parallel those of the free cell fermentations to some extent [6,7]. A progression from this would involve the mixing of beads of immobilized fungus A in water with those of mould B in a single vessel, followed by feeding of the appropriate substrate. The expectation was the isolation of additional metabolites that were different from those obtained from the individual single immobilized cell fermentations. Each of these new metabolites would be the product of "crossover"; that is, a compound formed when the transformed

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Table 1
^{13}C NMR data (δ) for compounds **2a–9a** determined in $CDCl_3$.

Carbon	2a	3a	4a	5a	6a	7a	8a	9a
1	36.1	36.5	36.6	36.5	35.8	35.5	36.5	35.5
2	35.8	27.5	27.5	27.4	33.9	33.9	27.7	33.9
3	199.9	73.0	73.1	72.9	199.4	198.7	72.9	198.8
4	124.4	37.8	37.5	37.8	124.1	125.6	37.5	126.5
5	171.4	146.7	144.2	146.9	170.2	165.5	144.4	166.3
6	33.2	120.6	122.0	120.4	32.5	38.1	121.9	37.3
7	31.9	67.6	75.3	67.1	26.2	75.5	74.9	70.3
8	34.3	35.6	36.3	35.5	38.6	39.5	36.2	38.2
9	54.1	43.1	47.9	43.3	46.7	50.5	48.1	46.4
10	39.0	37.3	36.3	37.5	38.7	37.9	36.7	38.4
11	20.9	20.2	20.5	19.9	19.6	20.5	20.4	20.4
12	37.0	36.1	36.5	30.9	28.9	36.3	31.1	36.1
13	42.9	42.2	42.7	47.3	46.8	43.1	47.7	42.5
14	50.7	43.7	49.8	44.7	82.9	49.2	50.6	44.8
15	23.9	23.3	24.5	21.8	32.8	25.3	23.1	22.9
16	27.9	27.4	27.5	35.7	26.9	27.6	35.7	27.3
17	82.9	82.5	82.2	220.3	80.9	81.9	220.3	82.2
18	12.4	11.5	11.8	13.1	15.9	12.0	13.6	11.8
19	17.8	18.2	18.9	18.2	17.3	17.2	19.0	17.1
OAc	21.6	21.2	21.1	21.3	21.3	21.2	21.4	21.1
OAc	171.6	21.3	21.3	21.4	171.2	21.6	21.6	21.2
OAc	–	21.4	21.5	170.4	–	170.2	170.3	170.5
OAc	–	170.4	170.3	170.6	–	171.1	170.9	171.2
OAc	–	170.7	170.9	–	–	–	–	–
OAc	–	171.3	171.1	–	–	–	–	–

Table 2
^{13}C NMR data (δ) for compounds **10a**–**16a** and **6** determined in $CDCl_3$.

Carbon	10a	11a	12a	13a	14a	15a	16a	6
1	35.9	26.0	31.8	33.0	31.9	31.9	32.0	35.7
2	27.4	24.4	26.6	27.0	26.6	26.6	26.6	33.9
3	72.2	70.6	69.9	70.4	70.1	70.3	70.4	201.0
4	38.0	31.2	35.9	37.0	37.2	37.0	36.9	123.6
5	166.0	76.7	75.8	75.7	75.9	75.9	74.9	172.8
6	126.7	72.7	73.0	76.0	74.9	76.0	76.2	32.8
7	201.8	71.4	71.6	31.3	73.0	30.1	25.8	26.2
8	48.8	36.6	34.2	30.2	35.6	26.7	34.0	38.7
9	44.4	37.0	39.4	48.0	44.2	45.1	38.2	46.8
10	38.6	41.0	39.1	40.3	38.2	38.9	38.9	39.0
11	20.0	20.9	20.4	70.3	20.8	20.4	19.5	19.0
12	27.9	36.1	36.3	43.5	36.8	38.0	29.4	28.6
13	46.9	42.6	42.8	42.9	43.5	42.7	47.1	47.0
14	80.4	44.7	44.3	48.9	49.1	53.0	83.0	83.3
15	35.2	23.1	23.1	24.4	25.3	71.7	32.3	32.3
16	27.2	27.3	27.3	27.4	27.7	38.3	26.9	29.2
17	80.5	82.3	82.2	81.6	82.4	81.2	81.0	78.3
18	16.1	11.7	11.9	12.8	12.3	14.1	16.1	15.0
19	17.2	16.9	16.5	16.4	17.2	16.4	16.3	17.3
OAc	21.2	21.1	20.9	21.2	21.2	20.8	21.1	–
OAc	21.4	21.1	21.0	21.4	21.3	21.1	21.3	–
OAc	170.2	21.4	21.1	21.5	21.4	21.3	21.4	–
OAc	171.2	169.3	21.4	21.9	21.5	21.3	170.0	–
OAc	–	170.9	168.7	170.1	170.2	169.7	170.7	–
OAc	–	171.4	168.8	170.5	170.3	170.3	171.4	–
OAc	–	–	170.5	170.6	170.8	170.7	–	–
OAc	–	–	171.4	171.2	171.2	171.0	–	–

Table 3
^{13}C NMR data (δ) for compounds **7**, **9** and **17**–**22** determined in $CDCl_3$.

Carbon	7	9	17	18	19	20	21	22	23
1	35.7	35.5	37.1	37.4	37.2	37.4	33.9	35.6	35.4
2	33.9	34.0	34.2	34.2	34.3	34.2	33.8	34.0	33.9
3	199.3	200.7	200.3	199.9	200.4	202.1	199.7	199.2	198.5
4	124.6	126.3	126.5	124.8	126.4	124.2	123.7	126.4	127.2
5	167.5	170.7	167.9	170.1	168.3	173.9	163.7	166.7	166.4
6	42.3	41.2	72.8	33.4	73.0	34.0	128.0	37.4	41.0
7	74.9	67.5	37.2	30.3	38.1	31.4	140.4	70.5	67.1
8	43.1	40.0	29.6	34.6	29.8	35.6	37.6	38.6	39.3
9	50.7	45.4	53.7	59.2	53.8	59.1	50.7	45.5	45.4
10	38.0	38.8	38.1	40.0	38.1	40.4	36.1	38.4	38.5
11	20.6	20.6	20.3	68.7	20.6	69.5	20.3	20.5	20.1
12	36.3	36.2	31.3	43.0	36.4	48.0	36.3	36.0	30.9
13	43.5	42.9	47.7	48.0	42.9	43.6	43.8	42.9	47.3
14	49.9	45.1	50.9	50.1	50.6	50.0	48.2	45.0	45.6
15	26.3	22.8	21.7	21.7	23.3	23.4	23.0	22.7	21.3
16	30.7	29.9	35.8	35.7	30.5	29.9	30.4	30.4	35.6
17	81.1	81.3	220.5	218.4	81.7	80.8	81.3	81.4	220.2
18	11.1	11.0	13.8	14.7	11.1	12.3	11.0	10.9	13.5
19	17.3	17.2	19.6	18.4	19.6	18.4	16.3	17.2	17.0
OAc	–	–	–	–	–	–	–	21.2	–
OAc	–	–	–	–	–	–	–	170.6	–

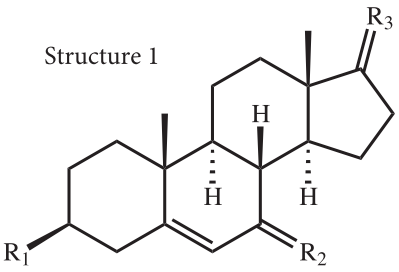
congener from one fungus would be used as the substrate by the second microorganism. This would lead to the production of multiple compounds. The results of our experiments show this to be the case. In previous work [6,7]. we reported on the use of six encapsulated fungi (*Rhizopus oryzae*, *Mucor plumbeus*, *Cunninghamella echinulata* var. *elegans*, *Aspergillus niger*, *Phanerochaete chrysosporium* and *Whetzelinia sclerotiorum* in the transformation of two steroidal substrates, 3β,17β-dihydroxyandrost-5-ene (**1**) and 17β-hydroxyandrost-4-en-3-one (**2**). In general, it was observed that the transformations of **1** and **2** by encapsulated *A. niger* and *P. chrysosporium* gave modest results. Therefore, only *R. oryzae*, *M. plumbeus*, *C. echinulata* var. *elegans* and *W. sclerotiorum* were chosen for the crossover experiments. 3β,17β-Dihydroxyandrost-5-ene (**1**) and 17β-hydroxyandrost-4-en-3-one (**2**) were used as substrates, as the current work is an extension of earlier studies [6,7]. With four fungi available for crossover, there were six possible permutations with each substrate, giving a total of 12 possible mixed bead fermentations. Once it was determined that one could generate new compounds from **1** using four combinations of fungi, only two mixed bead experiments using **2** as substrate were attempted. Products of transformation derived from **1** were acetylated. This aided in their purification. It also generated compounds that were readily soluble in $CDCl_3$. Furthermore, comparison of NMR spectra (for structure elucidation purposes) was facilitated when the biotransformation products from **1** had been derivitized in this way.

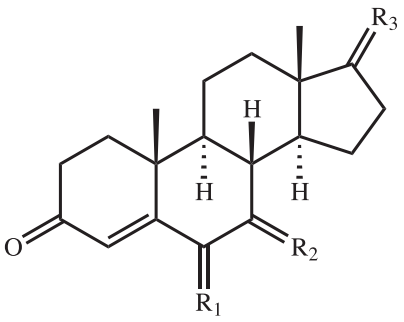
2. Experimental

2.1. General procedure

Melting points were obtained using a Reichert Hot Stage melting point apparatus and are uncorrected. Infrared data was acquired using a PerkinElmer Fourier transform infrared spectrophotometer 1000 using sodium chloride disks. NMR spectra were obtained on Bruker Avance 200, Bruker Avance 500 and Varian Unity 500 spectrometers. Compounds were analyzed using $CDCl_3$, unless otherwise stated, with tetramethylsilane as an internal standard. ^{13}C NMR data for all compounds are reported in Tables 1–3 . 1D and 2D NMR data for new compounds are reported in Tables S1–S7 (Supplementary data). 1D and 2D NMR data for compounds that were not previously fully characterized are in Tables S8–S11 (Supplementary data). Optical rotations were performed using a PerkinElmer 241 MC polarimeter and solutions were prepared using CH_2Cl_2 unless otherwise stated. Purifications were done by column chromatography using silica gel (230–400 mesh) as the stationary phase. Additionally, preparative thin layer chromatography (PLC) glass backed plates with silica gel (60 Å, 250 μm and 1000 μm thicknesses) were used. Thin layer chromatographic (TLC) analyses were done using polyester backed plates. Both the TLC and PLC plates were visualized under ultraviolet light or by spraying with ammonium molybdate-sulfuric acid or methanol-sulfuric acid reagents. After spraying the plates were warmed for colour development using a heat gun. Petrol refers to the petroleum fraction boiling between 60 and 80°. 3β-Hydroxyandrost-5-en-17-one (dehydroepiandrosterone, DHEA) was obtained from Productos Químicos Naturales,

Structure 1





1 R_1=OH, R_2=H_2, R_3=βOH,αH

2 R_1=R_2=H_2, R_3=βOH,αH

3 R_1=OH, R_2=αOH,βH, R_3=βOH,αH

2a R_1=R_2=H_2, R_3=βOAc,αH

3a R_1=OAc, R_2=αOAc,βH, R_3=βOAc,αH

7 R_1=H_2, R_2=R_3=βOH,αH

4 R_1=OH, R_2=R_3=βOH,αH

7a R_1=H_2, R_2=R_3=βOAc,αH

4a R_1=OAc, R_2=R_3=βOAc,αH

9 R_1=H_2, R_2=αOH,βH, R_3=βOH,αH

5 R_1=OH, R_2=αOH,βH, R_3=O

9a R_1=H_2, R_2=αOAc,βH, R_3=βOAc,αH

5a R_1=OAc, R_2=αOAc,βH, R_3=O

17 R_1=βOH,αH, R_2=H_2, R_3=O

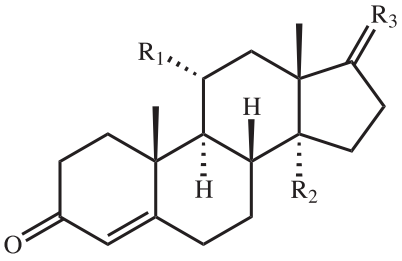
8 R_1=OH, R_2=βOH,αH, R_3=O

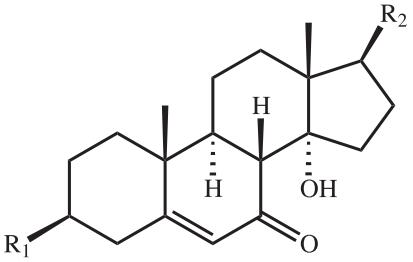
19 R_1=βOH,αH, R_2=H_2, R_3=βOH,αH

8a R_1=OAc, R_2=βOAc,αH, R_3=O

22 R_1=H_2, R_2=αOAc,βH, R_3=βOH,αH

23 R_1=H_2, R_2=αOH,βH, R_3=O





6 R_1=H, R_2=OH, R_3=βOH,αH

10 R_1=R_2=OH

6a R_1=H, R_2=OH, R_3=βOAc,αH

10a R_1=R_2=OAc

18 R_1=OH, R_2=H, R_3=O

20 R_1=OH, R_2=H, R_3=βOH,αH

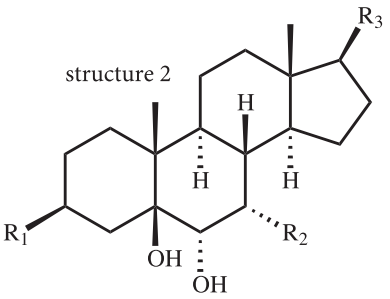
S.A. de C.V. (Orizaba, Mexico). 3β,17β-Dihydroxyandrost-5-ene (**1**) was prepared from DHEA in methanol using sodium borohydride. 17β-Hydroxyandrost-4-en-3-one (testosterone) (**2**) was acquired from Steraloids, Inc. (Wilton, NH, USA). The fungi used for these experiments were obtained from the American Type Culture Collection, Rockville, MD, USA.

2.2. Media for growth of fungi

The media and culture conditions for the following fungi have already been reported [6,7]: *Rhizopus oryzae* ATCC 11145 [8], *M. plumbeus* ATCC 4740 [9], *C. echinulata* var. *elegans* ATCC 8688a [10] and *W. sclerotiorum* ATCC 18687 [11].

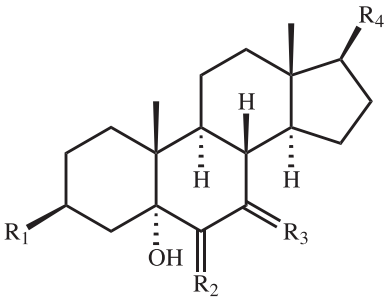
2.3. Preparation of immobilized fungal cells

One slant was used to inoculate four Erlenmeyer flasks each containing 125 mL liquid culture medium. The fungus was allowed to grow for 3 d. At the end of the incubation period the cells were harvested by filtration. The cells were suspended in water (40 mL) and 3% sodium alginate solution (140 mL) was added. The cells were macerated for 1 min at 8000 rpm using a IKA Ultra-Turrax T25 homogenizer. The cell-alginate suspension was then added dropwise to a stirred chilled solution of 0.1 M calcium chloride (200 mL). The alginate beads formed were allowed to harden for 30 min in the aqueous calcium chloride solution. The calcium chloride was decanted and the beads were rinsed with water. The beads were stored under water at 4° until used.



11 R_1=R_2=R_3=OH

11a R_1=R_2=R_3=OAc

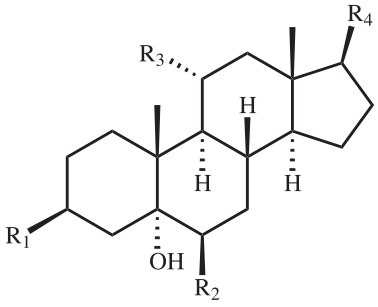


12 R_1=R_4=OH, R_2=βOH,αH, R_3=αOH,βH

12a R_1=R_4=OAc, R_2=βOAc,αH, R_3=αOAc,βH

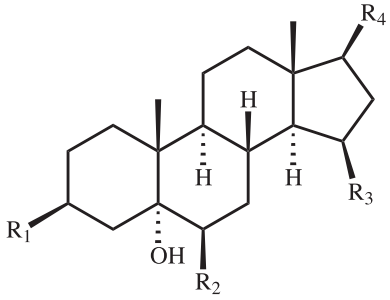
14 R_1=R_4=OH, R_2=R_3=βOH,αH

14a R_1=R_4=OAc, R_2=R_3=βOAc,αH



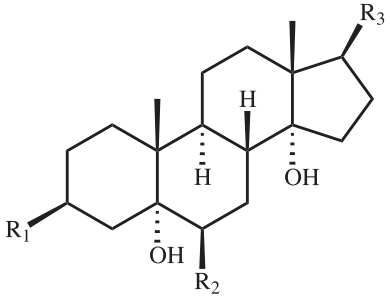
13 R_1=R_2=R_3=R_4=OH

13a R_1=R_2=R_3=R_4=OAc



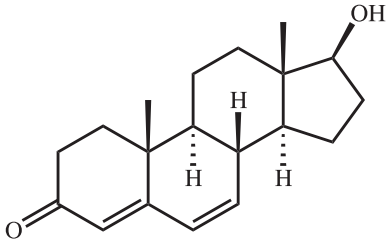
15 R_1=R_2=R_3=R_4=OH

15a R_1=R_2=R_3=R_4=OAc



16 R_1=R_2=R_3=OH

16a R_1=R_2=R_3=OAc



2.4. Mixed culture immobilized cell fermentation conditions

Equal portions of alginate beads of two fungi immobilized using the method above were placed into four 500 mL Erlenmeyer flasks, each containing water (125 mL). The substrate (200 mg) in ethanol (5 mL) was distributed among the flasks. The flasks were shaken at 180 rpm for 5 d. After the fermentation was complete the aqueous solution was decanted from the beads and the former was extracted using ethyl acetate (2 × 300 mL). The organic solution was dried using sodium sulfate, filtered, and the solvent was removed *in vacuo*. The residue was analyzed by TLC and purified by column chromatography. The products from the transformations of 3β,17β-dihydroxyandrost-5-ene (**1**) were poorly resolved. Acetylation of these metabolites aided in their purification by chromatography.

2.5. Biotransformations using 3β,17β-dihydroxyandrost-5-ene (*1*)

2.5.1. Products from Rhizopus oryzae and Cunninghamella echinulata var. elegans

Partial purification of the extract (177 mg) by column chromatography using acetone/dichloromethane (1:19 v/v) yielded the fed substrate (**1**) (58.6 mg). Further purifications using the same solvent system, followed by acetylation, afforded four metabolites. The first was 3β,7α,17β-trihydroxyandrost-5-ene (**3**). This was characterized as the triacetate (**3a**) (43.6 mg), which crystallized from acetone-methanol as plates, Rf = 0.89, acetone/dichloromethane (1:19 v/v), m.p. 142–144° $[\alpha]_D$ −176.8° (c 0.22), lit [12]. 156–158°, $[\alpha]_D$ −152°; IR: ν_{max} 1747, 1723, 1246 cm^{-1}; ^1H NMR: δ 0.80 (3H, s, H-18), 1.03 (3H, s, H-19), 2.04

(9H, s, 3 × CH_3CO_2), 4.68 (1H, m, w/2 = 12 Hz, H-17α), 4.69 (1H, dd, *J* = 9, 8 Hz, H-3α), 4.98 (1H, t, *J* = 4.5 Hz, H-7β), 5.59 (1H, d, *J* = 5 Hz, H-6). The second transformed compound was 3β,7β,17β-trihydroxyandrost-5-ene (**4**). This product was characterized as the triacetate **4a** (22.7 mg), which crystallized as needles from acetone-methanol, Rf = 0.88, acetone/dichloromethane (1:19 v/v), m.p. 178–179°, $[\alpha]_D$ +42.9° (c 0.15), lit [13]. 210–211°, $[\alpha]_D$ +52°; IR: ν_{max} 2947, 1739, 1733, 1370, 1238 cm^{-1}; ^1H NMR: δ 0.79 (3H, s, H-18), 1.05 (3H, s, H-19), 2.10 (9H, s, 3 × CH_3CO_2), 4.60 (2H, d, *J* = 8.1 Hz, H-3α, H-17α), 5.08 (1H, d, *J* = 8.2 Hz, H-7α), 5.37 (1H, s, H-6). The third product was 3β,7α-dihydroxyandrost-5-en-17-one (**5**). This was characterized as the diacetate **5a** (20.8 mg), which resisted crystallization, Rf = 0.81, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ −228° (c 0.21), lit [14]. m.p. 168–170°, $[\alpha]_D$ −178°; IR: ν_{max} 1722, 1622, 1228 cm^{-1}; ^1H NMR: δ 0.88 (3H, s, H-18), 1.05 (3H, s, H-19), 2.05 (6H, s, 2 × CH_3CO_2), 4.68 (1H, m, w/2 = 16.5 Hz, H-3α), 5.12 (1H, dt, *J* = 8.7, 2.1 Hz, H-7β), 5.62 (1H, dd, *J* = 5.3, 1.5 Hz, H-6). The final metabolite was 17β-hydroxyandrost-4-en-3-one (**2**). This was characterized as the acetate **2a** (1.8 mg), which crystallized from acetone as needles, Rf = 0.14, acetone/dichloromethane (1:19 v/v), m.p. 130–132°, $[\alpha]_D$ +92° (c 0.01), lit [15]. 141–142°, $[\alpha]_D$ +96.2°; IR: ν_{max} 2932, 1737, 1675, 1247 cm^{-1}; ^1H NMR: δ 0.84 (3H, s, H-18), 1.20 (3H, s, H-19), 2.05 (3H, s, CH_3CO_2-17), 4.60 (1H, dd, *J* = 7.7, 8.8 Hz, H-17α), 5.74 (1H, s, H-4).

2.5.2. *Products from* Rhizopus oryzae *and* Mucor plumbeus

The extract (173 mg) was purified using column chromatography. Elution with acetone/dichloromethane (1:9 v/v) afforded the fed compound (**1**) (57.6 mg). Further purifications using the same solvent system and acetylation resulted in the recovery of acetylated derivatives of three compounds which were previously isolated from the single bead fermentations: 3β,7α,17β-triacetoxyandrost-5-ene (**3a**) (11.4 mg), 3β,7β,17β-triacetoxyandrost-5-ene (**4a**) (8 mg), and 3β,7α-diacetoxyandrost-5-en-17-one (**5a**) (10 mg). Further purifications using PLC allowed for the isolation of two compounds that were not previously found in the single bead fermentations using either microorganism. The first metabolite was 14α,17β-dihydroxyandrost-4-en-3-one (**6**). This was characterized as the monoacetate **6a** (3.9 mg), which resisted crystallization, Rf = 0.19, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +133.8° (c 0.16); IR: ν_{max} 3509, 2946, 1733, 1671, 1375, 1248 cm^{-1}; HREIMS: *m/z*: 362.2149 M^+ ($C_{21}H_{28}O_4$ requires 362.2144); ^1H NMR: δ 0.96 (3H, s, H-18), 1.24 (3H, s, H-19), 2.06 (3H, s, CH_3CO_2-17), 5.18 (1H, dd, *J* = 6.5, 9.0 Hz, H-17α), 5.76 (1H, bs, H-4). The second compound was 7β,17β-dihydroxyandrost-4-en-3-one (**7**). This was characterized as the diacetate **7a** (6.6 mg), which resisted crystallization, Rf = 0.60, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +136.4° (c 0.28); IR: ν_{max} 2947, 1736, 1373, 1241, 1032 cm^{-1}; HREIMS: *m/z*: 411.2160 $[MNa]^+$ ($C_{23}H_{32}O_5Na$ requires 411.2141); ^1H NMR: δ 0.86 (3H, s, H-18), 1.23 (3H, s, H-19), 2.04 (3H, s, CH_3CO_2-7), 2.05 (3H, s, CH_3CO_2-17), 4.58 (1H, dd, *J* = 8.0, 9.0 Hz, H-17α), 4.63 (1H, td, *J* = 5, 10 Hz, H-7α), 5.77 (1H, d, *J* = 1.9 Hz, H-4).

2.5.3. *Products from* Rhizopus oryzae *and* Whetzelinia sclerotiorum

The extract (214 mg) was purified by column chromatography. Elution with acetone/dichloromethane (1:9 v/v) afforded the fed compound (**1**) (110 mg). Acetylation and further purification yielded three derivitized metabolites, 3β,7α,17β-triacetoxyandrost-5-ene (**3a**) (51.4 mg), 3β,7β,17β-triacetoxyandrost-5-ene (**4a**) (20.4 mg) and 3β,7β-diacetoxyandrost-5-en-17-one (**8a**) (3 mg), that were derived from compounds **3**, **4** and **8** respectively, and all of which were previously isolated from the individual immobilized cell fermentations [6]. A lone acylated metabolite, not found in the individual single bead fermentations, 17β-acetoxyandrost-4-en-3-one (**2a**) (1.7 mg) was also isolated.

2.5.4. *Products from* Mucor plumbeus *and* Cunninghamella echinulata *var.* elegans

The extract was purified using column chromatography. Elution with acetone/dichloromethane (1:9 v/v) afforded the fed compound (**1**) (6.2 mg). Further purification and acetylation resulted in the recovery of three derivitized metabolites that were found in the individual immobilized cell fermentations [6]: 3β,7α,17β-triacetoxyandrost-5-ene (**3a**) (7.6 mg), 3β,7β,17β-triacetoxyandrost-5-ene (**4a**) (31.1 mg), and 3β,7α-diacetoxyandrost-5-en-17-one (**5a**) (7.7 mg). Further purifications using PLC gave eight metabolites that were not previously isolated from the immobilized cell fermentation of either fungus. The first metabolite was 7α,17β-dihydroxyandrost-4-en-3-one (**9**). This was characterized as the diacetate **9a** (17.9 mg), which resisted crystallization, Rf = 0.62, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +93.3° (c 0.18); IR: ν_{max} 2948, 1737, 1676, 1376, 1245 cm^{-1}; HREIMS: *m/z*: 411.2162 $[MNa]^+$ ($C_{23}H_{32}O_5Na$ requires 411.2141); ^1H NMR: δ 0.84 (3H, s, H-18), 1.22 (3H, s, H-19), 2.03 (3H, s, CH_3CO_2-7), 2.05 (3H, s, CH_3CO_2-17), 4.63 (1H, dd, *J* = 7.9, 9.0 Hz, H-17α), 5.02 (1H, q, *J* = 3 Hz, H-7α), 5.70 (1H, bs, H-4). The second derivative was 3β,14α,17β-trihydroxyandrost-5-en-7-one (**10**). This was characterized as the diacetate **10a** (3.2 mg), which resisted crystallization, Rf = 0.34, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +102.7° (c 0.22); IR: ν_{max} 3630, 2924, 1735, 1374, 1245 cm^{-1}; HREIMS: *m/z*: 427.2074 $[MNa]^+$ ($C_{23}H_{36}O_6Na$ requires 427.2091); ^1H NMR: δ 0.89 (3H, s, H-18), 1.24 (3H, s, H-19), 2.04 (3H, s, CH_3CO_2-3), 2.05 (3H, s, CH_3CO_2-17), 4.72 (1H, tt, *J* = 5, 11 Hz, H-3α), 5.19 (1H, dd, J = 6.5, 9 Hz, H-17α), 5.73 (1H, d, *J* = 1.5 Hz, H-6). The third transformed product was 3β,5β,6α,7α,17β-pentahydroxyandrostane (**11**). This was characterized as the triacetate **11a** (3.8 mg), which resisted crystallization, Rf = 0.12, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +86.9° (c 0.26); IR: ν_{max} 3649, 2936, 1733, 1375, 1244 cm^{-1}; HREIMS: *m/z* (rel. int.): 489.2471 (**100**) $[MNa]^+$ ($C_{25}H_{38}O_8Na$ requires 489.2458); ^1H NMR: δ 0.79 (3H, s, H-18), 0.99 (3H, s, H-19), 2.05 (3H, s, CH_3CO_2-17), 2.09 (3H, s, CH_3CO_2-3), 2.10 (3H, s, CH_3CO_2-7), 3.85 (1H, d, *J* = 3.8 Hz, H-6β), 4.61 (1H, t, *J* = 8.5 Hz, H-17α), 5.27 (1H, bs, H-3α), 5.32 (1H, t, *J* = 3.6 Hz, H-7β). The fourth product was 3β,5α,6β,7α,17β-pentahydroxyandrostane (**12**). This compound was characterized as the tetraacetate **12a** (1.8 mg), which resisted crystallization, Rf = 0.28, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +163.8° (c 0.16), IR: ν_{max} 3727, 2940, 1733, 1371, 1243 cm^{-1}; HREIMS: *m/z* (rel. int.): 531.2562 (**100**) $[MNa]^+$ ($C_{27}H_{40}O_9Na$ requires 531.2564); ^1H NMR: δ 0.83 (3H, s, H-18), 1.17 (3H, s, H-19), 2.01 (3H, s, CH_3CO_2-7), 2.05 (3H, s, CH_3CO_2-17), 2.11 (3H, s, CH_3CO_2-6), 2.12 (CH_3CO_2-3), 4.62 (1H, t, *J* = 8.5 Hz, H-17α), 4.80 (1H, d, *J* = 2.3 Hz, H-6α), 4.89 (1H, t, *J* = 3 Hz, H-7β), 5.18 (1H, tt, *J* = 5.5, 11 Hz, H-3α). The fifth metabolite was 3β,5α,6β,11α,17β-pentahydroxyandrostane (**13**). This was characterized as the tetraacetate **13a** (7.6 mg), which resisted crystallization, Rf = 0.22, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +75.8° (c 0.24); IR: ν_{max} 3449, 2923, 1733, 1245 cm^{-1}; HREIMS: *m/z* (rel. int.): 531.2563 (**100**) $[MNa]^+$ ($C_{27}H_{40}O_9Na$ requires 531.2564); ^1H NMR: δ 0.86 (3H, s, H-18), 1.27 (3H, s, H-19), 1.97 (3H, s, CH_3CO_2-11), 2.02 (3H, s, CH_3CO_2-6), 2.03 (3H, s, CH_3CO_2-17), 2.09 (CH_3CO_2-3), 4.64 (1H, t, *J* = 8.5 Hz, H-17α), 4.68 (1H, bs, H-6α), 5.09 (1H, m, w/2 = 12 Hz, H-3α), 5.17 (1H, td, *J* = 5.5, 10.5 Hz, H-11β). The sixth congener was 3β,5α,6β,7β,17β-pentahydroxyandrostane (**14**). This was characterized as the tetraacetate **14a** (4.2 mg), which resisted crystallization, Rf = 0.12, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +75.4° (c 0.13); IR: ν_{max} 3449, 2925, 1733, 1245 cm^{-1}; HREIMS: *m/z* (rel. int.): 531.2562 (**100**) $[MNa]^+$ ($C_{27}H_{40}O_9Na$ requires 531.2564); ^1H NMR: δ 0.84 (3H, s, H-18), 1.19 (3H, s, H-19), 1.92 (3H, s, CH_3CO_2-6), 2.01

(3H, s, CH_3CO_2-7), 2.04 (3H, s, CH_3CO_2-17), 2.11 (CH_3CO_2-3), 4.56 (1H, t, J = 8.5 Hz, H-17α), 4.99 (1H, d, J = 4 Hz, H-6α), 5.12 (1H, m, w/2 = 22 Hz, H-3α), 5.20 (1H, dd, J = 4, 11 Hz, H-7α). The seventh compound was 3β,5α,6β,15β,17β-pentahydroxyandrostane (**15**). This was characterized as the tetraacetate **15a** (3.8 mg), which resisted crystallization, Rf = 0.05, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +160° (c 0.06); IR: ν_{max} 3630, 2926, 1735, 1375, 1244 cm^{-1}; HREIMS: *m/z* (rel. int.): 531.2569 (**100**) $[MNa]^+$ ($C_{27}H_{40}O_9Na$ requires 531.2564); ^1H NMR: δ 1.02 (3H, s, H-18), 1.21 (3H, s, H-19), 1.97 (3H, s, CH_3CO_2-15), 2.02 (3H, s, CH_3CO_2-6), 2.04 (3H, s, CH_3CO_2-17), 2.05 (CH_3CO_2-3), 4.58 (1H, t, J = 8.5 Hz, H-17α), 4.65 (1H, t, J = 2.5 Hz, H-6α), 4.96 (1H, ddd, J = 2, 5, 6 Hz, H-15α), 5.13 (1H, tt, J = 5, 10 Hz, H-3α). The eighth derivative was 3β,5α,6β,14α,17β-pentahydroxyandrostane (**16**). This was characterized as the triacetate **16a** (3.4 mg), which resisted crystallization, Rf = 0.03, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +162° (c 0.08); IR: ν_{max} 3630, 2936, 1734, 1458, 1374 cm^{-1}; HREIMS: *m/z* (rel. int.): 489.2451 (**100**) $[MNa]^+$ ($C_{25}H_{38}O_8Na$ requires 489.2458); ^1H NMR: δ 0.92 (3H, s, H-18), 1.18 (3H, s, H-19), 2.01 (3H, s, CH_3CO_2-6), 2.04 (3H, s, CH_3CO_2-17), 2.08 (3H, s, CH_3CO_2-3), 4.77 (1H, t, J = 3 Hz, H-6α), 5.12 (1H, tt, J = 5, 11 Hz, H-3α), 5.16 (1H, dd, J = 9.0, 6.6 Hz, H-17α).

2.6. Biotransformations using 17β-hydroxyandrost-4-en-3-one (**2**)

2.6.1. *Products from* Rhizopus oryzae *and* Mucor plumbeus

Partial purification of the extract (204 mg) allowed for the recovery of the fed substrate (**2**) (6.9 mg). Elution with acetone/dichloromethane (1:19 v/v) afforded the first metabolite, 6β-hydroxyandrost-4-ene-3,17-dione (**17**) (25.8 mg), which crystallized from acetone as amorphous crystals, Rf = 0.16, acetone/dichloromethane (1:19 v/v), m.p. 80–81°, $[\alpha]_D$ +46.3° (c 0.31), lit [16]. m.p. 154–162°; IR: ν_{max} 3429, 2360, 1733, 1679, 1230 cm^{-1}; ^1H NMR: δ 0.93 (3H, s, H-18), 1.41 (3H, s, H-19), 4.41 (1H, t, J = 2.8 Hz, H-6α), 5.84 (1H, bs, H-4). The second compound was collected in fractions that were eluted with acetone/dichloromethane (1:9 v/v). 11α-Hydroxyandrost-4-ene-3,17-dione (**18**) (10.7 mg) crystallized as needles from acetone, Rf = 0.19, acetone/dichloromethane (1:19 v/v), m.p. 182–184°, $[\alpha]_D$ +68.6° (0.17), lit [17]. m.p. 240–241°; IR: ν_{max} 3447, 2936, 1734, 1654, 1457 cm^{-1}; ^1H NMR: δ 0.95 (3H, s, H-18), 1.35 (3H, s, H-19), 4.08 (1H, dt, J = 5.2, 10.5 Hz, H-11β), 5.76 (1H, s, H-4). The third metabolite was eluted with acetone/dichloromethane (1:9 v/v). 6β,17β-Dihydroxyandrost-4-en-3-one (**19**) (29.1 mg) crystallized as needles from acetone, Rf = 0.14, 10% acetone in dichloromethane, m.p. 207–210°, $[\alpha]_D$ +9.41° (c 0.17), lit [16]. m.p. 206–210°; IR: ν_{max} 3396, 2946, 1661, 1230 cm^{-1}; ^1H NMR: δ 0.82 (3H, s, H-18), 1.39 (3H, s, H-19), 3.66 (1H, t, J = 8.5 Hz, H-17α), 4.39 (1H, t, J = 2.8 Hz, H-6α), 5.85 (1H, bs, H-4). Further elution with acetone/dichloromethane (1:9 v/v) allowed for the recovery of a fourth product, 14α,17β-dihydroxyandrost-4-en-3-one (**6**) (15.9 mg), which crystallized from acetone as amorphous crystals, Rf = 0.10, 10% acetone in dichloromethane (1:9 v/v), m.p. 183–188°, $[\alpha]_D$ +77.9° (c 0.78), lit [16]. m.p. 181–184°, $[\alpha]_D$ +121.8°; IR: ν_{max} 3425, 2944, 1709, 1660 cm^{-1}; ^1H NMR: δ 0.90 (3H, s, H-18), 1.22 (3H, s, H-19), 4.25 (1H, dd, J = 9.0, 6.8 Hz, H-17α), 5.73 (1H, bs, H-4). PLC purification using acetone/dichloromethane (3:17 v/v) yielded the final transformed product, 11α,17β-dihydroxyandrost-4-en-3-one (**20**) (20.5 mg), which crystallized as cubes from acetone, Rf = 0.08, acetone/dichloromethane (1:9 v/v), m.p. 168–172°, $[\alpha]_D$ +36.5° (c 1.13), lit [17]. m.p. 218–219°; IR: ν_{max} 3397, 2938, 1657, 1356, 1238 cm^{-1}; ^1H NMR: δ 0.81 (3H, s, H-18), 1.34 (3H, s, H-19), 3.69 (1H, t, J = 8.6 Hz, H-17α), 3.98 (1H, td, J = 10.5, 4.8 Hz, H-11β), 5.73 (1H, bs, H-4).

2.6.2. *Products from* Mucor plumbeus *and* Cunninghamella echinulata *var.* elegans

The extract (200 mg) underwent partial purification by column chromatography. This allowed for the recovery of the fed substrate (**2**) (14.6 mg). The first metabolite, isolated by PLC using acetone/dichloromethane (1:49 v/v) (×3), was 17β-hydroxyandrosta-4,6-dien-3-one (**21**) (1.9 mg), which resisted crystallization, Rf = 0.28, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +25.2° (c 0.14), lit [18,19]. m.p. 209–211°, $[\alpha]_D$ +80°; IR: ν_{max} 3426, 2930, 1661, 1452, 1225 cm^{-1}; ^1H NMR: δ 0.85 (3H, s, H-18), 1.13 (3H, s, H-19), 3.70 (1H, t, J = 8.5 Hz, H-17α), 5.68 (1H, s, H-4), 6.10 (1H, dd, J = 1.5, 4 Hz, H-7), 6.11 (1H, dd, J = 1, 3 Hz, H-6). The second transformed product was purified by PLC using acetone/dichloromethane (1:49 v/v). This gave 7α-acetoxy-17β-hydroxyandrost-4-en-3-one (**22**) (3.2 mg), which resisted crystallization, Rf = 0.18, acetone/dichloromethane (1:19 v/v), $[\alpha]_D$ +32.5° (c 0.08); IR: ν_{max} 3445, 1646, 1230 cm^{-1}; HREIMS: *m/z* (rel. int.): 369.2046 (**100**) $[MNa]^+$ ($C_{21}H_{30}O_4Na$ requires 369.2036); ^1H NMR: δ 0.80 (3H, s, H-18), 1.22 (3H, s, H-19), 2.03 (3H, s, CH_3CO_2-7) 3.68 (1H, t, J = 8.5 Hz, H-17α), 5.01 (1H, d, J = 2.8 Hz, H-7β), 5.70 (1H, bs, H-4). The third congener was 6β-hydroxyandrost-4-ene-3,17-dione (**17**) (3 mg). The fourth metabolite, purified via PLC using acetone/dichloromethane (1:9 v/v) (× 3), was 7α-hydroxyandrost-4-ene-3,17-dione (**23**) (6.6 mg), which crystallized from acetone as needles, Rf = 0.26, acetone/dichloromethane (1:19 v/v), m.p. 104–108°, $[\alpha]_D$ +20.1° (c 0.07), lit [20]. $[\alpha]_D$ +202°; IR: ν_{max} 3447, 2944, 1653, 1223 cm^{-1}; ^1H NMR: δ 0.92 (3H, s, H-18), 1.22 (3H, s, H-19), 4.09 (1H, m, w/2 = 10 Hz, H-7β), 5.83 (1H, s, H-4). The fifth biotransformed compound, isolated with acetone/dichloromethane (1:9 v/v), was 6β,17β-dihydroxyandrost-4-en-3-one (**19**) (16.3 mg). The sixth product of transformation, obtained in fractions that were eluted with acetone/dichloromethane (3:17 v/v), was 7β,17β-dihydroxyandrost-4-en-3-one (**7**) (9.9 mg), which crystallized from dichloromethane as plates, Rf = 0.11, acetone/dichloromethane (1:9 v/v), m.p. 178–180°, $[\alpha]_D$ +67.1° (c 0.07), lit [21]. m.p. 183.5–185.5°, $[\alpha]_D$ +101°; IR: ν_{max} 3395, 2941, 1640, 1435, 1233 cm^{-1}; ^1H NMR: δ 0.82 (3H, s, H-18), 1.22 (3H, s, H-19), 3.63 (1H, t, J = 8.7 Hz, H-17α), 3.45 (1H, m, w/2 = 15 Hz, H-7α), 5.77 (1H, s, H-4). Further elution with acetone/dichloromethane (3:17 v/v) yielded the seventh metabolite, 14α,17β-dihydroxyandrost-4-en-3-one (**6**) (33.4 mg). The final product of transformation was also obtained in fractions that were eluted from acetone/dichloromethane (3:17 v/v). 7α,17β-Dihydroxyandrost-4-en-3-one (**9**) (19.5 mg) crystallized from dichloromethane as plates, Rf = 0.12, acetone/dichloromethane (3:17 v/v), m.p. 205–206°, $[\alpha]_D$ +33.3° (c 0.42), lit [22]. m.p. 212–215°, $[\alpha]_D$ +88°; IR: ν_{max} 3411, 2941, 1661, 1232 cm^{-1}; HREIMS: *m/z*: 304.2038 M^+ ($C_{19}H_{28}O_3$ requires 304.2038); ^1H NMR: δ 0.79 (3H, s, H-18), 1.22 (3H, s, H-19), 3.65 (1H, t, J = 8.8 Hz, H-17α), 3.94 (1H, d, J = 2.5 Hz, H-7β), 5.78 (1H, s, H-4).

3. Results and discussion

3.1. Biotransformations using 3β,17β-dihydroxyandrost-5-ene (**1**)

3.1.1. *Products from* Rhizopus oryzae *and* Cunninghamella echinulata *var.* elegans

The incubation of 3β,17β-dihydroxyandrost-5-ene (**1**) with immobilized cells of *R. oryzae* and *C. echinulata* var. *elegans* yielded four metabolites, two of which were found in the single bead fermentations: 3β,7α,17β-trihydroxyandrost-5-ene (**3**) and 3β,7β,17β-trihydroxyandrost-5-ene (**4**) [6]. The other compounds, 3β,7α-dihydroxyandrost-5-en-17-one (**5**) and 17β-

hydroxyandrost-4-en-3-one (**2**), were not found in the single bead fermentation using either fungus. These compounds were characterized as their acetates (**3a**, **4a**, **5a** and **2a** respectively). It is likely that steroid **5** was generated by oxidation of the C-17 alcohol of **3** by one of the fungi. The formation of **2** possibly occurred via oxidation of the 3-hydroxyl of **1**, followed by rearrangement of the 5,6-double bond to give the conjugated enone.

3.1.2. Products from Rhizopus oryzae *and* Mucor plumbeus

This fermentation afforded five metabolites, three of which were also isolated from the single bead incubations: 3β,7α,17β-trihydroxyandrost-5-ene (**3**), 3β,7β,17β-trihydroxyandrost-5-ene (**4**) and 3β,7α-dihydroxyandrost-5-en-17-one (**5**) [6]. The ^{13}C NMR data for the monoacetate (**6a**) of one of the 'new' metabolites (**6**) showed the appearance of a nonprotonated carbon at δ 199.4, which was indicative of the presence of a conjugated carbonyl moiety. There was a significant downfield shift of 30.1 ppm in the resonance value for C-5. This pointed towards a migration of the carbon–carbon double bond from C-5,6 to C-4,5, after oxidation of the C-3 alcohol. Further inspection of the NMR data revealed that there was also another nonprotonated carbon present at δ 82.9. The HMBC spectrum showed couplings between H-12, H-18 and this new resonance, effectively placing the site of oxygen insertion in ring D. The only position that met these requirements was C-14. This compound was determined to be 17β-acetoxy-14α-hydroxyandrost-4-en-3-one (**6a**), which was derived from 14α,17β-dihydroxyandrost-4-en-3-one (**6**) [6]. Oxidation of **1** with double bond rearrangement to **2** (as seen above), followed by 14α-hydroxylation could be a possible route to **6**. The NMR data for the diacetate (**7a**) of the final product of transformation (**7**) showed a similar feature to that of compound **6a**, where there was a new nonprotonated carbon present at δ 198.7, pointing towards the presence of a conjugated ketone. There was also a new methine that resonated at δ 75.5. The COSY data showed correlations between H-8 and the new methine proton, indicating that oxygen insertion had occurred at C-7. This was further corroborated by the HMBC data that showed couplings between H-5, H-6, H-8, H-9 and this new methine. T-ROESY data indicated that H-7 was correlated to H-6α, H-9 and H-14; hence the stereochemistry of the alcohol was determined to be β. This analogue was deduced to be 7β,17β-diacetoxyandrost-4-en-3-one (**7a**), which was derived from **7** [6]. Compound **7** is envisaged as the 7β-hydroxylation product of **2**.

3.1.3. Products from Rhizopus oryzae *and* Whetzelinia sclerotiorum

This biotransformation afforded four metabolites, three of which were previously isolated from the individual immobilized cell fermentations: 3β,7α,17β-trihydroxyandrost-5-ene (**3**), 3β,7β,17β-trihydroxyandrost-5-ene (**4**) and 3β,7β-dihydroxyandrost-5-en-17-one (**8**). These compounds were characterized as their acetates (**3a**, **4a**, and **8a**) respectively [6]. The sole metabolite which was not previously found in the single bead fermentation was 17β-hydroxyandrost-4-en-3-one (**2**), characterized as its acetate (**2a**). The proposed path to this compound has been outlined above.

3.1.4. Products from Mucor plumbeus *and* Cunninghamella echinulata *var.* elegans

Eleven metabolites were isolated, eight of which were not found in the single fungal bead fermentations. The three congeners that were previously isolated were 3β,7α,17β-trihydroxyandrost-5-ene (**3**), 3β,7β,17β-trihydroxyandrost-5-ene (**4**) and 3β,7α-dihydroxyandrost-5-en-17-one (**5**), all characterized as their respective acetates (**3a**–**5a**) [6]. The first "new" metabolite (**9**) was characterized as the acetate (**9a**). The NMR data of the latter was similar to that of compound **7a**. However, the new methine resonance at δ 70.3 was approximately 5 ppm upfield of that in compound **7a**. The T-ROESY data showed that H-7 was coupled to H-8 and H-15β, indicating that the stereochemistry of the hydroxyl group was α. Hence, this compound was deduced to be 7α,17β-diacetoxyandrost-4-en-3-one (**9a**). This was derived from 7α,17β-dihydroxyandrost-4-en-3-one (**9**). Steroid **9** was probably formed by 7α-hydroxylation of **2**, the generation of which was proposed above. The ^{13}C NMR spectrum of compound **10a** revealed the appearance of a new nonprotonated carbon at δ 201.8. This pointed towards the presence of a conjugated carbonyl; however, the alcohol at C-3 remained, and this was a clear indication that the conjugated ketone was not located in ring A. The HMBC data showed that H-8 was coupled to this new carbonyl; therefore, this ketone moiety had to be located at C-7. Additionally, there was a new nonprotonated carbon, resonating at δ 80.4. This was indicative of oxygen insertion at a methine position. The HMBC data also placed this new alcohol in ring D, as there was coupling between H-18 and the nonprotonated carbon signal. It was deduced that this compound was 3β,17β-diacetoxy-14α-hydroxyandrost-5-en-7-one (**10a**), which was derived from **10**. The latter would have been formed by 14α-hydroxylation of **1**. The NMR data of the triacetate (**11a**) of the third metabolite (**11**) displayed the loss of the C-5,6 double bond. This was accompanied by the appearance of three new functionalized carbons at δ 71.4, 72.7 and 76.7. The HSQC spectrum showed that the methine at δ 71.4 was bonded to a proton resonating at δ 5.32. This proton signal was correlated to H-8 in the COSY spectrum. Consequently, the site of oxygen insertion was located at C-7. The T-ROESY spectrum showed couplings between H-7 and H-6β, H-8, H-15β, and as such the stereochemistry of the acetoxy group was determined to be α. From the HSQC data it was extrapolated that the resonance at δ 76.7 represented the carbon of a tertiary alcohol. It was determined that this hydroxyl moiety was located at C-5. The T-ROESY data showed no cross peaks between H-4 and H-19. It was determined that this was due to the presence of a *cis*-fused ring junction. Therefore, the stereochemistry of the C-5 hydroxyl must have been β. Subsequently, the methine at δ 72.7 was proposed to be located at C-6. This was supported by the HMBC data that showed the corresponding proton to be coupled to C-4, C-5 and C-7. T-ROESY data also revealed that H-6 was coupled to H-7β, H-8 and H-19. As such the stereochemistry of the hydroxyl group was deduced to be α. Compound **11a** was 3β,7α,17β-triacetoxy-5β,6α-dihydroxyandrostane, a previously unreported compound, which was derived from compound **11**. The fourth metabolite (**12**) was characterized the acetate (**12a**). The ^{13}C NMR of the latter showed similarities to that of **11a**. The C-5,6 double bond was no longer present, and there were three new functionalized carbons. There was a new nonprotonated carbon signal at δ 75.8, which was deduced to be the resonance for C-5. The COSY data showed that the signals at δ 71.6 (^{13}C) and δ 4.89 (^{1}H) were coupled to H-6 and H-8. Therefore, this acetoxy group was located at C-7. The HSQC spectrum indicated that the carbon with resonance at δ 73.0 was bonded to the proton at δ 4.80. In the COSY spectrum coupling was observed between this proton and H-7. It was inferred that this methine was at C-6. This was further supported by the HMBC data that showed that H-6 correlated to C-5, C-7, C-8, and C-10. This compound was deduced to be 3β,6β,7α,17β-tetraacetoxy-5α-hydroxyandrostane (**12a**), which was derived from 3β,5α,6β,7α,17β-pentahydroxyandrostane (**12**). Analogues **11** and **12** are probably formed by 7α-hydroxylation of **1** to give **3**, that is then epoxidized. Enzyme-catalyzed hydration of the resulting 5,6-epoxide could lead to either **11** or **12**. The spectral data of compound **13a**, derived from the fifth metabolite (**13**), showed similarities to those of the preceding compounds. The C-5,6 double bond was no longer present. The ^{13}C NMR spectrum displayed three new resonances which corresponded to functionalized carbons. The signal at δ 75.7 represented a non-

protonated carbon, and as such was assigned to the C-5 position. Based on information obtained from the HSQC and COSY spectra it was deduced that the carbon at δ 76.0 was located at C-6. The T-ROESY data revealed that H-6 was correlated to H-4α. This indicated that the stereochemistry of the acetoxyl group was β. The carbon (methine) signal at δ 70.3 was attached to the proton at δ 5.11. The COSY spectrum showed that the latter was coupled to H-9 and H-12. Hence, the position of hydroxylation was determined to have been at C-11. The T-ROESY spectrum showed H-11 was correlated to H-18 and H-19, and as such the stereochemistry of the acetoxy group was determined to be α. This compound was designated to be the novel 3β,6β,11α,17β-tetraacetoxy-5α-hydroxyandrostane (**13a**), which was derived from 3β,5α,6β,11α,17β-pentahydroxyandrostane (**13**). The formation of 13 is imagined to involve 11α-hydroxylation of **1**. The resulting triol is then epoxidized and hydrated as described previously to yield **13**. The tetraacetate (**14a**) of the sixth metabolite (**14**) exhibited the same features as the preceding congeners in the NMR spectra. The double bond was no longer present and there were three new carbons bearing oxygen funtionalities. The acetoxy bearing carbon with resonance at δ 75.9 was nonprotonated, and was determined to be located at C-5. The methine at δ 5.02 and δ 73.0 was coupled to H-6 and H-8, which indicated it was located at C-7. The proton attached to the methine carbon at δ 74.9 showed couplings to C-5, C-7 and C-8 in the HMBC spectrum; therefore, this methine was located at C-6. The coupling constant for H-6 was 4 Hz. Based on this the proton would be equatorial (α). Thus, the stereochemistry of the acetoxyl group must be β. H-7 appeared as a doublet of doublets with couplings constants of 4, 11 Hz. Based on these values it was determined that this proton was axial. The stereochemistry of H-7 was deduced to be α and as such the acetoxyl group must be β. This compound was determined to be 3β,6β,7β,17β-tetraacetoxy-5α-hydroxyandrostane (**14a**), which was derived from compound (**14**). There are no previous reports in the literature of this compound. Steroid **14** is probably formed from **4**, that is then epoxidized and hydrated. As was found with the previous metabolites the tetraacetate of seventh transformed product (**15a**) no longer had a carbon–carbon double bond present, and there were three new functionalized carbons. The nonprotonated carbon bearing a hydroxyl and resonating at δ 75.9 was denoted as C-5. The HSQC and COSY data revealed that the methine at δ 76.0 was located at C-6. The T-ROESY data showed that H-6 was coupled to H-4α and H-7α. Therefore, the stereochemistry of the acetoxy group was β. The proton of the final methine was coupled to H-14 and H-16, based on the information from the COSY spectrum, and therefore the position of hydroxylation must have been C-15. The T-ROESY data was used to establish the stereochemistry as being β. Correlations were observed between H-14, H-16α and H-15. Compound **15a** was determined to be the hitherto unreported 3β,6β,15β,17β-tetraacetoxy-5α-hydroxyandrostane, which was derived from 3β,5α,6β,15β,17β-pentahydroxyandrostane (**15**). The latter could be formed firstly by hydroxylation at C-15β, followed by epoxidation and hydration. For the triacetate (**16a**) of the final product of transformation (**16**) it was observed, as with previous analogues, that the double bond was absent. However, the compound possessed two tertiary alcohols. The HMBC data showed that the methine resonating at δ 74.9 was coupled to H-4, H-6 and H-19. This indicated that this carbon was located between rings A and B, and therefore it was reasoned to be at C-5. The second nonprotonated oxygen bearing carbon was correlated to H-18; hence, the site of oxygen insertion was at C-14. The final site of hydroxylation was determined to be at C-6. This was supported by the coupling observed between the proton and H-7 (COSY data). H-6 appeared as a triplet with a coupling constant of 3 Hz. This indicated that this proton was equatorial, therefore, the stereochemistry of the acetoxyl moiety was β. This compound was determined to be 3β,6β,17β-triacetoxy-5α,14α-dihydroxyandrostane (**16a**), which was derived from compound **16**. This compound was not previously reported in the literature. Its formation would possibly involve the generation of **11** and that is sequentially epoxidized and hydrated.

3.2. Biotransformations using 17β-hydroxyandrost-4-en-3-one (*2*)

3.2.1. *Products from* Rhizopus oryzae *and* Mucor plumbeus

This incubation afforded five metabolites, four of which were isolated from the single bead fermentations: 14α,17β-dihydroxyandrost-4-en-3-one (**6**), 6β-hydroxyandrost-4-ene-3,17-dione (**17**), 6β,17β-dihydroxyandrost-4-en-3-one (**19**) and 11α,17β-dihydroxyandrost-4-en-3-one (**20**) [7]. The ^{13}C NMR for the lone 'new' metabolite showed the appearance of a nonprotonated carbon, resonating at δ 218.4. This change was accompanied by the loss of the methine at δ 81.9, which pointed towards oxidation of the C-17 alcohol. This was supported by HMBC correlations between the carbon at δ 218.4 and H-12, H-15, H-16 and H-18. In the ^{1}H NMR spectrum there was a new resonance at δ 4.08, which was coupled to H-9 and H-12 (COSY). From this it was interpreted that a new methine was located at C-11. The T-ROESY data was used to establish the stereochemistry of the hydroxyl as α, due to the correlations observed between H-16β, H-18, H-19 and H-11β. This congener was determined to be 11α-hydroxyandrost-4-ene-3,17-dione (**18**). This would have been formed by oxidation of **20** at C-17.

3.2.2. *Products from* Mucor plumbeus *and* Cunninghamella echinulata *var.* elegans

This bioconversion yielded eight metabolites, four of which were found in the single bead fermentations: 14α,17β-dihydroxyandrost-4-en-3-one (**6**), 6β-hydroxyandrost-4-ene-3,17-dione (**17**), 6β,17β-dihydroxyandrost-4-en-3-one (**19**) and 7α,17β-dihydroxyandrost-4-en-3-one (**9**) [7]. The ^{1}H NMR spectrum of the first 'new' product (**21**) revealed the appearance of two new protons in the olefinic region at δ 6.10 and 6.11. The COSY data showed that they were coupled to each other, while the proton at δ 6.10 was also coupled to H-8. Therefore, it was determined that a double bond was located between C-6 and C-7. There were no other significant changes in the NMR data and as such compound **21** was deduced to be 17β-hydroxyandrosta-4,6-dien-3-one. This steroid may have been formed from **17**, which was then dehydrated. ^{1}H NMR data for the second compound (**22**) revealed a new singlet with resonance at δ 2.08 which was bonded to the carbon at δ 21.2 (HSQC data). The DEPT experiments revealed that this was a new methyl and its resonance frequency suggested that it was part of an acetyl group. This was supported by the presence of a new nonprotonated carbon resonating at δ 170.6. Further inspection of the NMR spectra showed that there was a new methine at δ 70.5 (^{13}C) and δ 5.01 (^{1}H). This proton was coupled with H-6 and H-8, and this indicated that oxygen insertion had taken place at C-7, followed by biological acetylation. H-7 was correlated to H-6β, H-8, H-15β and H-19, indicating that the stereochemistry of the acetoxy group was α. This analogue was determined to be 7α-acetoxy-17β-hydroxyandrost-4-en-3-one (**22**). Compound **22** is envisaged to be formed by acetylation of **9**. The ^{13}C NMR data of the third compound (**23**) showed the loss of the methine signal at δ 81.9, which was accompanied by the appearance of a new nonprotonated carbon at δ 220.1. This suggested that there was oxidation of the C-17 hydroxyl group. There was a new proton at δ 4.09, which was coupled to H-6 and H-8 in the COSY spectrum. This indicated that a new alcohol was located at C-7. The T-ROESY data revealed that H-7 was coupled H-1β, H-6β, H-8 and H-15β. Therefore, the stereochemistry of the hydroxyl was determined to be α. This metabolite was deduced

to be 7α-hydroxyandrost-4-ene-3,17-dione (**23**). This compound probably is formed by oxidation of **9** at C-17. The spectral data of the final new metabolite (**7**) was quite similar to that of compound **23**, however, the resonance value for the new methine showed a significant difference of 12.6 ppm. The proton of this methine was coupled to H-6 and H-8 in the COSY spectrum indicating that the site of hydroxylation was C-7. The T-ROESY data showed that H-7 was correlated to H-6α, H-9 and H-14. Consequently, the stereochemistry of this hydroxyl moiety must have been β. This analogue was elucidated to be 7β,17β-dihydroxyandrost-4-en-3-one (**7**). It is possible that **7** comes from **9** via redox chemistry at C-7.

4. Conclusion

The results show that the mixture of beads derived from two different fungi can effect transformation of steroid substrates to yield compounds not seen in the fermentation utilizing immobilized cells from either organism. In this study the aim was not to optimize product yield, but to determine if crossover occurred. Therefore, fermentations were not run until the fed substrate was completely consumed. There is great potential in employing this method for generation of small libraries of compounds. This was particularly evident in the case of the fermentation using *M. plumbeus* and *C. echinulata* var. *elegans* for the bioconversion of 3β,17β-dihydroxyandrost-5-ene (**1**), where eight metabolites, not present in the individual fermentations were observed. Seven of these were novel compounds. Similarly, when **2** was incubated with beads derived from *M. plumbeus* and *C. echinulata* var. *elegans*, four additional metabolites were formed, one not previously reported in the literature. The fact that supplementary compounds were formed in the mixed fermentations suggests that in some cases the substrate is first functionalized by one microorganism and the product thus formed is further processed by the second fungus. In other instances the mechanisms operating are not as obvious. It is not easy to elucidate the pathways by which the products of crossover are formed. Experiments in which products from a fermentation using beads of fungus A are then incubated with fungus B might provide some insight into what is happening; particularly if this is followed by an examination of the results when biotransformed compounds from encapsulated fungus B are then incubated with fungus A. Extensions of this work could involve mixtures of beads generated from three or more microorganisms. Moreover, preliminary results in our laboratory show that this method has application in terpene transformation as well.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.11.026.

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