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Anna Fryszkowska,[†,¶] Justine Peterson,[†] Nichola L. Davies[†,‡] Colin Dewar,[†] George Evans,[†] Matthew Bycroft,[†] Neil Triggs,[†] Toni Fleming,[†] Srikanth Sarat Chandra Gorantla,[‡,‡‡] Garrett Hoge,[†,††] Michael Quirmbach,[§,§§] Upadhya Timmanna,[‡,¶¶] Srinivas Reddy Poreddy,[‡] D Naresh Kumar Reddy,[‡] Vilas Dahanukar,[‡] Karen E. Holt-Tiffin[†]*

[†] Dr. Reddy's Laboratories EU Ltd, Chirotech Technology Centre, 410 Cambridge Science Park, Cambridge CB4 0PE, United Kingdom; [‡] Dr. Reddy's Laboratories Ltd, Custom Pharmaceutical Services, Bollaram Road, Miyapur, Hyderabad, 500049, India; [§] Dr. Reddy's Laboratories SA Elisabethenanlage, 11CH-4051 Basel, Switzerland.

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4-Androsten-3,17-dione **1**

3-steps

Dehydroepiandrosterone acetate **3**

Abiraterone acetate **4**
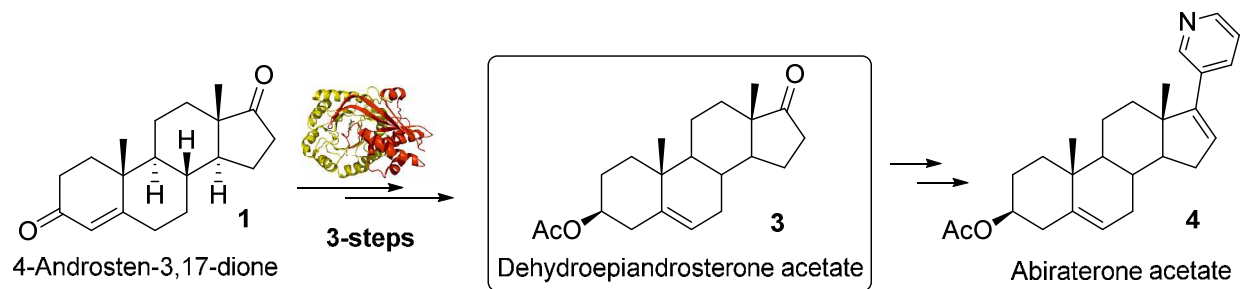
We report a novel chemoenzymatic process to prepare dehydroepiandrosterone acetate (**3**), a key building block in the synthesis of anti-prostate cancer drug abiraterone acetate (**4**) from 4-androstene-3,17-dione (**1**). The key step of our route is a robust and stereoselective enzymatic reduction of 5–androstene-3,17-dione (**5**) to dehydroepiandrosterone (**2**, >99% *de*) at high substrate concentration (100-150 g/L), highlighting the potential of ketoreductases in industrial steroid drug manufacture.

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Abstract

Dehydroepiandrosterone (DHEA, **2**) is an important endogenous steroid hormone in mammals used in the treatment of a variety of dysfunctions in female and male health,[1] as well as an intermediate in the synthesis of steroidal drugs, such as abiraterone acetate which is used for the treatment of prostate cancer.[2–4] In this manuscript we describe a novel, concise and cost-efficient route towards DHEA (**2**) and DHEA acetate (**3**) from 4–androstene-3,17-dione (4-AD, **1**). Crucial to success was the identification of a ketoreductase from *Sphingomonas wittichii* for the highly regio- and stereoselective reduction of the C3-carbonyl group of 5-androstene-3,17-dione (**5**) to the required 3β-alcohol (**2**, >99% *de*). The enzyme displayed excellent robustness and solvent stability under high substrate concentrations (up to 150 g/L).

KEYWORDS: Steroids, stereoselective synthesis, reduction, biocatalysis, ketoreductases, screening.

1. Introduction

3β-Hydroxyandrost-5-en-17-one (**2**), also known as dehydroepiandrosterone (DHEA) or prasterone is an important endogenous steroid hormone[1] and a precursor in the synthesis of other steroidal drugs such as abiraterone acetate (Zytiga®), a drug developed for the treatment of castration-resistant prostate cancer.[2–4] Current routes towards DHEA (**2**) and DHEA acetate (**3**) are typically based on the selective degradation of yam-derived diosgenin to 16-dehydropregnenolone acetate (**29**),[5,6] followed by oxime formation and rearrangement (Scheme 1).[5–8]

Scheme 1. Diosegenin (**28**) and stigmasterol (**31**) as precursors for DHEA acetate (**3**) synthesis.
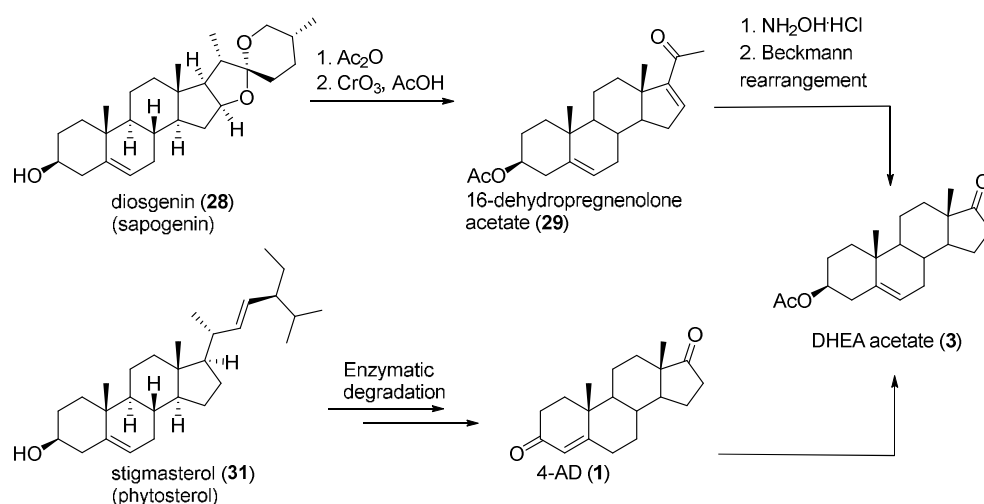
Dependence on raw-materials with variable availability often drives the development of alternative processes for steroid drug manufacture.[5] Here, we aimed to develop a novel and scalable route to DHEA acetate (**3**) from 4-androstene-3,17-dione (4-AD, **1**), which is derived from phytosterols obtained from soybeans (e.g. stigmasterol, **31**; Scheme 1).[5,9–11]

Nature offers a vast array of enzymes that can be used to access the structural and functional diversity of steroids[10,11]and they often display high substrate specificity, as well as remarkable regio- and stereoselectivity. During the initial phase of route scouting for possible ways to make

dehydroepiandrosterone acetate (**3**) from 4-AD (**1**) we considered several possible chemoenzymatic approaches, including routes A and B depicted in Scheme 2.

Scheme 2. Proposed synthetic strategies to convert 4-AD (**1**) to DHEA acetate (**3**).
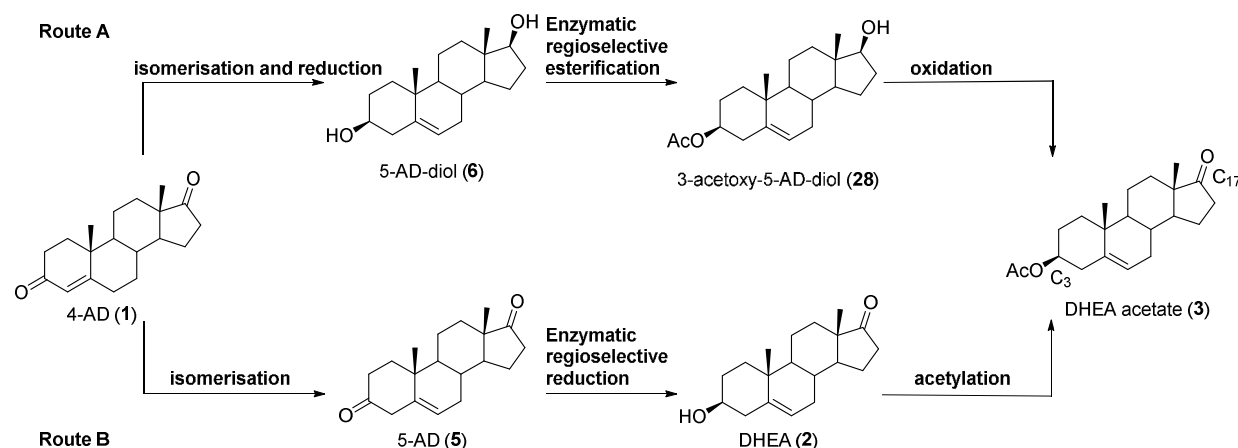
Although hydrolases can be used for the regioselective esterification of polyhydroxylated steroid derivatives,[12,13] our initial trials with 5-androstene-3,17-diol (5-AD-diol, **6,** Scheme 2, Route A) indicated that with Novozyme 435 (*Candida antarctica* Lipase B) the reaction progressed slowly and required high enzyme loading (60h, 100% w/w enzyme, complete conversion to **28**). Inspired by natural metabolic pathways for androgens, we turned our attention to oxidoreductases (Route B, Scheme 2) .[14–20] In mammals 4-AD (**4**) is synthesised from DHEA (**2**) by the action of two enzymes: 3β-hydroxysteroid dehydrogenase (3β-HSD) catalysing its oxidation to 5-androstene-3,17-dione (5-AD, **5**)[14,21–26] and ketosteroid isomerase (KSI), which catalyses the thermodynamically favoured isomerisation of the double bond to the conjugated α,β-unsaturated ketone **1** (K_{eq} = 2400).[20,27–30] Thus, we hoped to take advantage of the reversibility of biooxidation and use an enzyme for the stereo- and regioselective reduction of the C3-carbonyl group – a reverse sequence to the natural mammalian metabolic pathway. Ultimately this proved successful and the results are described hereafter.

2. Results and Discussion

In order to successfully convert 4-AD (**1**) into DHEA acetate (**3**), we envisaged a three step process. Firstly, we planned to convert 4-AD to 5-AD (**5**) through a selective $\Delta^4 - \Delta^5 -$ isomerisation.[31–33] Secondly, we needed to identify a highly stereoselective ketoreductase with appropriate industrial properties to selectively reduce 5-AD (**5**) to DHEA (**2**), which was the key to success of this project. As ketoreductases and hydroxysteroid dehydrogenases may display vastly-different degrees of regioselectivity (3- *vs*. 17-positions) and stereoselectivity (α *vs*. β),[14,21–26] finding the enzyme with exclusive 3β-stereoselectivity was crucial. Finally, we planned to convert DHEA (**2**) into DHEA acetate (**3**) and to develop an efficient acetylation and crystallisation to provide the material with the required purity ($\geq$99.5% by HPLC area).

2.1 Isomerisation

Several approaches to prepare 5-AD (**5**) are described in the literature,[19,34,35] however these were considered sub-optimal for our process requirements, as they required multi-step syntheses. Our attention was drawn by short communications on deconjugation of α,β-unsaturated ketones,[31–33] including an example of a low yielding potassium hydroxide-catalysed isomerisation of 4-AD (**1**).[32] After an initial screen, we were able to identify a promising initial set of reaction conditions that afforded 5-AD (**5**) in >80% yield (Scheme 3). Treatment of 4-AD (**1**) with 10 eq. of potassium *tert*-butoxide in *tert*-butanol for 90 min, followed by the kinetic quench of the extended enolate (**32**) into 10% v/v aqueous acetic acid allowed precipitation of the product **5,** which was then isolated by filtration.

**Scheme 3.** Isomerisation of 4-AD (**1**) to 5-AD (**5**)
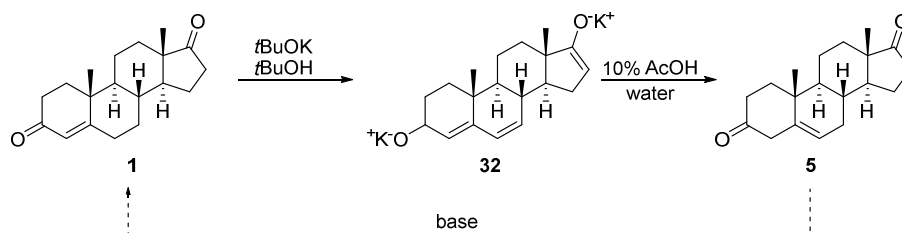
Encouraged by these results, we proceeded to develop the process further. Initially, we observed great variability in the reaction outcome, with the relative amount of the desired 5-AD (**5**) varying from 50 to 95% (by HPLC area%). From consideration of the isomerisation mechanism (Scheme 3), the root cause of this problem became evident. The initial formation of the dianion **32** occurs readily (<30 min) using 2.0-2.5 equivalents of potassium *tert*-butoxide. (*Note*: while we have drawn this as the dianion, this is purely based on mechanism proposed in the literature[31] and the observation that we required > 2 eq of base and we did not obtain any additional confirmation of the structure). The subsequent kinetic protonation results in the desired 5-AD (**5**) isomer, which is sensitive to even mildly basic conditions and readily isomerises back to 4-AD (**1**). Fortunately, the exposure of the 5-AD (**5**) to basic conditions could be minimised by quenching the enolate into an excess of acetic acid, rather than by adding the acid to the reaction mass. The addition rate, efficient mixing and temperature (<30 °C) have to be carefully controlled to achieve high yields and to prevent localised high pH 'hotspots' during the quench. Failure to control these physical parameters leads to significant isomerisation and apparent poor conversion. Moreover, 5-AD (**5**) quenched this way precipitated from the solution and could be readily isolated by filtration, which is a significant process advantage.

While the controlled quench addressed one of the issues with the reaction, we observed formation of several other impurities, as evidenced by additional peaks in the HPLC chromatogram and NMR spectra. The levels of these impurities varied from batch to batch and

their content changed upon storage of the 5-AD (**5**). Moreover, the formation of the same

impurities was then also observed during the bioreduction step (*vide infra*).

Scheme 4. Impurities generated during isomerisation of 4-AD (**1**) to 5-AD (**5**)
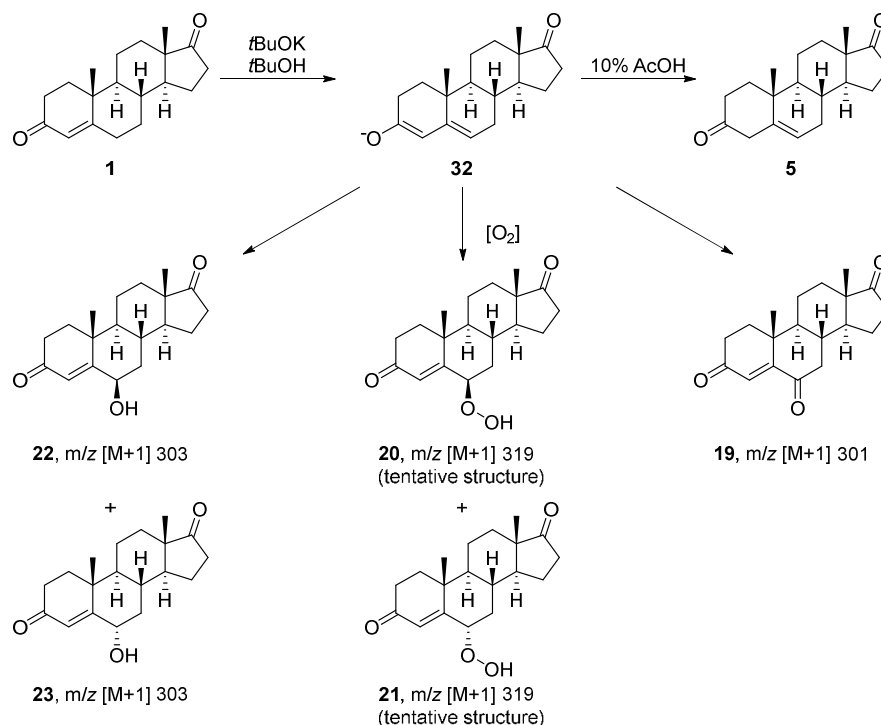
The main impurities were isolated chromatographically and characterised by NMR and MS

(see supplemental info for details). The spectroscopic data obtained for one of the main side-

products was in agreement with the structure of androst-4-ene-3,6,17-trione (**19**, m/*z* 301

[M+1]$^{+}$). This oxygenated impurity was reported previously by Pollack *et al*. and its formation

ascribed to autooxidation of the steroid backbone by molecular oxygen present in the reaction.[19]

We identified two further impurities, 6β- and 6α-hydroxyandrost-4-ene-3,17-dione (**22** and **23**,

respectively, m/*z* 303 [M+1]$^{+}$).[36] In addition we were able to isolate small quantities of two

further compounds, which proved unstable and we observed their conversion to trione **19** over

the course of the NMR experiments. Based on the similarity of their ^{1}H NMR spectra to the

isomeric 6-hydroxyandrost-4-ene-3,17-diones (**22** and **23**) and mass spectrometry data (both had

m/z of 319), these compounds were tentatively assigned as 6β- and 6α-perhydroxy–androst-4-ene-3,17-diones (**20** and **21**), respectively. Their presence would be in agreement with the involvement of oxygen in the side-product formation; however no further elucidation of the mechanism of autoxidation and possible interconversion between the compounds **19-23** was performed. Nevertheless, this highlighted the need to carry out the isomerisation under oxygen-free conditions. Thus, the introduction of a rigorous nitrogen sparging protocol during the reactions reduced the levels of these impurities sufficiently; on 300g scale we were able to prepare crude 5-AD (**5**) of acceptable purity: typically 89-93% by HPLC area, containing 3-5% of 4-AD (**1**) and 3-4% oxygenated impurities **19-23**.

In summary, our efforts resulted in an efficient 4-AD (**1**) isomerisation procedure, giving 5-AD (**5**) in approx. 80% yield and acceptable purity. We reduced the base requirement from 10 to 2 equivalents of potassium tert-butoxide, providing significant cost reduction for this step. The material generated under the optimised conditions was used directly in the subsequent biocatalytic step, as it was not suitable for prolonged storage.

2.2 Development of enzymatic step

Ketoreductase screening. The key step of our proposed route relied on identifying a highly regio- and stereoselective ketoreductase enzyme to convert 5-AD (**5**) to the desired 3β-hydroxyandrost-5-ene-17-one (DHEA, **2**) exclusively. The bioreduction of diketone **5** could theoretically result in up to 16 possible products: 4 possible diastereoisomers of monohydroxysteroids (C3-OH or C17-OH) and further 4 possible diols (**6**). An additional 8 isomeric products could be expected, if the double bond moves back to C4. The complexity of the chromatographic analysis of such a mixture and lack of commercially available reference materials added to the challenging nature of the enzymatic screen. Therefore to simplify and

accelerate the identification of regioselective enzymes, we took advantage of the reversibility of

the ketoreductase-catalysed reactions. Specifically, we screened the enzyme libraries towards the

oxidation of hydroxysteroids as depicted in Figure 1. The screen was performed using a routine

colourimetric formazan-linked assay, which detects reduced NAD(P)H cofactor, formed during

the biooxidation.[37] In parallel, we tested ketoreductases for the oxidation of commercially

available DHEA (**2**), testosterone (**9**) and androst-5-ene-3β,17β-diol (5-AD-diol, **6**), observing

red-colouration in the wells with biocatalyst active towards individual compounds after 60 min at

30 °C (the low substrate solubility in aqueous media did not allow monitoring of the reactions

spectrophotometrically and the plates were inspected visually).

Figure 1. The principle of screening for regioselective ketoreductases in the oxidative direction

using a NAD(P)H-linked colourimetric assay.
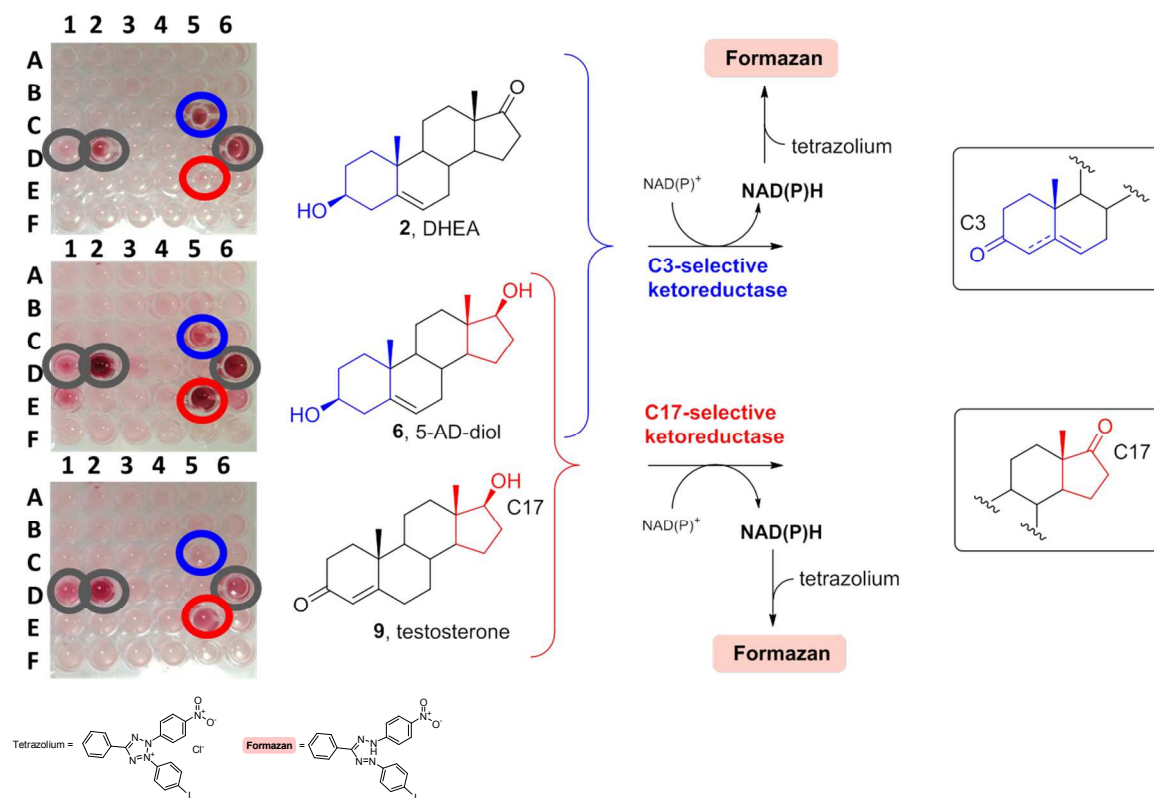
The pictures show snapshots of typical screening plates tested with three different hydroxysteroids: top panel – DHEA (**2**); middle panel – 5-AD-diol (**6**); bottom panel – testosterone (**9**). NAD(P)H cofactor formed in the oxidation in the presence of tetrazolium (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride - INT) and diaphorase causes red-colouration of the wells with an active enzyme. Highlighted in blue circles – an enzyme with exclusive activity towards C3β-hydroxyl group; in red circles – an enzyme with exclusive activity towards C17β-hydroxyl group; in grey circles – enzymes active towards both C17- and C3-hydroxyl groups (non-selective).

Using this screening strategy we quickly discriminated between the enzymes displaying 3β- *vs.* 17β-regioselectivity (see Figure 1, top *vs.* bottom panel). Androst-5-ene-3β,17β-diol (**6**, Figure 1, middle panel) was added to the screening program as a control to confirm the regioselectivity towards C3 and C17 hydroxyl groups.

The colourimetric screening strategy simplified and accelerated the screening for regioselective enzymes, without the need to put effort into complex and lengthy chromatographic analysis. We efficiently tested a collection of almost 400 proprietary and commercial ketoreductases to identify a short-chain dehydrogenase/reductase from *Sphingomonas wittichii*[38] as **the sole enzyme** with the desired 3β-stereoselectivity. Subsequent studies on the enzymatic reduction of 5-AD (**5**) demonstrated that the *Sphingomonas wittichii* ketoreductase catalysed formation of DHEA with high diastereoselectivity (**2**, >99% *de*) – spiking experiments showed no detectable formation of 3α-epimer **13** by HPLC. Moreover, the enzyme showed almost no activity towards the isomer 4-AD (**1**).

Reaction optimisation.

We optimised process parameters, such as substrate and ketoreductase loading, co-solvent,
phase ratio, pH, temperature in a series of experiments (details are shown in Table 1). We aimed
to maximise the substrate concentration and to minimise the enzyme loading in order to improve
the volumetric efficiency and the cost of the process. In parallel, we also improved the gene and
the protein expression of the ketoreductase to minimise the fermentation requirement (*vide
infra*).

Table 1. Optimisation studies of 5-AD (**5**) bioreduction.

Parameter	Evaluated range	Set value
Cofactor	NAD^+/$NADP^+$	NAD^+
pH	6.0-8.0	6.3
Enzyme loading & form Enzyme addition	0.25-10% (w/w or v/w) One batch *vs.* multiple additions	1st generation ketoreductase: 3% w/w (powder, 1 batch)[a] 2nd generation ketoreductase: 5% v/w (liquid, 4 batches)[a] 3rd generation ketoreductase: 0.25% w/w (powder, 1 batch)[b]
Temperature	20-50 °C	33 °C
Cosolvent[c]	2-MeTHF, EtOAc, MTBE, DIIPE, BuOAc, toluene, *n*-BuOH, THF, DMSO, DMF	2-MeTHF, EtOAc
Cosolvent volume	0–66% v/v	50-60% v/v
Buffer	TRIS, phosphate	phosphate
Substrate conc.	3-150 g/L	100 g/L
$MgCl_2$[d]	0-5 mM	0 mM
Base	2.0 M NaOH, 1.7 M Na_2CO_3, 1.5 M K_2CO_3	1.5 M K_2CO_3

[a] used for the >200g-scale campaign; [b] loading for the optimised enzyme powder; [c] optimised
water immiscible co-solvents were initially tested at 30% v/v while water miscible ones at 6%

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v/v; [d] magnesium chloride was added to see if it would improve the protein stability/activity under the reaction conditions, however no difference was observed

All the bioreductions were conducted in a Multimax-system, using 50 mL reactors with overhead stirring, temperature control and pH-control. We used glucose and glucose dehydrogenase to recycle the NAD(P)H-cofactors, adjusting the pH with a base (2.0 M NaOH or 1.7 M Na_2CO_3). The best conversions were obtained at 25-35 °C and pH 6.0-7.0. Very early into the process development, we concluded that biotransformations proceeded more efficiently under bi-phasic reaction conditions, using 2-MeTHF or EtOAc – this increased the substrate solubility in the reaction media and allowed much higher conversions and substrate loadings than the addition of water miscible co-solvents. Consequently we were able to run >200 g-scale batches at concentrations up to 150 g/L, achieving full conversion. EtOAc was selected as the best solvent overall, since it was significantly cheaper, although it required 10-20% higher enzyme loadings. Moreover, EtOAc offered a process safety advantage, as 2-MeTHF may form explosive peroxides. We replaced 2 M NaOH with 1.7 M Na_2CO_3 to avoid localised high pH 'hotspots' leading to side-product formation. Ultimately, we preferred 1.4 M K_2CO_3 solution as a base, as it possesses higher solubility in water in comparison to Na_2CO_3 (112 g/100 mL *vs*. 21.5 g/100 mL at 20 °C), which improved process reproducibility and eliminated the risk of line blockage in the pH control apparatus seen occasionally with saturated sodium carbonate solutions.

Under the optimised conditions >200 g-scale enzymatic reductions proceeded efficiently using our 2nd generation biocatalyst (liquid cell-free extract of the gene and expression optimised ketoreductase). Such biotransformations reached full conversions in 24-28h, giving crude DHEA (**2**) in 88-90% purity by HPLC. Further improvements of biocatalyst form (powder, 3rd

generation enzyme) further reduced the time required to reach >95% conversion to 6-8h at 0.25-0.30% w/w enzyme loading.

During these bioreductions, we observed the formation of similar impurities to the isomerisation step. Unsurprisingly, their content increased significantly at elevated temperatures (>40 °C). Under such conditions, at pH 7.5 and in the presence of air, 5-AD (**5**) decomposed almost completely over 3 days. The most prominent impurities were 4-AD (**1**), trione (**19**) as well as hydroxy- and perhydroxysteroids **20-23**. Nevertheless, maintaining the pH at 6-7 and temperature <35 °C ensured an efficient biotransformation and minimised the reverse isomerisation. The content of 4-AD (**1**) present in starting 5-AD (**5**) material did not increase by more than 2-3%. Sparging of the bioreduction solutions with nitrogen was necessary to keep the level of the oxygenated impurities below 8% from the initial 4-6% present in the input crude 5-AD (**5**).

In addition, we observed the formation of some impurities specific to the bioreduction itself. We detected low levels of 5-AD-diol (**6**, <0.5% by HPLC area), possibly arising from reduction at C17 catalysed by the *Sphingomonas wittichii* ketoreductase and/or other endogenous, non-selective *E. coli* derived ketoreductase enzymes present in either the ketoreductase or glucose dehydrogenase preparations. This impurity could be minimised by monitoring conversion and stopping the reaction as soon as it reached completion.

Molecular biology and fermentation. The 1st generation ketoreductase preparation used in the screen and the initial process development had relatively low activity, 17-35 U/g of enzyme powder, as it contained a large amount of insolubly expressed protein. Consequently, high enzyme loadings (3-5% w/w, 2U per g of substrate) were required to achieve full conversion of 5-AD (**5**). Therefore, we decided to improve the protein expression, fermentation and

downstream processing of the enzyme. This resulted in 10-15 fold improved enzyme productivity, lowering enzyme loadings, which also resulted in better process parameters (elimination of the emulsions we observed with the first generation enzyme preparations). It is worth noting that such improvements in biocatalyst production can be very effective in improving the efficiency of a biotransformation and are rapidly implemented.[39]

Firstly, we optimised the gene encoding the ketoreductase for heterologous expression in a phage-resistant *E. coli* BL21(DE3) strain containing pET26b vector. This afforded an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *E. coli* strain, in which 90% of the ketoreductase was solubly expressed. Secondly, we developed high optical density fermentation (20L, glucose feed, 176 OD, see Supplemental Information for details), which after clarification and ultrafiltration (10 kDa membrane) resulted in 2nd generation enzyme produced as liquid cell free extract of 39 U/mL activity.

Unfortunately, upon storage for 1 month at 2-8 °C we observed that the enzyme preparation lost 75% of its activity and the later batches of the bioreduction required higher than expected enzyme loading. We discovered that lyophilisation improved its storage and stability over time. Consequently, later batches of the enzyme were lyophilised to give 3rd generation ketoreductase powder with 400-500 U/g activity. This represented an overall 10-15 fold improvement in comparison to the 1st generation biocatalyst (17-35 U/g) and it allowed a decrease in the enzyme loading from 3-5% to 0.25-0.30% w/w to achieve 95-99% conversion in 6h. Not only did the reduction in biocatalyst loading lower the cost of the biocatalyst, but it improved the ease of the workup, as quick phase separation was observed after the biotransformation.

Purification of DHEA. Initially we intended to develop an efficient crystallisation of crude DHEA (**2**) after the biotransformation step (79-91% purity by HPLC). A literature evaluation

indicated that five polymorphs of DHEA (**2**) are known (forms I-V), as well as three monohydrate polymorphs and numerous solvates with solvents like alcohols and ethers.[40,41] Crystallisation studies of the crude DHEA (**2**) isolated from the biotransformation gave product of >98% purity using a variety of solvents, but the recoveries were low (10-55%, results not shown). Therefore, we decided to telescope the crude DHEA to the subsequent acetylation-step and focus on developing an efficient crystallisation of DHEA acetate (**3**) instead.

2.3 Acetylation and final crystallisation

The acetylation of DHEA (**2**) to its acetate (**3**) proved to be a facile reaction and proceeded quantitatively under a variety of reaction conditions tested. Successful reaction conditions included: (i) dichloromethane/acetic anhydride/pyridine/DMAP; (ii) dichloromethane/acetic anhydride/boron trifluoride diethyl etherate; (iii) acetic acid/acetic anhydride/sodium acetate; (iv) toluene/acetic anhydride/triethylamine/DMAP (details not shown). In the early stages of development DHEA acetate (**3**) was prepared using acetic anhydride, triethylamine with catalytic DMAP in toluene.[38] These conditions gave a fast, robust reaction with low cost raw materials and acetate (**3**) was obtained in >98% purity (by HPLC) as 2 crops in 85-87% yield.

During further process development and optimisation work we aimed to reduce the number of unit operations and to develop a facile acetylation procedure that could be telescoped into the final crystallisation process. After some further optimisation we achieved full conversion of DHEA (**2**) using acetic anhydride in the presence of sodium acetate in acetic acid over 16h. Quench of the reaction into a mixture of water and methanol (9:1, 1.5 vol) precipitated crude DHEA acetate (**3**) in 88% yield and 98% purity. This material was subsequently recrystallised from methanol (4 vol), affording final product **3** in 74% yield and >99.5% purity (by HPLC),

meeting the product specifications. These modifications considerably simplified the process and provided both cost and operational benefits.

Scheme 5. Optimised conditions for the >200 g-scale process to covert 4-AD (**1**) to DHEA acetate (**3**).
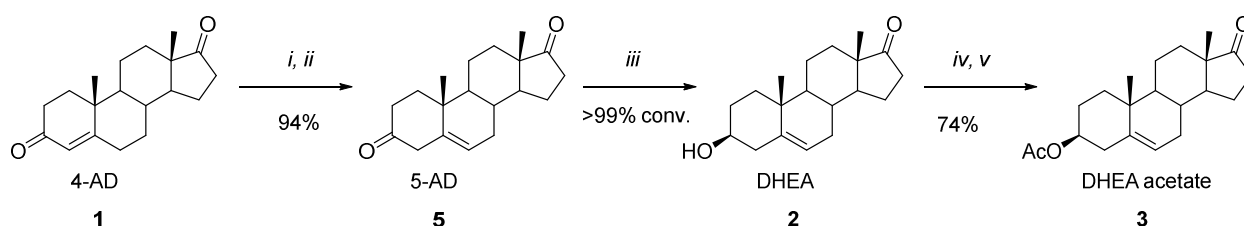
(*i*) *t*BuOK (2.1 eq), *t*BuOH (6 vol), 30-35 °C, N$_2$, 20 min; (*ii*) 2.5% AcOH in water (20 vol), 20-30 °C, N$_2$, 60 min, 94% yield, 93% pure; (*iii*) 100 g/L, EtOAc:phosphate buffer (40 mM, pH 6.5) 60:40 v/v, NAD$^+$ (0.075% w/w), glucose (1.1 eq), glucose dehydrogenase (0.044% w/w), ketoreductase from *Sphingomonas wittichii* (liquid preparation, 2 U per g of 5-AD), temp. 33 °C, 29h, N$_2$, >99% conversion, 91% purity (*iv*) 2.0 eq Ac$_2$O, AcOH (3 vol), NaOAc (1.25 eq), 60 °C, N$_2$, 16h, quench with MeOH: water (1.5 vol); (*v*) crystallisation from hot MeOH (4 vol), 74%, purity >99.5% (by HPLC). 64% overall yield.

3. Summary

We developed a novel, high yielding chemoenzymatic route to DHEA acetate (**3**) from 4-AD (**1**). The key enabling reaction of this process was a highly regio- and stereoselective bioreduction of 5-AD (**5**) to DHEA (**3**). A fast colourimetric assay was crucial in the identification of a short-chain dehydrogenase from *Sphingomonas wittichii,* which possessed the desired selectivity. The enzyme displayed good robustness and performance under the bi-phasic reaction conditions,[38] which enabled the biotransformations to be conducted at high substrate concentrations (up to 150 g/L). Optimisation of the gene expression as well as the fermentation conditions of an *E. coli* host strain resulted in 10-15 fold improvement of the enzyme productivity and gave us access to a cost-effective biocatalyst. Development of both the 4-AD to

5-AD isomerisation and the final acetylation and purification provided a robust process, suitable for further scale-up. Identification of the impurities generated in the process enabled the development of control strategies and was crucial to obtain high purity final product.

4. Experimental

Materials and methods

4-AD (**1**) was supplied by Dr. Reddy's de Mexico, Cuernavaca-Cuautla, 62578 Civac Jiutepec Morelos, Mexico. Testosterone (**9**) was obtained from Sigma. Epitestosterone (**10**) was obtained from LGC Standards, Queens Road, Teddington, Middlesex, TW11 0LY, UK. 3β,17β-5-AD-diol (**6**) was provided by Dr Reddy's Laboratories Ltd., Custom Pharmaceutical Services, TDC-1 Bollaram Road, Miyapur, Hyderabad 500049, Telangana, India. 3α-DHEA acetate (**14**) was obtained from Bujno Synthesis, Dorodna 16, Warsaw, Poland. 3α-DHEA (**13**) and 3α-DHEA acetate (**14**) were also synthesised from 3β-DHEA (**2**) using a Mitsunobu reaction sequence according to a previously reported method[42] (See Supplemental Info for details). All enzymes used in the screen were proprietary to Dr Reddy's Laboratories or obtained from commercial suppliers (Almac, Codexis, Daicel, Evocatal, Johnson Matthey, Prozomix and Syncore). Glucose dehydrogenase GDH CDX-901 (66 U/mg) was supplied by Codexis. Nicotinamide cofactors NAD^+ and $NADP^+$ were obtained from Europa Bioproducts. Routine NMR spectra were recorded on a Bruker DRX 400MHz spectrometer and chemical shifts for ^1H spectra were referenced relative to internal tetramethylsilane or residual solvent peaks. If additional resolution and sensitivity was required, compounds were analysed on a 700 and 500 MHz NMR instrument with a cryoprobe, School of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK.

Biology and fermentation: For detailed information of the methods, please see the Supplemental Information.

Analytical methods

The isomerisation and biotransformations were monitored by HPLC: **Method A** using ELSD and UV (λ = 200 nm) detectors and Luna C18 column 150 × 4.6 mm, column temp. 50 °C, flow 1 mL/min, MP 50:50 A:B, A: water, B: MeOH:MeCN 30:70, inj. vol: 10 µL. The progress of the biotransformation reactions was also monitored by the base consumption as well as by ^{1}H NMR spectroscopy. LC-MS data was collected using ESI(+) ion detector and HPLC method A, where 0.1% v/v was added to water.

The purity of the DHEA acetate (**3**) was determined by HPLC: **Method B** using UV detector (λ = 205 nm) and Kinetex C8 column 2.6 µm 100Å 150 × 4.6mm, flow 1 mL/min, column temp. 40 °C, using mobile phase gradient (A = 80:20 water:MeCN, B = MeCN, see **Table 2**), inj. vol: 10 µL.

Table 2. Mobile phase gradient for HPLC analysis of DHEA acetate (**3**) using Kinetex C8 column (Method B).

Time (min)	0	5	20	25	35	50	55	60
Mobile phase A	65	65	35	10	5	5	65	65
Mobile phase B	35	35	65	90	95	95	35	35

Enzyme activity assay. The activity of the ketoreductase from *Sphingomonas wittichii* was determined in 5 g-use tests or spectrophotometrically using 4-heptanol as a substrate, by following its oxidation at 340 nm, monitoring the conversion of the NAD(P)$^+$ cofactor to NAD(P)H. One unit of activity is defined as sufficient enzyme to catalyse the oxidation of 1

µmol/min of 4-heptanol under the assay conditions (see the Supplemental Information for details).

Colourimetric enzyme screening using INT/diaphorase

In a spectrophotometric 96-well plate ketoreductase (0.1-0.6 mg/mL), diaphorase (0.1-0.6 mg/mL), NAD^+ (0.4 mg/mL) and $NADP^+$ (0.4 mg/mL) were re-hydrated with a solution of INT in deionised water (1 mg/mL, 190 µL). 10 µL of a DMSO solution of the respective hydroxysteroid (20 mg/mL stock solution of **2**, **6** or **9**) was added to commence the reaction. The plates were sealed and incubated in a Heidolph shaker at 30 °C, 750 rpm and they were visually inspected after 1h, 5h and 24h.

Biotransformation reactions (optimisation studies) - *General procedure:*

In a Multimax reactor vessel 5-AD (**5**, 0.3-3.0 g) was pre-dissolved in water immiscible co-solvent (5-25 mL, MTBE, 2-MeTHF, EtOAc). Buffer cocktail (10-25 mL) containing ketoreductase (1-3% w/w), glucose (1.1-2.0 equivalents), NAD^+ (7-10 mg) and $NADP^+$ (7-10 mg) and glucose dehydrogenase (CDX-901, 3-10 mg, 66 U/mg) in phosphate buffer (50 mM, 10-25 mL) was added and the reaction mixtures were stirred for 21h at 20-50 °C. The reaction pH (5.5-8.5) was maintained by autotitration with 2 M NaOH or 1.7 M sodium carbonate solution. The products were analysed by HPLC and NMR spectroscopy.

Chemistry

The initial reaction conditions were performed as described previously.[38] The optimised process was performed as described below.

Preparation of 5-androsten-3,17-dione (5)

Tert-butanol (2.00 L) was sparged with nitrogen for 90 min at 30-35°C. Potassium *tert*-butoxide (235 g, 2.09 mol) was added and the mixture was stirred with nitrogen sparging for a

further 90 min at 30-35 °C. 4-AD (**1**, 300 g, 1.00 mol) was added and the reaction was stirred for

a further 1h with nitrogen sparging. Separately, acetic acid (150 mL) was added to a 20 L vessel

containing water (6.00 L) that had been sparged with nitrogen for 24 h and the mixture was

stirred at 20 °C under nitrogen. The reaction mixture was then slowly added to the acetic acid

solution *via* cannula over 10-15 min. Once the addition was complete, the mixture was cooled to

12 °C and stirred for 1h. The precipitate obtained was filtered, washed with water (3 × 2.00 L)

and dried under vacuum at 40 °C. The product is obtained as a free flowing white powder (283 g,

94% yield), purity by HPLC: 93%, 4-AD (**1**) - 3%, oxygenated impurities (**19-23**) – 4%). ^{1}H

NMR (CDCl$_3$, 400 MHz) δ 5.38 (d, 1H), 3.28-3.33 (m, 1H), 2.86 (dd, 1H), 2.45-2.50 (2H), 1.05-

2.60 (m, 15H), 1.22 (s, 3H), 0.92 (s, 3H).

Preparation of dehydroepiandrosterone (2)

5-AD (**5**, 8.0 g, 27.9 mmol, 94% pure by HPLC, 2% 4-AD, 4% impurities **19-23**) was

dissolved in EtOAc (40 mL) at 33 °C under nitrogen atmosphere and stirred vigorously. To this

was added a solution of phosphate buffer (50 mM, 40 mL, pH 6.3) containing glucose (6.0 g,

33.3 mmol, 1.2 eq), NAD$^+$ (15 mg), glucose dehydrogenase (CDX-901, 8 mg, 528 U) and

ketoreductase powder (3rd generation, 20 mg, 0.25% w/w, 9 U) was added. The reaction

temperature was maintained at 32.5 °C (jacket temp 33 °C), while the pH 6.3-6.5 was kept

constant by addition of 1.5 M K$_2$CO$_3$ solution in water. The reaction conversion was monitored

by base consumption as well as by HPLC and ^{1}H NMR spectroscopy. After 6h the reaction

reached ~95% conversion. It was stirred for a total of 21 h to give >99% conversion. The

mixture was warmed up to 70 °C to fully dissolve partially precipitated DHEA and the phases

were separated. The separated organic layer was dried over magnesium sulfate and evaporated to

give crude DHEA as solid residue (7.2 g, 24.2 mmol, 90% yield). Purity by HPLC: 93% (5-AD

(**5**, 0.5%), 4-AD (**1**, 1.9%), 5-AD-diol (**6**, 0.5%)). ^{1}H NMR (CDCl$_3$, 400 mHz) δ 5.38 (d, 1 H), 3.50-3.58 (m, 1 H), 2.47 (dd, 1 H), 2.22-2.39 (m, 2H), 2.04-2.12 (m, 2H), 1.92-1.98 (m, 1 H), 1.83-1.89 (m, 3H), 1.44-1.71 (m, 6H), 1.22-1.29 (m, 2H), 0.97-1.14 (m, 2H), 1.04 (s, 3H), 0.89 (s, 3H).

Preparation of DHEA acetate (3) from 5-AD (5) on 200 g-scale (telescoped process)

5-AD (**5**, 235 g, 0.82 mol) was loaded into a 5 L reactor and sparged with nitrogen for 30 min upon gentle stirring. EtOAc (1.15 L) was added and the mixture was stirred at 33 °C under nitrogen until the substrate was fully dissolved. In a separate flask KH$_2$PO$_4$ (7.85 g) and glucose (162 g, 0.90 mol) were dissolved in 1.65 L of deionised water at 20 °C and the pH was adjusted to 6.3 using solid KOH (1.40 g, ≥85% assay). The solution was sparged with nitrogen for 2-5h. NAD$^+$ (0.20 g), glucose dehydrogenase (CDX-901, 165 mg, 10.9 kU) and ketoreductase cell free extract (2nd generation, 4.0 mL, 40 U) were added and stirred until fully dissolved. The buffer solution of enzymes was added to the vigorously stirred solution of 5-AD in EtOAc at 32.5 °C. The reaction was stirred at 32.5 °C and pH 6.3-6.5 was maintained by autotitration with 1.7 M Na$_2$CO$_3$ solution in water. The reaction conversion was monitored by base consumption as well as by ^{1}H NMR spectroscopy. After 4 h the reaction reached 43% conversion and additional portions of ketoreductase (3 mL, 30 U), NAD$^+$ (300 mg) and GDH (160 mg, 10.9 kU) were added; after 8 h more ketoreductase (4 mL, 40 U), NAD$^+$ (300 mg) and GDH (160 mg, 10.9 kU) were added. The mixture was stirred for 22 h and reached full conversion. The reaction mixture was warmed up to 55 °C to fully dissolve the partially precipitated DHEA and the phases were separated. The aqueous layer was extracted with EtOAc (1 L) at 55 °C. The combined organic layers were washed with brine (0.4 L), water (0.4 L) and passed through a magnesium sulfate plug, washed with additional EtOAc (0.4 L). The solvent was partially evaporated under vacuum

(100 mbar/ 50 °C water baths) to give 643 g of DHEA (**2**) in EtOAc, which solidified. Sample analysis indicated 36% potency by HPLC, equivalent to 0.80 mol, yield 97% (91% purity by HPLC, 4-AD – 7%, oxygenated impurities – 2%). This material was suspended in acetic acid (400 mL) and the residual EtOAc was removed under reduced pressure. Additional 320 mL of acetic acid were added (720 mL total, 3 vols), acetic anhydride (150 mL, 1.60 mol) and sodium acetate (80.0 g, 0.98 mol). The reaction was stirred at 60 °C for 16 h under a nitrogen atmosphere. Upon completion (by ^{1}H NMR) the reaction was cooled to 25 °C and a mixture of water (1125 mL) and methanol (125 mL) was added to the reaction mixture over 10 min and it was stirred for 1h. The precipitate was filtered and washed with further 3:1 water/methanol solution (1000 mL). The wet cake (320 g) was dissolved in hot methanol (1000 mL) and this solution was allowed to cool to 20 °C, then cooled further in an ice bath. The solid was collected and washed with additional methanol:water mixture (1:2, 300 mL). The solid was placed in a drying oven at 40 °C overnight to give DHEA acetate (**3**) as a white solid (171.4 g, 74% yield for 2 steps, and purity 99.5% by HPLC). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 5.41 (d, 1H), 4.57-3.65 (m, 1H), 2.46 (dd, 1H), 2.28-2.36 (m, 2H), 2.04 (s, 3H), 1.80-2.20 (m, 6H), 0.95-1.75 (m, 10), 1.05 (s, 3H), 0.89 (s, 3H).

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ASSOCIATED CONTENT

Supporting Information. Synthesis and characterisation of the main process impurities; structural assignment data for the oxygenated compounds **19-23**; experimental for molecular

biology and fermentation; enzyme activity assay and sample chromatograms. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* email: keholt.tiffin@drreddys.com

Notes

The authors declare no competing financial interest.

Present Addresses

¶ Department of Process Research, Merck Research Laboratories Merck & Co., Inc., P.O. Box 2000, Rahway, New Jersey 07065, USA

‡ AstraZeneca, 310 Cambridge Science Park, Milton Road, Cambridge CB4 0FZ, UK

‡‡ Zydus Cadila Ltd, Sarkhej-Bavla N. H. No. 8A, Moraiya Tal: Sanand, Dist: Ahmedabad-382210, India.

§§ CordenPharma International GmbH, Otto Hahn Strasse 1, 68723 Plankstadt, Germany

¶¶ Inogent Laboratories Private Ltd, 28A, IDA Nacharam, Hyderabad- 500 076, India

†† Almac Group, 25 Fretz Road, Souderton, PA 18964, USA

Author Contributions

The manuscript was written by Anna Fryszkowska through equal contributions of all authors. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

DHEA − dehydroepiandrosterone, 5-AD − androst-5-ene-3,17-dione, 4-AD − androst-4-ene-3,17-dione, HSD − hydroxysteroid dehydrogenase, KSI − ketosteroid isomerase, NAD^+ − nicotinamide adenine dinucleotide (oxidised form), NADH − nicotinamide adenine dinucleotide (reduced form), $NADP^+$− nicotinamide adenine dinucleotide phosphate (oxidised form), NADPH − nicotinamide adenine dinucleotide phosphate (reduced form), GDH − glucose dehydrogenase, IPTG − isopropyl β-D-1-thiogalactopyranoside.

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