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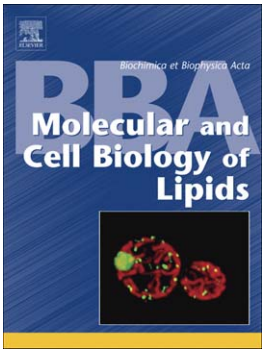

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Metabolism of steroidal lactones by the fungus *Corynespora cassiicola* CBS 161.60 results in a mechanistically unique intramolecular ring-D cyclization resulting in C-14 spiro-lactones

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Abstract

The fungus *Corynespora cassiicola* metabolises exogenous steroids in a unique and highly specific manner. Central to this, is the ability of this organism to functionalise substrates (androgens, progestogens) at the highly stereochemically hindered 8β-position of the steroid nucleus. A recent study has identified that 8β-hydroxylation occurs through inverted binding in a 9α-hydroxylase. In order to discern the metabolic fate of more symmetrical molecules, we have investigated the metabolism of a range of steroidal analogues functionalised with ring-D lactones, but differing in their functional group stereochemistry at carbon-3. Remarkably, the 3α-functionalised steroidal lactones underwent a mechanistically unique two step intramolecular cyclisation resulting in the generation of a ring-D spiro-carbolactone. This rapid rearrangement initiated with hydroxylation at carbon 14 followed by transesterification, resulting in ring contraction with formation of a butyrolactone at carbon-14. Remarkably this rearrangement was found to be highly dependent on the stereochemistry at carbon-3, with the β-analogues only undergoing 9α-hydroxylation. The implications of these findings and their mechanistic bases are discussed.

1. Introduction

Spiro-carbon containing compounds are formed in a range of different organisms that include bacteria, yeast, fungi, plants and insects [1-4]. These secondary metabolites are generated through polyketide, as well as dual shunt pathways [5]. They are structurally diverse and frequently have important biological activity [6-8]. Here we report for the first time, the generation of a 5-membered steroidal ring-D spiro-lactone, through a novel biocatalytic pathway by the fungus *Corynespora cassiicola*. This organism is unique amongst fungi, in that it can monohydroxylate androgens and progestogens at the highly hindered 8β-position on the steroid nucleus [9-11]. Mechanistically, the 8β-hydroxylation of androgens and progestogens occurs through inverted binding within an endogenous 9α-hydroxylase [9]. In contrast to many steroidal transformations by fungi [12] monohydroxylations observed to date with this organism predominate with axial stereochemistry, the precise position of attack being dependent on the type of steroid nucleus [9-11]. For example, androgens undergo axial attack both above and below the plane of the steroidal ring system. In contrast, progestogens must have C-17α functionality (alcohol or epoxide) to undergo enzymatic attack at the 8β-position.

In order to further reveal the factors determining steroid metabolism by this organism, we have investigated ring-D steroidal lactones which differ in functional group (alcohol, acetate) stereochemistry at C-3. Previous studies on the metabolic fate of these molecules by the filamentous fungus *Aspergillus tamarii* [13] demonstrated that these structural architectures can be hydroxylated in all four possible binding orientations within the steroid hydroxylase, with monohydroxylation occurring at both axial and equatorial positions. This flexibility of steroidal lactone/hydroxylase binding orientation is facilitated by increased symmetry imparted by having a six-membered ring-D. Structurally this results in the C-17

carbonyl group being transposed in to a more central position in the plane of the steroid [14] nucleus, as compared to steroids with a C-17 keto architecture.

The metabolism of the steroidal lactones by *Corynespora cassiicola* has revealed a unique rearrangement reaction (Fig.1). This is mechanistically (Fig.2) based on a two-step enzyme catalysed transformation resulting in C-14 steroidal spiro-lactones, the formation of which has highly specific stereochemical requirements.

2. Materials and methods

2.1 Chemicals and Reagents

Steroidal lactones **1**-**4** were synthesized [14] and analysed as previously described [13,15]. They were all found to be in excess 99% purity following elemental analysis, 3α-Acetoxy-17a-oxa-D-homo-5α-androstan-17-one **1** [found: C, 72.35; H, 9.36. $C_{21}H_{32}O_4$ requires C, 72.38; H, 9.26%]; 3α-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one **2** [found: C, 74.14; H, 10.04. $C_{19}H_{30}O_3$ requires C, 74.47; H, 9.87%]; 3β-Acetoxy-17a-oxa-D-homo-5α-androstan-17-one **3** [found: C, 72.38; H, 9.26. $C_{21}H_{32}O_4$ requires C, 71.92; H, 9.56%]; 3β-Hydroxy-17a-oxa-D-homo-5α-androstan-17-one **4** [found: C, 73.05, H, 10.22 $C_{19}H_{30}O_3$. 0.33.H_2O requires C73.02; H 9.9%]. All other chemicals and reagents were supplied by the Aldrich Chemical Company (UK). Solvents were of analytical grade; light petroleum ether refers to the fraction with a boiling point 60-80 ^oC. Silica for column chromatography was Merck 9385 and TLC was performed with Macherey-Nagel Alugram® SIL G/UV_{254}.

2.2 Microorganism

Corynespora cassiicola CBS 161.60 was obtained from the collection at the Centraalbureau voor Schimmelcultures (Ned). Stock cultures were grown on potato dextrose agar (Oxoid, UK) slopes (3 days) and maintained at 4^{o}C until use. Steroid transformation studies were carried out in 3% malt extract medium (Oxoid, UK).

2.3 Conditions of cultivation and transformation

Spores were transferred aseptically in a containment level 2 biological safety cabinet into 500 ml Erlenmeyer flasks containing 300 ml of sterile media and were incubated for 72 h at 25 ^{o}C. The cultures were shaken at 180 rpm on an orbital shaker. Aliquots (5 ml) from the seed flask were transferred aseptically to 5 flasks and grown for a further 72 h as above, at the end of which the fungus is in log phase growth. After this time period, steroid dissolved in dimethylformamide (DMF) was evenly distributed between the flasks (1mg/mL) under sterile conditions and incubated for a further 5 days after which the metabolites were extracted from the broth.

2.4 Extraction and separation of metabolites

The fungal mycelium was separated from the broth by filtration under vacuum. Following completion, the mycelium was rinsed with ethyl acetate (0.5 L) to ensure the entire available steroid was removed. The mycelial broth was then extracted twice with ethyl acetate (1.5 L). The organic extract was dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo* to give a gum. This gum was adsorbed onto silica and chromatographed on a column of silica; the steroidal metabolites were eluted with increasing concentrations of ethyl acetate in light petroleum ether. The solvent was collected in aliquots (10 ml) and analysed by thin layer chromatography (TLC) to identify the separated metabolite fractions. The solvent systems used for running the TLC plates were 50:50 light petroleum ether in

ethyl acetate or pure ethyl acetate. A 50:50 sulfuric acid in methanol spray was used to develop the TLC plates.

2.5 Analysis and identification of metabolites

Characteristic shift values [16, 17] in the ^{1}H and ^{13}C NMR spectra from the starting compounds were used to determine metabolite structure and used in combination with DEPT analysis to identify the nature of the carbon (Tables 1 and 2). Spectra were recorded on a B400 Bruker Advance III 400 MHz spectrometer, all samples were analysed in deuteriochloroform using tetramethylsilane as the internal standard. Infra-red spectra were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®.

2.6 *Time course experiment*

Experimental conditions were identical to those in section 2.3 except that steroids (600mg) dissolved in DMF (6 mL) was evenly distributed between 6 flasks (each containing 100 mL media) (**1**, **2**, **3, 4**). One flask was harvested after 12 h, then one every 24 h from the initiation of the experiment. These were extracted as in section 2.4. Following 6 h under high vacuum, the product ^{1}H NMR spectrum was determined in $CDCl_3$ to confirm the presence and steroidal nature of the extracts.

2.7 *Crystallographic structure determination of 3α,13α-dihydroxy-nor-15,16,17-5α-androst-14α-carbolactone* ***5***

$C_{19}H_{30}O_4$, M_r: 322.43, orthorhombic space group $P2_12_12_1$ (No.19), a = 6.0682(3), b = 16.4779(8), c = 19.0484(4) Å, $\alpha = \beta = \gamma = 90^{o}$, V =1735.06(14)Å^3, Z = 4, $D_{calc'}$ = 1.234 mg/m^3, u = 0.679mm^{-1}, F(000)704. Data were collected using a crystal of size 0.25 mm x 0.21 mm x 0.09 mm on a Bruker APEX CCD X-ray diffractometer by using graphite monochromated,

MoKα radiation (wavelength = 0.71073 Å). A total of 5084 reflections were collected for 3.70 < θ < 72.19° and -7 ≤ h ≤ 4, -20 ≤ k ≤ 12, -18 ≤ l ≤ 2. There were 5084 independent reflections and 2859 reflections with $I > 2\sigma(I)$ were used in the refinement. No absorption correction was applied. The structures were solved by direct methods and refined by full-matrix least-squares on all F_0^2 data using SHELXL-97. The diagram used ORTEP-3 for windows. The final *R* indices (all data) were [I > 2σ(I)] *R*1 = 0.035, w*R*2 = 0.09 and *R* indices (all data) *R*1 = 0.037, w*R*2 = 0.092. The goodness-of-fit on F^2 was 1.070 and the largest difference peak and hole was 0.21 and -0.20 eÅ^{-3}. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, CCDC 1532852 contains the supplementary crystallographic data for this paper for compound 5. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_ request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallo- graphic Data Centre, 12, Union Road, Cambridge CB2 1EZ, U.K. Fax: +44 1223 336033."

3. Results

3.1 Products isolated following transformation

*3.1.1 Transformation of 3α-acetoxy-17a-oxa-D-homo-5α-androstan-17-one **1***

Starting material **1** (127 mg, 8.5%) was recovered from the chromatography column with 30% ethyl acetate in light petroleum ether, comparison of the ^{1}H and ^{13}C NMR spectra to an authentic sample confirmed structural identity [15]. Elution with 55% ethyl acetate in light petroleum ether afforded a product of hydrolysis, 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one **2** (107 mg, 7%)**.** Comparison of this structure to the ^{1}H and ^{13}C NMR spectra of an authentic sample confirmed structural identity [15]. 3α,13α-dihydroxy-nor-15,16,17-5α-androst-14α-carbolactone **5** (353mg, 24%) was isolated from the chromatography column at a concentration of 60% ethyl acetate in light petroleum ether and was crystalized from ethyl

acetate in light petroleum ether as needles m.p. 243-246 ^{o}C (found: C, 70.12; H, 9.48 $C_{19}H_{30}O_4$ requires C, 70.76, H, 9.38 %); $IR_{\upsilon max}$ cm^{-1} = 3405 br, 1751; ^{1}H NMR ($CDCl_3$) 0.73 (3H, s, 19-H), 1.17 (3H, s, 18-H), 4.40 (1H, d, *J* =2 Hz, 3β-H)

*3.1.2 Transformation of 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one **2***

Starting material **2** (91 mg, 18%) was recovered from the chromatography column with 55% ethyl acetate in light petroleum ether, comparison of the ^{1}H and ^{13}C NMR spectra to an authentic sample confirmed structural identity [15]. Elution with 60% ethyl acetate in light petroleum ether afforded 3α,13α-dihydroxy-nor-15,16,17-5α-androst-14α-carbolactone **5** (130mg, 26%). Comparison of the ^{1}H and ^{13}C NMR spectra of this product with that of an authentic sample confirmed its' structure. 3α,9α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**6**) eluted from the chromatography column at 80% ethyl acetate in petroleum ether as a gum (HRMS ESI: Calc for M+Na^+: 345.203 obsd. 345.204); $IR_{\upsilon max}cm^{-1}$= 3398br and 1792; ^{1}H NMR ($CDCl_3$) 0.88 (3H, s, 19-H), 1.30 (3H, s, 18-H), 4.02 (1H, t, J = 2.7 Hz, 3β-H).

3.1.3 *Transformation of 3β-acetoxy-17a-oxa-D-homo-5α-androstan-17-one **3***

Starting material **3** (194 mg, 19%%) was recovered from the chromatography column with 30% ethyl acetate in light petroleum ether, comparison of the ^{1}H and ^{13}C NMR spectra to an authentic sample confirmed structural identity [13,14]. Elution with 45% ethyl acetate in petroleum ether afforded 9α-hydroxy-17a-oxa-D-homo-5α-androstan-3,17-dione **8** (214mg, 21%) which, crystalized from ethyl acetate in light petroleum ether as needles m.p. 216 ^{o}C with decomposition. (found: C, 70.89; H, 9.22 $C_{19}H_{28}O_4$ requires C, 71.22, H, 8.81 %); (HRMS ESI: Calc. for M+Na^+: 343.187 obsd. 343.187); $IR_{\upsilon max}$ cm^{-1} = 3411, 1703br; ^{1}H NMR ($CDCl_3$) 1.10 (3H, s, 19-H), 1.33 (3H, s, 18-H). 3β,9α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one **7** (396mg, 39%) eluted at a concentration of 65% ethyl acetate in petroleum

ether as a gum (HRMS ESI: Calc. for M+Na^+: 345.203 obsd. 345.204); $IR_{\upsilon max}cm^{-1}$= 3398br and 1792; ^1H NMR ($CDCl_3$) 0.90 (3H, s, 19-H), 1.31 (3H, s, 18-H), 3.60 (1H, tt, J = 5 Hz, J = 9 Hz, 3α-H).

*3.1.4 Transformation of 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one **4***

Starting material **4** (400 mg, 40%) was recovered from the chromatography column with 40% ethyl acetate in light petroleum ether, comparison of the ^1H and ^{13}C NMR spectra to an authentic sample confirmed structural identity [13,14]. Elution with 60% ethyl acetate in petroleum ether afforded 3β,9α-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one **8** (76mg, 8%) 7 which was identified by comparison of the spectral data to that of an authentic sample.

3.2 Identification of metabolites

Transformation of **1** yielded two products of transformation. Comparison of the ^1H NMR spectrum of the first product to that of the starting material demonstrated loss of the acetate signal at 2.06 ppm (3H, s) and an upfield shift for the 3β-proton, indicating that hydrolysis had taken place. This was further supported by the product ^{13}C NMR which was devoid of the C-20 (169.88 ppm) and C-21 (21.51 ppm) resonance signals. All of these product signals were consistent with that of an authentic sample of 3α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one **2.**

The second product of transformation was identified as 3α,13α-Dihydroxy-nor-15,16,17-5α-androst-14α-carbolactone **5**. This molecule, in comparison to the starting material, had undergone hydrolysis at C-3, with loss of all acetate signals *vide supra*. Comparison of the product **5** ^1H NMR spectrum to that of **2** revealed a significant shift (0.12 ppm) upfield for the 18-methyl resonance possibly suggesting loss of the ethereal oxygen present in the ring-D lactone of **2**. The ^{13}C NMR spectrum of the product further supported

loss of the 6-ring ethereal oxygen, this was demonstrated by a new upfield shift (10.18 ppm) in the ^{13}C NMR spectrum, resulting in a resonance value of 73.31 ppm. This was consistent with the presence of a hydroxyl group thus indicating opening of the ring-D lactone and formation of a new carbon skeleton. A new signal in the product spectrum at 93.17 ppm was consistent with the presence of a spiro-carbon [18] as was the downfield shift (4.63 ppm) for carbon 8. This suggested that a more thermodynamically stable 5-ring lactone had been formed; this was fully supported by the downfield shift of 6.04 ppm for the C-17 non-protonated carbon. Elemental analysis and X-ray crystallography fully supported the proposed structure of **5** (Fig. 3)

Two products of transformation were isolated following incubation of compound **2**. The first metabolite was identified as 3α,13α-dihydroxy-nor-15,16,17-5α-androst-14α-carbolactone **5** by comparison of its' ^{1}H and ^{13}C NMR spectra with that of an authentic sample. The second product isolated demonstrated a significant downfield shift in the ^{1}H NMR for the 19-methyl group of 0.13 ppm. Absence of any new signals in this spectrum suggested hydroxylation at a methyne proton such as C-5 or C-9 generating a non-protonated quaternary resonance. Lack of observed downfield shift for the 3β-proton indicated hydroxylation at the axial C-9 position of the steroid. This notion was further supported by comparison of the product ^{13}C NMR spectrum with that of the starting material where all β-carbons to C-9 had undergone downfield shifts C-8 (2.02 ppm), C-10 (4.53 ppm) and C-11 (4.71 ppm) and γ-carbons upfield shifts were observed for C-5 (8.11 ppm), C-7 (2.79 ppm) and C-14 (7.86 ppm), all of which supported the proposed structure of 3α,9α-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one **6** as did accurate mass measurement.

The first product of biotransformation following incubation of **3** was identified as 3β,9α-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one **7**. The ^{1}H NMR spectrum of this

compound demonstrated a downfield shift (0.1 ppm) for the 19-methyl signal in the absence of any new resonance signals, this suggested hydroxylation at C-5 or C-9. The 3α-H signal at 4.69 ppm (3H, tt) present in the starting material ^{1}H NMR spectrum had undergone an upfield shift (1.0 ppm) to 3.60 ppm (1H, tt). This coupled with the loss of the resonance signal at 2.02 ppm from the product ^{1}H NMR spectrum indicated that the acetate group had been hydrolysed, resulting in a 3β-hydroxyl group. This structural change was further confirmed by absence of the C-20 (171.60 ppm) and C-21 (21.45 ppm) from the product ^{13}C NMR spectrum. Hydroxylation at C-9 was confirmed with β-carbon downfield shifts for C-8 (2.07 ppm), C-10 (4.64 ppm) and C-11 (2.98 ppm) and γ-carbon upfield shifts for C-5 (8.08 ppm), C-12 (10.40 ppm) and C-14 (6.27 ppm) all of which were consistent with the proposed structure. This was also fully supported by elemental analysis and accurate mass measurement.

The second product of biotransformation was identified as 9α-hydroxy-17a-oxa-D-homo-5α-androstan-3,17-dione **8**. The ^{1}H NMR spectrum for this product did not contain resonance signals consistent with the presence of the 3α-H (1H,tt). This was confirmed in the product ^{13}C NMR resonance where the acetate signals at C-20 (171.60 ppm) and C-21 (21.45 ppm) and been replaced by a non-protonated carbon resonance signal at 211.80 ppm. Hydroxylation at carbon 9 was confirmed by β-carbon downfield shifts for C8 (2.02 ppm), C-10 (4.71 ppm) and C-11 (2.65 ppm) and γ-carbon upfield shifts for C-1 (2.11 ppm), C-5 (4.03 ppm), C-12 (10.52 ppm) and C-14 (8.10 ppm). Accurate mass measurement also fully supported the proposed structure.

Transformation of **4** resulted in the isolation of 3β,9α-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one **7**. The structure of this compound was confirmed by comparison to that of an authentic sample.

*3.3 Determination of metabolic pathway for compounds **1**, **2, 3** and **4***

^{1}H NMR was used to follow the steroidal transformation [9,15,19,20] and thus enable the determination of the time sequence to the formation of the individual metabolites isolated in this study.

Following 24 hours incubation of **1,** complete hydrolysis of the 3α-acetate group to the 3α-alcohol **2** occurred. This was demonstrated by an upfield shift in the 3β-proton resonance signal (1H, t) from 5.1 ppm in the starting material **1** to 4.06 ppm (1H, t) in **2**. Formation of the spiro-lactone **5** was first observed at the 48 hour time point. This was identified in the ^{1}H NMR spectrum with the presence of new methyl resonances at 0.73 ppm (19-H) and 1.18 ppm (18-H) respectively which are consistent with formation of the molecular structure of **5**.

Transformation of **2** resulted in formation of the spiro-lactone **5** as well as the 9α-hydroxy lactone **6**. Examination of the ^{1}H NMR spectrum at 48 hours revealed signals consistent with **5** (*vide supra*). The presence of compound **6** was identified by a downfield resonance signal, in comparison to the starting material, for the 19-methyl group at 0.88 ppm.

Transformation of **3** demonstrated key structural changes to the starting material at the 48 hr time point. Hydrolysis of the 3β-acetate group was confirmed by the presence of a new resonance signal for the 3α-proton at 3.60 ppm (1H, tt) and a new 19-methyl resonance at 0.90 ppm (3H, s) confirmed the formation of **7.** Formation of **8** was confirmed by the presence of the 19-methy resonance signal at 1.10 ppm and this was present at the 48 hour time point. Hydroxylation of **4** at carbon-9 was observed following 48 hours with the presence of a new 19-methyl signal at 0.90 ppm.

4. Discussion

Formation of the spiro-lactone **5** is the first example of a hydroxylase catalysed trans-esterification of a steroid in any organism. The carbon skeleton generated is novel and mechanistically insightful (Fig. 2 and Fig. 3), especially as spiro-carbons are a rare metabolic phenomenon within xenobiotic pathways [21,22]. The unique bimodal base catalysed transformation initiates after 48 hours incubation with hydroxylation at C-14 alpha, a position on the steroid nucleus at the trans C/D ring junction (Fig. 2, Intermediates A-D). Opening of the ring-D lactone in the substrate presumably occurred through the activity of a lactonohydrolase, a transformation previously reported on exogenous steroids by fungi [23]. Ring opening of the lactone facilitates rearrangement through transesterification (Fig. 2, Intermediates C and D) resulting in the formation of the spiro-butyrolactone **5**.

The *in situ* generation of compound **5** was supported by the timed experiments where samples for ^{1}H NMR did not undergo column chromatography. The spectra of these samples contained the 19-methyl resonance (0.73 ppm) which was consistent with the rearrangement of **5**.

Furthermore, from these experiments there was no spectroscopic evidence for 18-methyl downfield shift values of 0.1ppm that would have been consistent with the presence of the 14α-hydroxylation product, with the 6-membered ring-D lactone in either open or closed constitution [23,24]. It is of note that some species of *Corynespora cassiicola* do produce macrolactone structures such as octa and deca-lactones [25-27], but in contrast to our reported mechanism, these lactones are polyketide derived secondary metabolites.

The mechanistic basis for the biosynthetic formation of austinol, a fungal meroterpenoid from *Aspergillus nidulans*, which contains spiro-ring formation was recently reported [28]. The mechanism leading to the spiro-carbon in this secondary metabolite had distinct similarities to our observed biosynthetic transformation. The rearrangement initiated through hydroxylation (albeit at a β-proton) followed by ring contraction (7 to 6 membered

ring), but differed in the final step, which required dehydrogenation resulting in double bond formation to the spiro-ring. It is of note that an alternative double bond participated radical rearrangement has been proposed [29].

In the case of formation of the spiro-ring in **5**, one would expect little difference in the thermodynamic stability between the 5- and 6-ring lactones, with the potential for rapid interconversion between them being supported by X-ray crystallography (where C-13 hydroxyl is 2.948 Å from the five membered ring lactone, Fig. 3). In spite of this, only the five membered spiro-ring-D structure was observed. The prevalence of this may in part be due to the favoured twist chair conformation of the six membered lactone (Fig. 4a) and the favourable kinetic preorganization (Fig. 4b) it affords formation of the five membered lactone. This may, in part, explain the observed cyclisation to the 5-ring lactone **5** in comparison to the 6-membered counterpart [30,31].

Hydroxylation at C-14 and subsequent rearrangement of the lactone demonstrated a strict stereospecific requirement for the presence of a 3α-hydroxyl group. In contrast, the 3β-hydroxylactones only underwent monohydroxylation at the C-9α position. This suggests that the 3β-hydroxyl group inhibits attack at C-14. This could be due to the equatorial stereochemistry of this functional group physically extending the distance of the steroid 14α-proton away from active site, resulting in the 9α-proton being positionally favoured for attack. In light of this, it could reasonably be argued that a single enzyme handles the respective 3α- and 3β-hydroxy steroidal lactones, as both structures underwent hydroxylation at carbon-9, but only the alpha alcohols underwent rearrangement. This would be consistent with previous studies that have indicated a high degree of structural flexibility, with both androgens and progestogens being hydroxylated at C-9α by this organism [9]. Only future studies with isolation and structural elucidation of the responsible hydroxylase(s) will provide a definitive answer. 9α-Hydroxylation is a pharmaceutically important transformation [32] which

facilitates intermediate compounds for 9α-halogenated corticosteroids [33] and 9,11-dehydro systems for 11-keto steroids [34]. The novel spiro-lactone structure may also be of pharmaceutical interest, as drugs with ring-D lactones for example, spironolactone and eplerenone are already in clinical use for treatment of cardiac disease [35,36] and potentially new applications in conditions such as HIV are on the horizon [37].

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Table 1 ^{13}C NMR data for starting materials determined in $CDCl_3$

	Compounds			
	1	**2**	**3**	**4**
Carbon atom				
1	32.62	31.94	36.48	36.73
2	25.95	28.92	27.26	31.20
3	69.77	66.29	73.34	71.08
4	32.58	35.55	33.66	37.68
5	39.40	38.47	43.96	44.17
6	27.92	28.12	28.11	28.25
7	30.42	30.52	30.47	30.58
8	37.85	37.89	37.81	37.88
9	52.93	53.07	52.89	53.05
10	35.06	36.08	35.46	35.48
11	19.73	19.75	21.49	22.02
12	28.64	28.68	39.20	39.27
13	82.66	83.49	83.35	83.50
14	46.34	46.33	46.15	46.25
15	21.58	21.59	19.75	19.76
16	39.30	39.32	28.62	28.62
17	170.84	171.67	170.68	171.85
18	20.11	20.16	20.13	20.12
19	11.19	11.05	12.04	12.15
20	169.88		171.60	
21	21.51		21.45	

Data for compounds **1** and **2** from references [13] and [15] respectively

Table 2 ^{13}C NMR data for transformation products

	Compounds			
	5	**6**	**7**	**8**
Carbon atom				
1	31.86	34.44	35.88	34.37
2	28.60	28.27	29.69	37.70
3	66.09	66.04	70.62	211.80
4	35.45	35.76	34.37	44.36
5	36.98	30.36	35.88	39.93
6	27.86	27.92	28.02	28.22
7	29.88	27.73	31.05	31.36
8	42.48	39.91	39.88	39.83
9	46.63	74.55	74.45	74.31
10	37.87	40.61	40.10	40.17
11	21.65	24.46	24.47	24.14
12	25.91	28.71	28.72	28.68
13	73.31	83.48	83.33	83.07
14	93.17	38.47	39.88	38.05
15	24.41	19.56	19.62	19.64
16	35,91	28.45	28.14	28.34
17	177.71	171.76	171.67	171.45
18	21.20	19.41	19.36	19.36
19	10.78	13.25	14.28	13.44

Figure 1

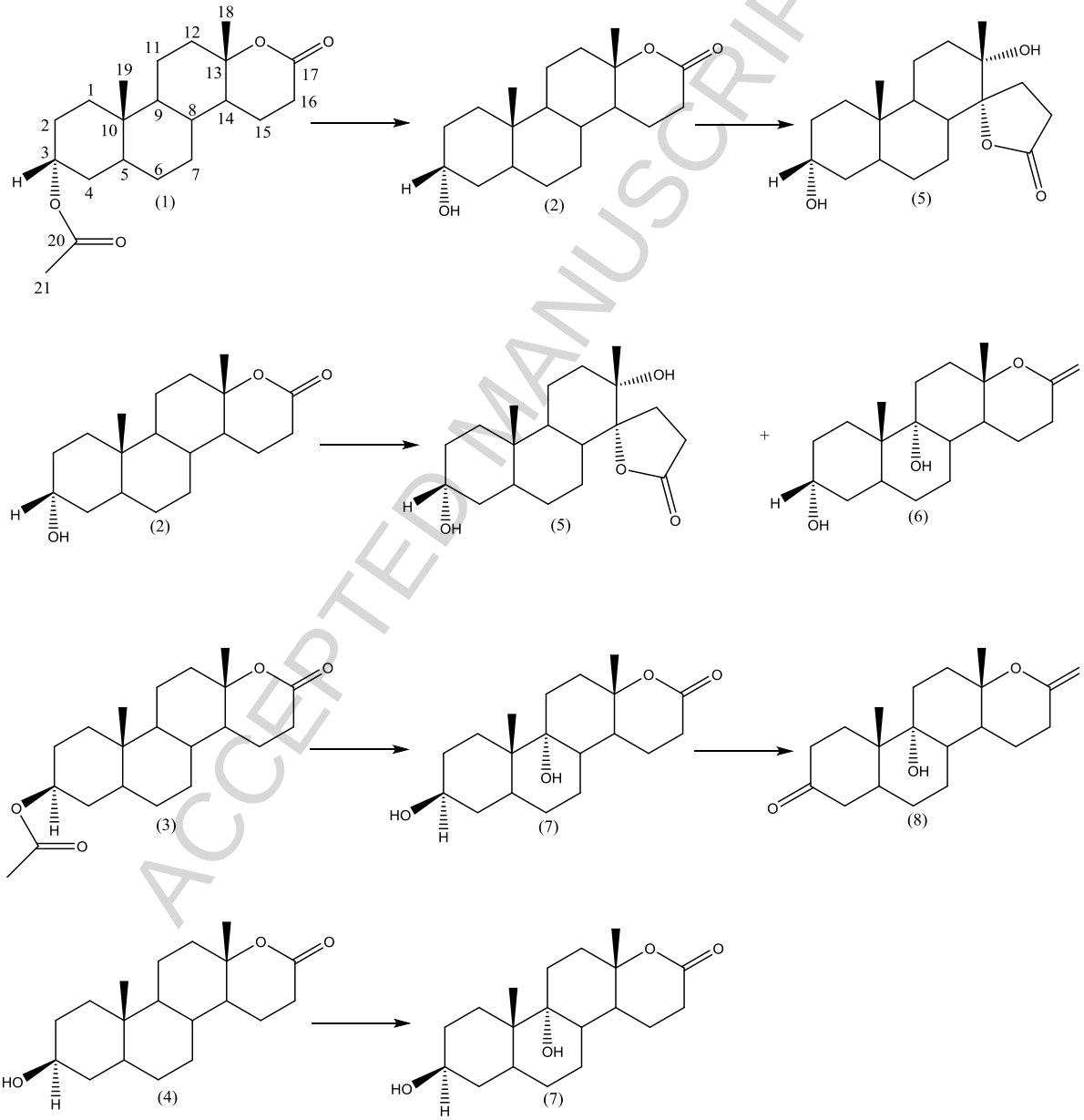


Figure 2.

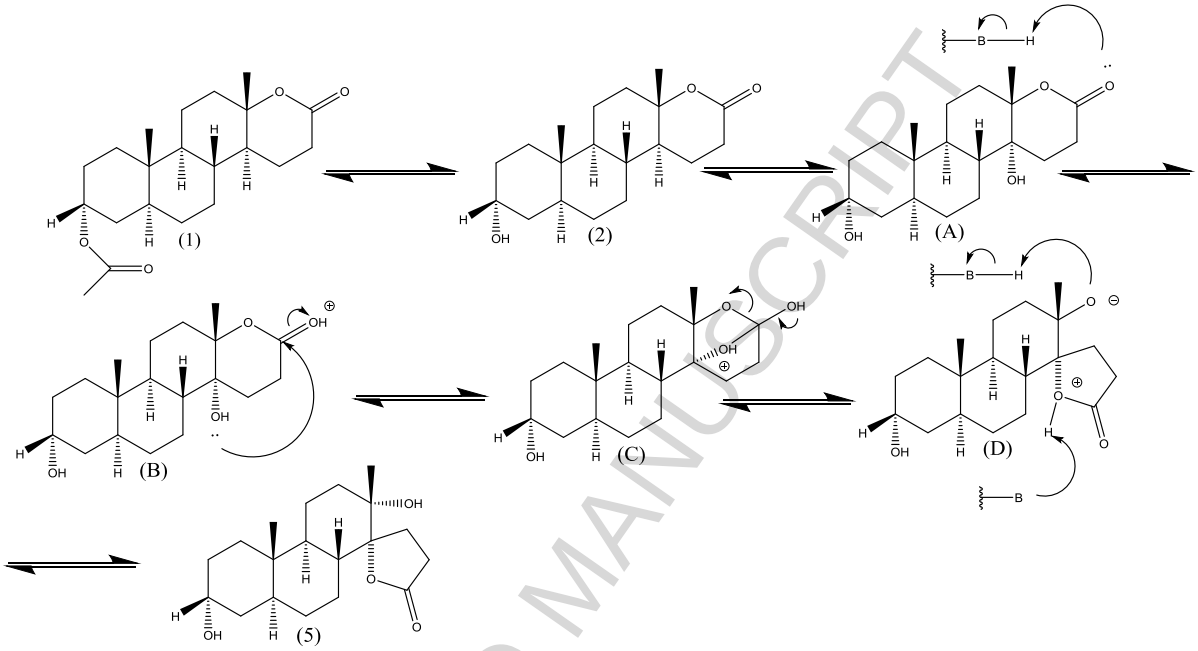


Figure 3.

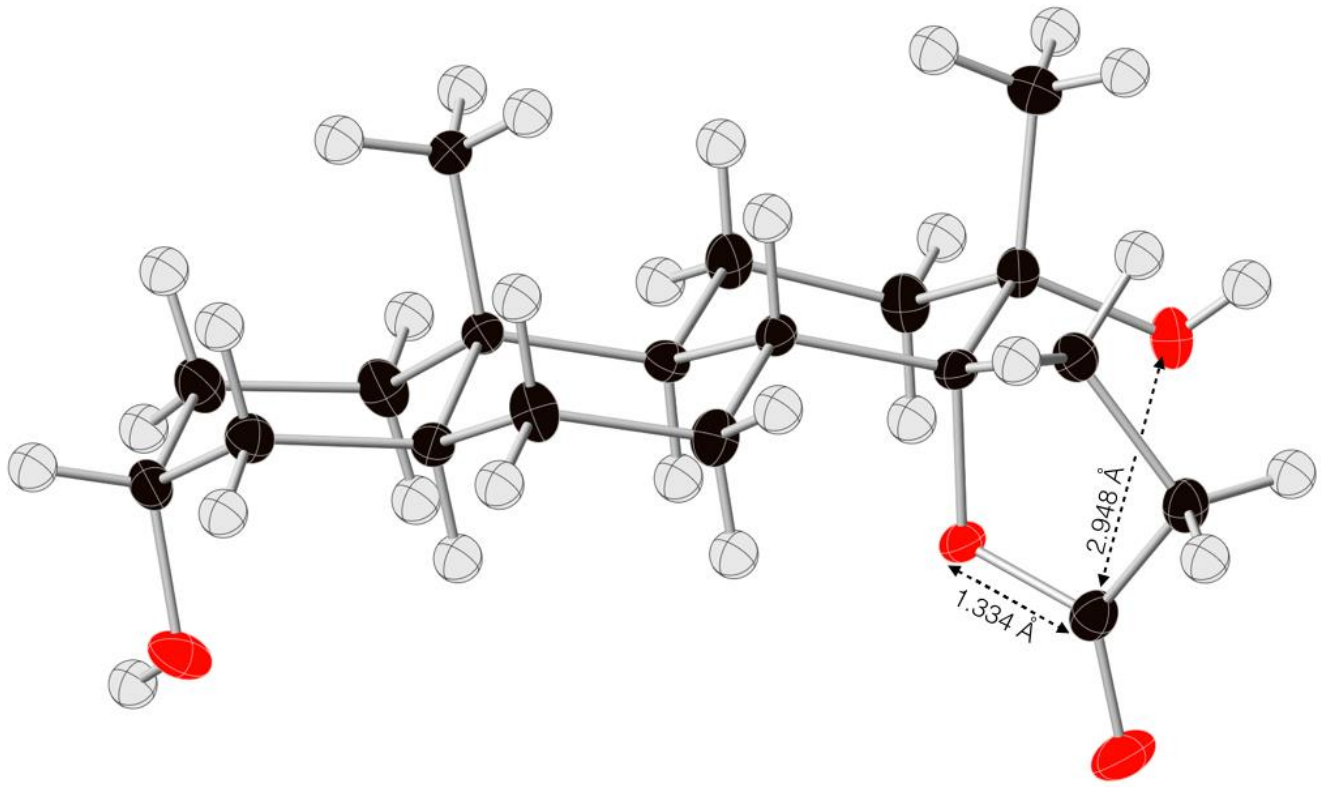


Figure 4

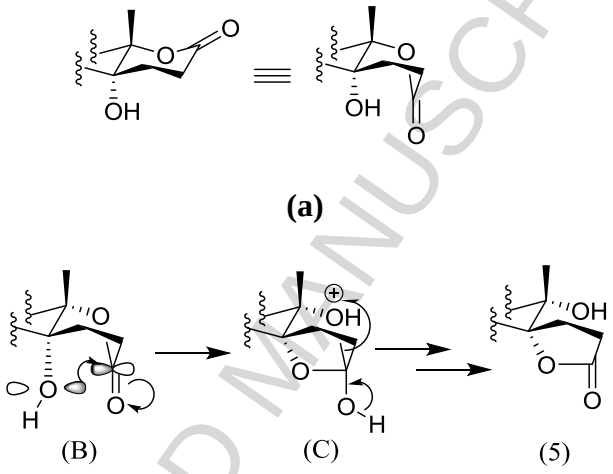


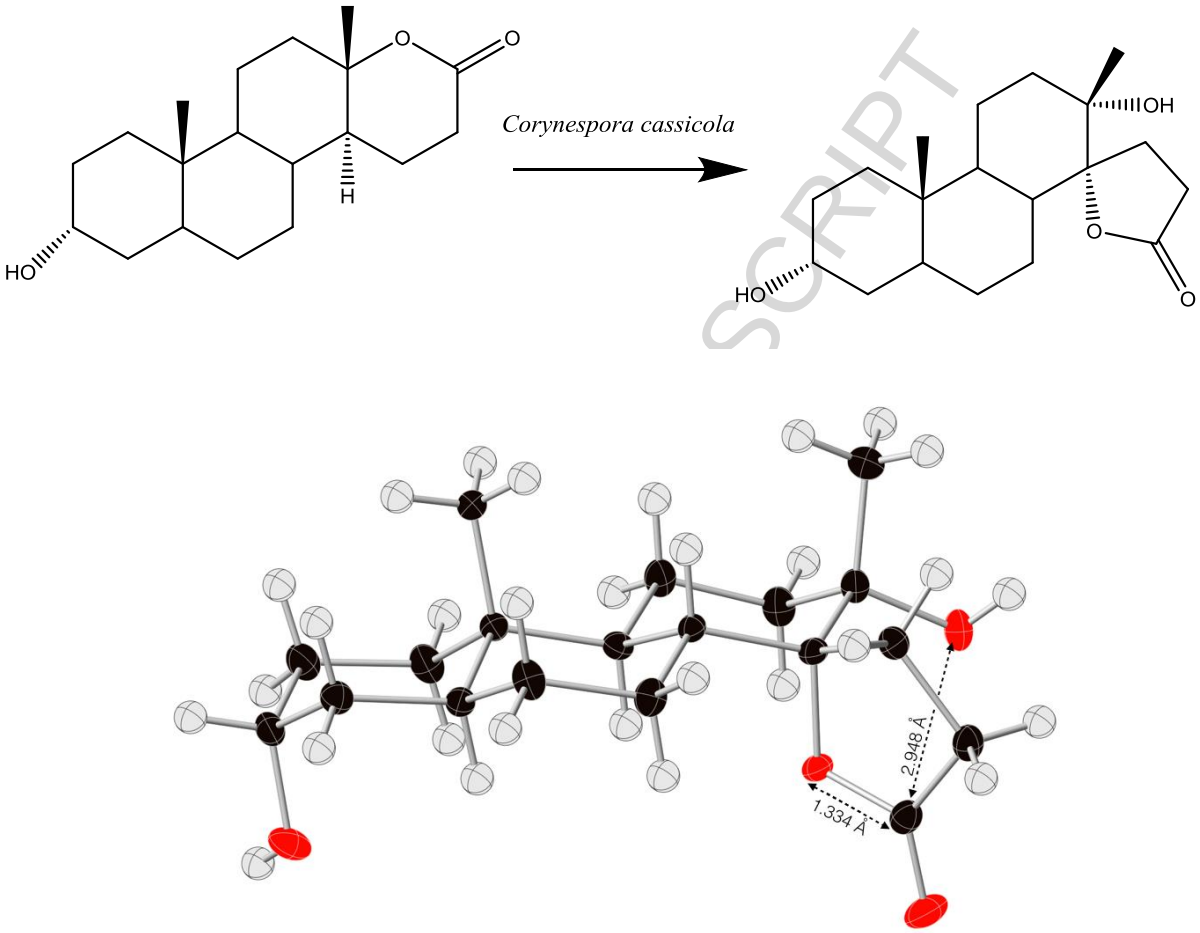
Figure legends

Figure 1. Transformation of a series of 3α- (**1** and 2**)** and 3β- (**3** and **4)** functionalized steroidal lactones and their products (**2**,**5**,**6**,**7**,**8**) following 5 days incubation with *Corynespora cassiicola*.

Fi**gure 2**. Acid catalysed mechanistic pathway for transesterification mechanism underlying the formation of **5.**

Figure 3. Single X-ray crystal structure of 3α,13α-dihydroxy-nor-15,16,17-5α-androst-14α-carbolactone **5** with proximity of oxygens attached to ring-D keto-carbon.

Figure 4. (a) Structural comparison of chair conformation and energetically favoured twist chair conformation. (b) Kinetic preorganization promoting formation of the 5-membered lactone **5**.



Graphical Abstract

Highlights of study

-Novel enzyme driven rearrangement resulting in unique spiro-lactone architecture
-Spiro-lactone formation based on rapid two step mechanism
-Rearrangement based on C-14 hydroxylation followed by transesterification
-Strict stereochemical requirements of C-3 functionality identified for rearrangement