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Hydroxyl groups at C-3 and at C-17 of the unnatural enantiomer, *ent*-androsta-5,9(11)-diene-3β,17β-diol are oxidised by cholesterol oxidase from *Rhodococcus erythropolis*

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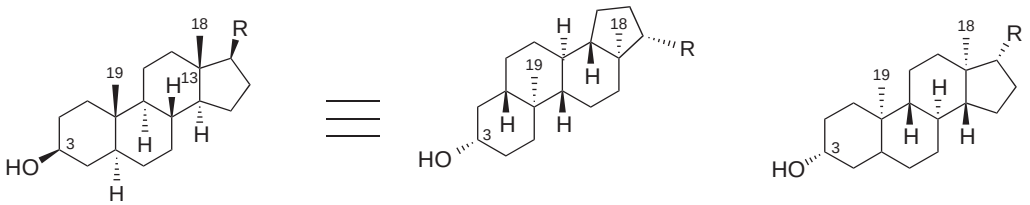
Abstract—The *ent*-androsta-4,9(11)-diene-3β,17β-diol **1b** and *ent*-androsta-5,9(11)-diene-3β,17β-diol **2b** prepared from chiral dione **3**, were oxidised by cholesterol oxidase with kinetic parameters close to those of the natural steroids **1a** and **2a**. In the preparative oxidation the final product was *ent*-androsta-4,9(11)-diene-3,17-dione **5**. So the enzyme catalysed the oxidation of the hydroxyl groups at C-3 and C-17, whereas the natural enantiomers were only oxidised at C-3.

Cholesterol oxidase (EC 1.1.3.6) catalyses the oxidation of cholesterol into cholest-5-en-3-one and the isomerisation of cholest-5-en-3-one to cholest-4-en-3-one.[1] Previously, we found that the enantioselectivity of the oxidation of bi- and tricyclic alcohols with cholesterol oxidase from *Rhodococcus erythropolis*,[2–4] was lower with the tricyclic than with the bicyclic alcohols.[5] We then realised that the enantiomeric steroids have more similarities than may be seen on first examination. If the structure of the natural steroid turned by 180° around an axis joining C-3 to C-13 ('upside-down') is compared to the structure of the *ent*-steroid, the hydroxyl group at C-3, rings A and C and the methyl groups at position C-18 and C-19 of both enantiomers are superimposed. The enantiomers differ by the position of rings B and D as shown in Fig. 1. Due to this partial symmetry, cholesterol oxidase may not be enantiospecific and could oxidise *ent*-steroids.

The 'natural' enantiomers **1a** and **2a** were prepared from cortisol acetate.[6] We also prepared *ent*-androsta-4,9(11)-diene-3β,17β-diol **1b** and *ent*-androsta-5,9(11)-diene-3β,17β-diol **2b** from chiral dione **3** (Scheme 1).[7–10] To improve yields we modified the reaction conditions of the published synthesis. The kinetic parameters (k_{cat}, K_m) for cholesterol oxidase were determined by a modification of the method of Smith and Brooks.[2,11] Androsta-4-ene-3β,17β-diol and androsta-5-ene-3β,17β-diol were used as references.



3β-hydroxy-sterol

ent-3β-hydroxy-sterol

Figure 1. Structures of the enantiomers of 3β-hydroxy-sterol.

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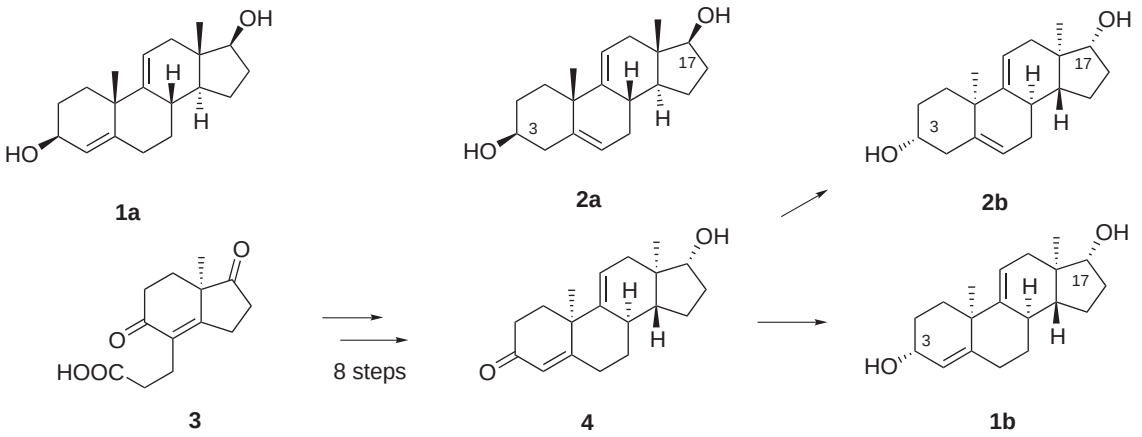

Scheme 1.

The first order rate constant for this process ($k = 0.00684$ min^{-1}; $t_{1/2} = 101$ min) as determined from the The unnatural enantiomers **1b** and **2b** were oxidised with rate constants close to those of the corresponding natural enantiomers **1a** and **2a** (Table 1). The enzyme was found to catalyse not only the oxidation at the hydroxyl group at C-3 of products **1b** and **2b**, but also the isomerisation of the double bond of compound **2b** as shown by the formation of the conjugated ketone, which is used to measure the enzymatic rate.

In the preparative oxidation in biphasic system,[5] the final reaction product from **1b** and **2b** was not the expected *ent*-dehydrotestosterone **4**, but *ent*-androsta-4,9(11)-diene-3,17-dione **5** (Scheme 2).[12] By chromatography we detected the *ent*-dehydrotestosterone **4** as an intermediate.

The oxidation rate of the hydroxyl group at C-17 must be significant, but slower than the oxidation at C-3 and the double bond isomerisation.

Another interesting feature is the isomerisation of the β,γ-enone to the α,β-enone on unnatural enantiomer **2b**. In cholesterol oxidase from *Brevibacterium sterolicum*,[13,14] the terminal carboxylate of Glu^{361}, the base involved in the isomerisation, is positioned over the β-face of the bound sterol and is quite mobile.[15] In the cholesterol oxidase used here, the base involved in the isomerisation, likely positioned over the β-face of the bound substrate, is also quite mobile, since both enantiomers of 3-keto-5-ene were isomerised to the corresponding 3-keto-4-ene. The mechanism of the isomerisation of the enantiomers remains to be studied.

The unnatural enantiomers **4**, **1b** and **2b** may bind again to the active site of cholesterol oxidase in the 'upside down' and 'backward' mode to account for the oxidation of the hydroxyl group at C-17. In contrast, the natural enantiomers **1a** and **2a** were thus not bound to the active site in the 'backward' mode. The resolution of the racemic steroidal diol with cholesterol oxidase could be performed owing to the enantiospecificity of the oxidation at C-17 but not at C-3. Other structures related to natural steroids might be substrates of cholesterol oxidase. The enantiospecificity should be determined for other enzymes of the steroid metabolism: such as 5-ene-3-keto steroid isomerase, hydroxysteroid dehydrogenase and hydroxysteroid sulfotransferase. These enzymes are neither regio- nor stereospecific due to the 'wrong-way' binding mode, i.e. the 'backward' and/or 'upside-down' mode.[16,17] *ent*-Steroids may act as inhibitors for some of these enzymes.

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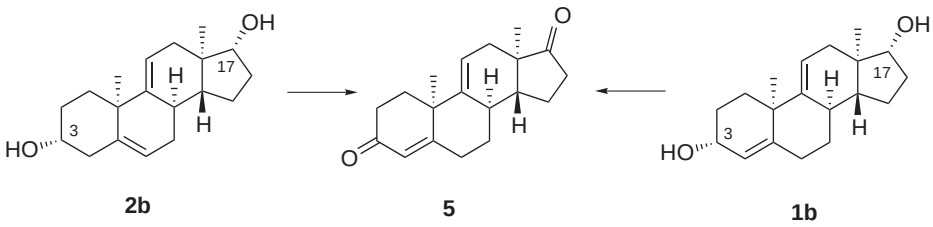
Table 1. Comparison of kinetic parameters of the natural and unnatural enantiomers of androstenediols for cholesterol oxidase from *Rhodococcus erythropolis*

Compounds (range of concentrations used, μM)	K_m (μM)	k_{cat} (μmol min^{-1} mg^{-1})	k_{cat}/K_m ($\times 10^3$ L min^{-1} mg^{-1})
Androsta-4-ene-3β,17β-diol (18–190)	66	0.37	5.6
Androsta-4,9(11)-diene-3β,17β-diol (**1a**) (49–396)	383	0.32	0.8
ent-Androsta-4,9(11)-diene-3β,17β-diol (**1b**) (136–820)	525	0.20	0.4
Androsta-5-ene-3β,17β-diol (3.7–158)	6.5	0.05	7.5
Androsta-5,9(11)-diene-3β,17β-diol (**2a**) (11–125)	36	0.04	1.1
ent-Androsta-5,9(11)-diene-3β,17β-diol (**2b**) (21–116)	52	0.03	0.6



2b **5** **1b**

Scheme 2.

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