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A simple two-step method for the conversion of [^{3}H]cortisol to [^{3}H]-11-ketotestosterone

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Despite the existence of several protocols, problems appear to persist in the small scale chemical synthesis of radiolabeled 11-ketotestosterone from cortisol. We investigated the possibilities of using the mild oxidant pyridinium dichromate for the oxidative cleavage of the dihydroxyacetone side chain of cortisol and 17β-hydroxysteroid dehydrogenase for the subsequent reduction of the resulting 17-keto group. Our protocol has resulted in consistently high yields of both the intermediate, adrenosterone (70–80%), and the product, 11-ketotestosterone (up to 60%). This, taken together with the convenience and relatively low cost of our method, recommends the protocol for its use for the synthesis of [^{3}H]-11-ketotestosterone for endocrine studies. (*Steroids* **62**:655–658, 1997) © *1997 by Elsevier Science Inc.*

Keywords: radiolabeled steroid; 11-ketotestosterone; adrenosterone; pyridinium dichromate; 17β-hydroxysteroid dehydrogenase; oxidative cleavage

Introduction

11-Ketotestosterone (11-KT, 17β-hydroxyandrost-4-ene-3,11-dione) is an androgen that plays an important role in spermatogenesis in male teleost fish.[1] This steroid hormone was also identified in the blood of female salmonid[2,3] and anguillid[4] fishes, but its function in these females remains unknown.

To perform radioimmunoassay and radioreceptor assays for 11-ketotestosterone, a supply of the radiolabeled hormone (such as [^{3}H]-11-KT) is required. However, commercially available [^{3}H]-11-KT is very expensive and available only in quantities that greatly exceed the requirements for most, if not all, laboratories. As a result, several methods have been developed for the in-house preparation of radiolabeled 11-KT which use sodium bismuthate for the oxidation of [^{3}H]cortisone (17,21-dihydroxypregn-4-ene-3,11,20-trione) to [^{3}H]adrenosterone (androst-4-ene-3,11,17-trione) and metal hydrides for the subsequent reduction to [^{3}H]-11-KT.[5–7] Inconsistent and uneconomical yields for these procedures, together with reduced routine availability of [^{3}H]cortisone, prompted Truscott[8] to modify the methodology for 11-KT synthesis, using tritiated cortisol (11β,17,21-trihydroxypregn-4-ene-3,20-dione) as a precursor (Scheme 1).

In the first step, the 11β-hydroxy group of cortisol is oxidized with CrO_3 to cortisone which is then reacted with 20β-hydroxysteroid dehydrogenase to produce 17,20β-21-trihydroxypregn-4-ene-3,11-dione. Oxidation of this intermediate with periodic acid cleaves the C-17–20 side chain, producing adrenosterone, which can be selectively reduced to 11-KT by 17β-hydroxysteroid dehydrogenase (17β-HSD) in the final step. Truscott's method is reported to consistently yield around 40% product. However, it requires three steps for the synthesis of the key intermediate adrenosterone (Scheme 1), thus making it very time-consuming with the potential for high product losses associated with adsorption on glassware and general procedural losses.

We therefore aimed to facilitate the protocol for the synthesis of [^{3}H]-11-KT by reducing the number of steps necessary for the conversion of cortisol to adrenosterone. This conversion requires cleavage of the hydroxyketone bond between C-17 and C-20 (see Scheme 1) for which several methods have been developed, including manganese dioxide,[9] sodium bismuthate,[5–7] calcium hypochlorite,[10] chromium trioxide,[11,12] lead tetraacetate, periodic acid,[13] and Jones reagent (containing $Cr(VI)O_3$[14]). These reagents have all been used with varying degrees of success for the

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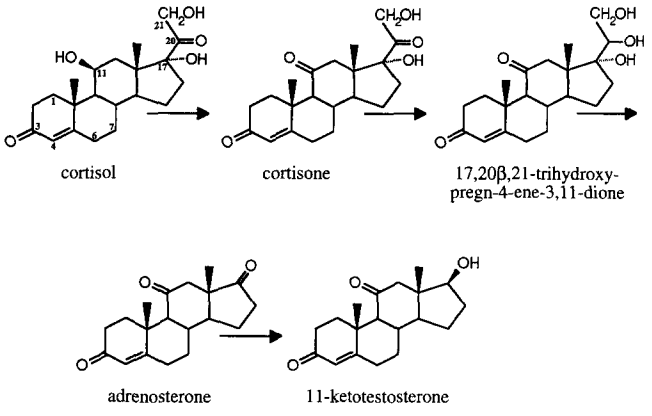
Parts of this study have been presented at the Third International Symposium on Fish Endocrinology, Hakodate, Japan, May 1996.
Received January 6, 1997; accepted May 21, 1997.

655 Avenue of the Americas, New York, NY 10010

0039-128X/97/$17.00
PII S0039-128X(97)00052-4



Scheme 1 Chemical synthesis of 11-ketotestosterone from cortisol, according to Truscott.[8] Numbering of the steroid skeleton is indicated for relevant carbons.

oxidative cleavage of α-hydroxyketones[15] and 17,20- or 17,20,21-oxygenated pregnanes (for a review see Ref. 13).

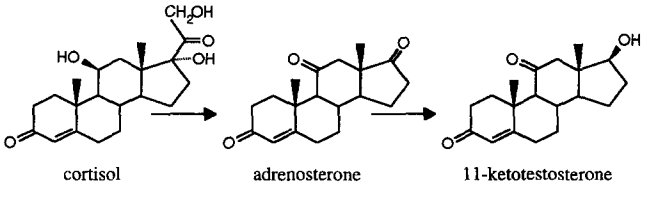
Among these reagents, CrO_3 most readily cleaves the C-17–20 bond, but it will also oxidize other alcohols and may react with the double bond (C-4–5[13]). In contrast, oxochromium–amine complexes, such as pyridinium dichromate (PDC; $Cr(VI)O_3$), are less acidic and more selective than either CrO_3 or Jones reagent.[16] Furthermore, PDC can be used under anhydrous conditions, allowing for mild oxidation,[17] and it has been successfully applied as a catalyst in the oxidative cleavage of α-hydroxyketones by sodium percarbonate and tetraalkylammonium chloride.[18]

Our paper describes the application of PDC to the conversion of cortisol to adrenosterone which, when combined with a subsequent enzymatic reduction step with 17β-HSD (see Scheme 2), constitutes a simple procedure for the conversion of [^{3}H]cortisol to [^{3}H]-11-KT.

Experimental

Chemicals

[1,2,6,7-^{3}H]Cortisol (specific activity 57 Ci/mmol; Amersham), dichloromethane (CH_2Cl_2), diethyl ether (Et_2O; BDH Laboratory Supplies, Poole, UK), silica gel (Merck kieselgel 60, Darmstadt, Germany), silica gel precoated thin layer chromatography (TLC) plates (Whatman, Maidstone, UK), 17β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*, β-NADH, and standards (cortisol, adrenosterone, and 11-KT; all from Sigma Chemical Co., St. Louis, Missouri) were purchased from the sources indicated. Pyridinium dichromate was either obtained commercially (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or produced according to the method of Corey and Schmidt.[17]



Scheme 2 Chemical synthesis of 11-ketotestosterone from cortisone, using PDC as the oxidizing agent.

Product identification

The identity of tritiated or radioinert products was confirmed by ^{1}H NMR, electrospray mass spectrometry (ESMS), or radioimmunoassay. Samples for NMR were prepared in deuterated chloroform ($CDCl_3$; Sigma) and referenced to the residual $CHCl_3$ signal at 7.24 ppm. The spectra were obtained using a JEOL GX 270 spectrometer or a Varian VXRS300 spectrometer operating at 270 and 299.909 MHz, respectively. Methanol, containing trifluoroacetic acid, was used as the solvent for ESMS using a VG Platform II instrument, according to the method of Henderson et al.[19] Radioimmunoassay was performed according to the method from Kagawa et al.[20] The antiserum (lot 561-2) for this assay was kindly donated by Professor Y. Nagahama, National Institute for Basic Biology, Okazaki, Japan.

Results

Conversion of cortisol to adrenosterone

Cortisol (25.1 mg) was dissolved in anhydrous CH_2Cl_2 (25 mL). Subsequently, PDC (267.9 mg) was added, and the mixture was stirred at room temperature under nitrogen for 40–44 h. The reaction was quenched with Et_2O (60 mL), and the solution was filtered through a silica gel column. Removal of the solvent resulted in a pure solid (20.8 mg) in 91% yield (18.9 mg), which gave one spot on TLC using CH_2Cl_2/methanol/distilled water (99:10:1; System I). The NMR and electrospray mass spectra were identical with that of an authentic sample of adrenosterone. δ_H ($CDCl_3$) 0.89 (s, 3H, $18CH_3$) 1.45 (s, 3H, $19CH_3$) 5.76 (s, 1H, 4H). ESMS MH^+ 301.5.

Conversion of [^{3}H]cortisol to [^{3}H]adrenosterone

The above-mentioned protocol for the synthesis of adrenosterone from cortisol was modified for trace quantities, as follows: 10–50 μCi of [1,2,6,7-^{3}H]cortisol were transferred to a large scintillation vial (20 mL), and the solvent was removed under a stream of nitrogen gas. Redistilled CH_2Cl_2 (5 mL) and PDC (54 mg) were added, and the solution was stirred at room temperature for 40–44 h, resulting in a brown-black mixture. To terminate the oxidation, distilled water (75 μL) was added and after 10 min of further stirring, a clear orange solution was produced containing black-brown precipitates. The oxidant residues and the product were separated by washing with distilled water (3–5 mL) for 20 s and removal of the water phase with a Pasteur pipette. This procedure was repeated until no more Cr(III) could be extracted from the CH_2Cl_2 phase, resulting in a light straw-colored solution. Finally, the flocculent particles at the interface between the phases were removed with a Pasteur pipette. Any CH_2Cl_2 drawn up in the pipette was returned to the bulk solution. The solvent was then evaporated under vacuum and [^{3}H]adrenosterone isolated by thin layer chromatography on silica gel using 5:2 CH_2Cl_2/Et_2O as the solvent (System II).

Following chromatography, the sample lane was divided into 1-cm bands, and radioactivity was located by liquid scintillation counting of 2–3 punches of silica (approximate-

ly 2 mm in diameter) collected with a Pasteur pipette. The retention time of the radioactive peak coincided approximately with that of a radioinert standard on an adjacent lane visualized under ultraviolet light (Figure 1a). Appropriate silica bands were identified and the silica was scraped off the plate. After addition of a small amount of distilled water, the [^{3}H]adrenosterone was extracted from the silica with Et_2O.

Conversion of adrenosterone to 11-ketotestosterone

Based on Truscott's method,[8] an enzymatic reduction of adrenosterone to 11-KT was carried out, using 17β-HSD. For this purpose, adrenosterone (2 mg) was dissolved in glycol (10 μL) and incubated in the dark at room temperature with 1 unit of 17β-HSD and β-NADH (5 mg) in 3 mL of 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.4). After 1 h, the steroids were extracted with Et_2O or CH_2Cl_2 and chromatographed by TLC using System II. The 11-KT band was identified under ultraviolet light and isolated from the silica gel plates as described above. The yield of 11-KT was approximately 300 μg or 15%. The NMR and electrospray mass spectra were identical with that of an authentic sample of 11-KT. $\delta_H(CDCl_3)$, 0.74 (s, 3H, $18CH_3$) 1.41 (s, 3H, $19CH_3$) 3.85 (t, 1H, 17αH) 5.70 (s, 1H, 4H). ESMS MH^+ 303.6.

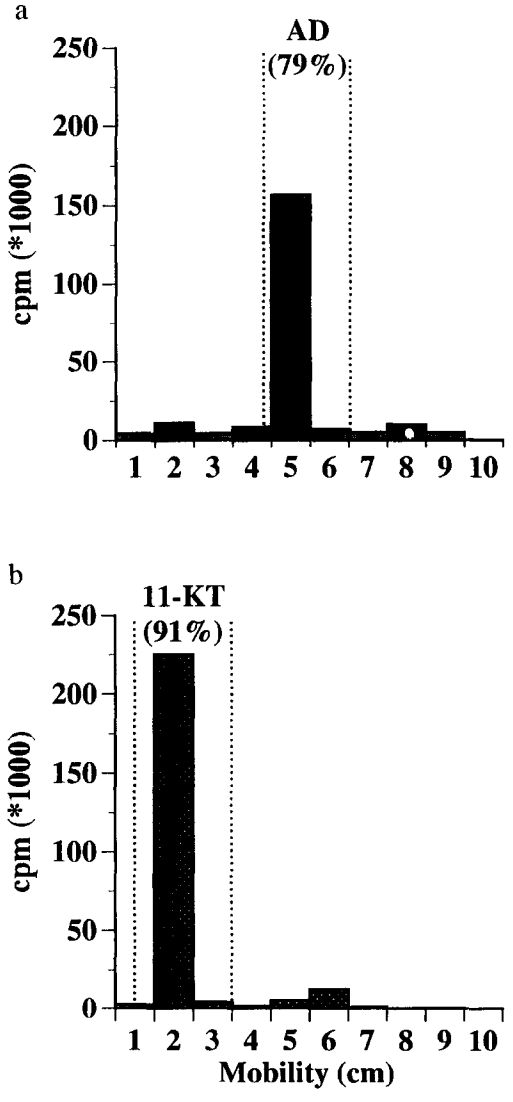


Figure 1 TLC chromatogram of sample mixtures following oxidation of cortisol (a) and subsequent reduction of adrenosterone (b). Run no. 4. AD, adrenosterone; KT, 11-ketotestosterone.

Conversion of [^{3}H]adrenosterone to [^{3}H]-11-ketotestosterone

Trace quantities of [^{3}H]adrenosterone were 17-reduced essentially as described by Truscott,[8] with minor modifications. Following evaporation of the solvent, [^{3}H]adrenosterone was reconstituted in 2 mL of 0.05 M Tris buffer (pH 7.4). This solution was mixed with 0.5 mL of 17β-hydroxysteroid dehydrogenase (0.5 unit/mL of Tris) and β-NADH (0.5 mL of a 5 mg/ml solution in Tris buffer) and incubated in the dark at room temperature. An incubation time of 1 h was chosen, since conversion of [^{3}H]androstenedione to [^{3}H]testosterone, a conversion analogous to that of adrenosterone into 11-KT, in a pilot experiment was highest (95%) after 60 min (using immunological identification of product in aliquots taken at 20-min intervals; data not shown). The steroids were extracted, chromatographed by TLC (Figure 1b), and isolated as described above. Finally, the residue was dissolved in 100% ethanol and kept at −20°C for storage.

Discussion

Conversion of cortisol to adrenosterone

We describe a novel two-step method for the synthesis of 11-KT. The three steps described by Truscott[8] for the conversion of cortisol to adrenosterone have been reduced to a single step in high yield, using PDC as the oxidizing agent. The success of this reaction was judged from the NMR and mass spectral data.

Oxidation of tritiated cortisol with PDC consistently yielded one main radioactive peak by TLC (Figure 1a) which was identified as adrenosterone by its location on the plate relative to that of an authentic sample. The average yield of the oxidation was 76 ± 6% (mean ± SEM) of all the counts recovered from the silica gel (range, 46–89%; $n = 6$). Successful conversion was observed in 6 of 7 trials, although yields varied due to the occasional presence of a second peak, additional to that of [^{3}H]adrenosterone. This product, averaging around 20% (range, 1–44%) of radioactivity, was very mobile in the nonpolar solvent, but its identity and the reaction conditions favoring its synthesis are unclear. The presence of this contamination peak may be correlated to our observations that with increasing quantities of labeled cortisol, a dramatic decrease of the conversion efficiency was observed for reasons that are as yet unknown.

Our yield of adrenosterone (70–80%) is comparable with that reported in several other studies, despite our rather uneconomical radiolocation method (losses of 5–10%). Shner and Dybailo,[9] using MnO_2, obtained 55–77% prod-

uct, depending on the functional group (R) at C-11 (77% for R = OH), while Kley et al.[7] recovered 88% after oxidation with sodium bismuthate. Oliveto,[13] reviewing several methods, and Truscott[8] also reported recoveries of around 80%.

Conversion of adrenosterone to 11-ketotestosterone

The second step (reduction of adrenosterone) by 17β-HSD in the presence of NADH resulted in high yields of product in a short time span. The 11-KT was clearly identified by the NMR data and the ESMS fragmentation pattern, with a peak at 303.5 Da/e, corresponding to protonated 11-KT (results not shown).

Enzymatic reduction of radiolabeled adrenosterone again resulted in one main, more polar, peak by TLC (Figure 1b), associated with 78 ± 8% of the total radioactivity on the gel (range, 51–98%; $n = 6$). The mass spectrum of the tritiated 11-KT sample confirmed its identity, although the spectra were somewhat difficult to interpret, possibly as a result of the small sample size. Finally, the identity of [^{3}H]-11-KT was confirmed immunologically, yielding assay figures (sample 11-KT levels, sensitivity, etc.) similar to those from a previous commercial batch of [^{3}H]-11-KT.

Enzymatic reduction of adrenosterone by 17β-HSD was introduced by Truscott,[8] who obtained conversions of around 90%, yielding on average 67% [^{3}H]-11-KT after chromatographic purification. His method has also proven very reliable in our experiments. High yields can be obtained, whereas reaction times are short. However, the use of 17β-HSD may be restricted to small quantities (e.g., microgram range) of substrate, since, probably as a result of low solubility of 11-KT in hydrophilic media, low conversion figures were obtained (about 15%) with milligram quantities of adrenosterone. Alternatively, longer incubation times may be required or metal hydrides used for the conversion of (sub)molar amounts of adrenosterone.

In conclusion, we have developed a new method for the concomitant oxidative cleavage of the dihydroxyacetone side chain and the 11β-hydroxy group of cortisol, using PDC to give adrenosterone. This method gives recoveries of adrenosterone similar to those reported elsewhere but appears to be more convenient and consistent. The relative ease of obtaining [^{3}H]adrenosterone from [^{3}H]cortisol allows for the subsequent synthesis of [^{3}H]-11-KT by enzymatic reduction and its use in endocrine research.

Acknowledgments

We thank Professor Brian Nicholson, Waikato University, Hamilton, New Zealand, for performing ESMS spectroscopy and for his help in interpreting the spectra. We also acknowledge the donation of radiolabeled 11-ketotestosterone by Professor Ned Pankhurst, University of Tasmania, Launceston, Australia, and the gift of 11-ketotestosterone antibodies by Professor Yoshitaka Nagahama, National Institute for Basic Biology, Okazaki, Japan. This study was supported by grants from the Foundation for Research, Science and Technology (PSD), Otago Research Committee (GY), and the Lottery Science Board (GY).

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