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Design and Synthesis of 6α-Corticosteroid Haptens and Their Bovine Serum Albumin (BSA) Conjugates

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Abstract: The site of attachment of protein carrier to corticosteroids has great influence on the specificity of produced antibody. In order to obtain highly specific and accurate antibodies for bioimmunoassay determination of cortisol, different tether lengths of 6α-corticosteroid haptens and their BSA conjugates were designed and synthesized.

Keywords: Cortisol, hapten, monocolonal antibody, bioimmunoassay.

As one of metabolic substances, cortisol **1** plays a key role as an indicator for some diseases. Therefore, it is highly desired to develop a rapid, specific, sensitive and accurate method for detecting cortisol in blood or urine. Immunoassay using monoclonal antibodies is widely used to determine the steroids. Antibodies against cortisol have been obtained by immunizing with the steroids coupled through the already posed functions (such as C-21)[1,2] or through the easier synthesized functions (such as C-4)[3] hemisuccinate to BSA. But such antibodies cross-react with other steroids because the functionalities using for coupling with BSA are belong to the molecule itself or close to the inherent functionalities. Empirically, the site of attachment to the steroid molecule by protein carrier greatly influences on the specificity of produced antibodies. Greater specificity towards the existing functionalities could be achieved by coupling the protein carrier to the position far from these functionalities[4]. From the 3D viewing of cortisol molecule, introduction of the protein carrier to 6α-position was one of the best selections for these purposes. Herein, we wish to report the synthesis of 6α- coticosteroid haptens and their BSA conjugates.

The synthesis of 6α-coticosteroid hapten had been reported[5], but the final hapten was unstable under their harsh acidic condition to convert the β-isomer to the desired α-isomer from our hand[6]. The produceable and more selective route is needed to achieve our purpose. **Scheme 1** outlined our synthesis towards these haptens.

The 21-OH was protected with acetic anhydride in pyridine to afford the acetate **2** in almost quantitive yield. Enalization of the conjugate ketone using $HC(OC_2H_5)_3$ in ethanol in the presence of TSA gave 65% yield of compound **3**. Auto-oxidization of

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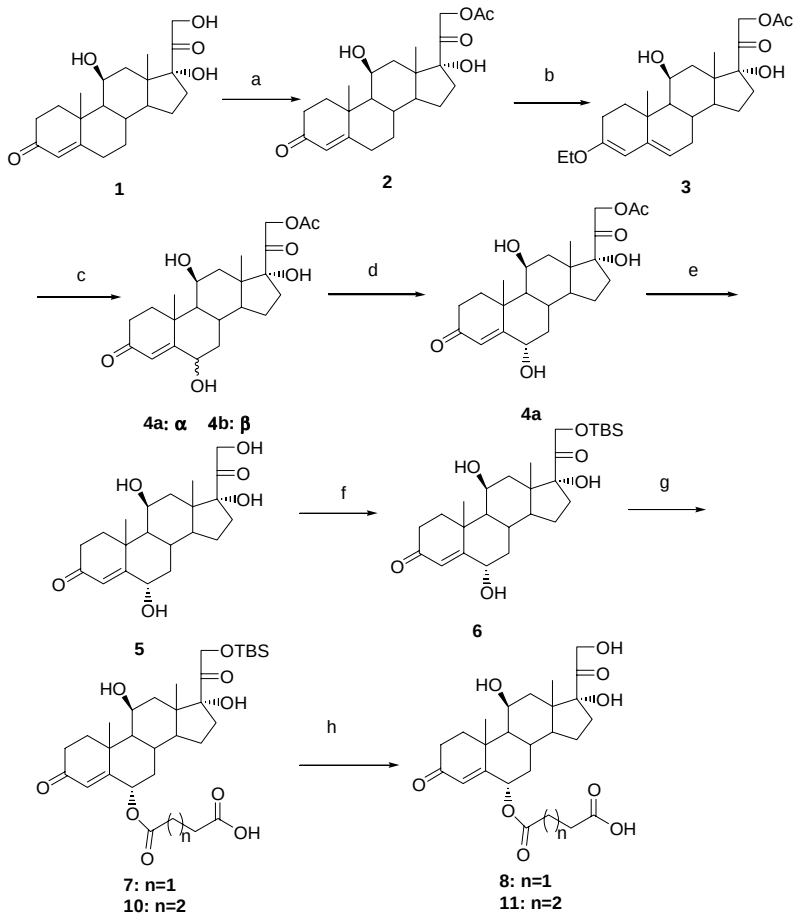
Scheme 1

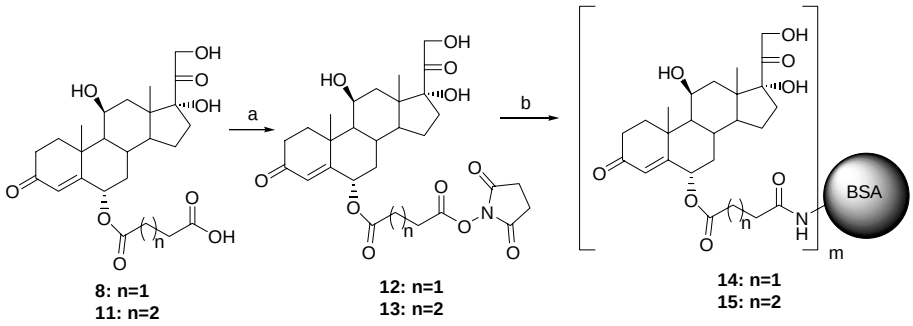


Reagents and conditions: a. Ac_2O, pyridine, r.t., 12h, 98%; b. $HC(OC_2H_5)_3$, TSA, EtOH, r.t., 4h, 65%; c. EtOH, 0°C, sunlight, 1h, 40% combined yield; d. Preparative TLC, 8%; e) 1mol/L KOH, r.t., 0.5h, 91%; f. TBSCl, imidazole, DMF, r.t., 1.5h, 89%; g. succinic anhydride or glutamic anhydride, pyridine, r.t., 12h, 50% for **7**, 56% for **10**; h. CSA, MeOH, 0°C, 45min, 77% for **8**, 56% for **11**.

compound **3** under the reported condition[7] resulted in almost all undesired thermostable 6β- isomer **4b**. Several conditions had been tried in order to improve the ratio of 6α-isomer in the product mixture. It was found that the amount of 6α-isomer **4a** could be highly improved by doing the reaction at low temperature (0°C) and quick reaction time (1h). Using this condition, we could obtain the 6α, 6β mixture in a ratio of 1:4 and a 40% combined yield. After separation by preparative TLC, 6α-isomer **4a** was obtained as a colorless solid. Removing the acetate in **4a** using 1mol/L KOH gave the free alcohol **5** in excellent yield. Compound **5** was highly polar because of four free hydroxyl groups. It was very difficulty to separate using usual method. We found it

was very easy to obtain this compound from the aqueous layer by pass through a column of Almberlyte resin. In order to couple **4a** at 6α- position, the C-21 primary OH should be protected firstly. We selected the TBS ether as protecting group for 21-OH because of its easy binding and removing. To our surprise, almost no reaction was occurred and only the starting material **5** was recovered when compound **5** was treated with standard condition (1.1eq TBSCl, 1.3eq imidazole in DMF). After rechecking compound **5** again, we found there were three co-crystalized water contained in **5** from MALDI-TOF-MS. The water would consume the TBS first and prevent the reaction to proceed. Therefore, more equivalents (4.5eq) reagents were added to the mixture, and to our glad, the reaction was very quick to give the primary TBS ether **6**. Directly reacted this compound with succinic anhydride would be introduce the linker only at 6α-position instead of the 11-position because these two hydroxyl groups were discriminate by the hinderness of 18 and 19-methyl group. Thus, treatment of compound **6** with succinic anhydride in pyridine, the hemisuccinate **7** was obtained in moderate yield. Deprotection of the TBS ether using standard condition (TBAF in THF) only resulted the decomposed starting material, while using the more acidic condition (TSA in MeOH) also produced an unidentified less polar byproduct[6]. The desired hapten **8** was obtained in good yield by reaction of the compound **7** with less acidic CSA in methanol at 0°C. Because the length of the linker sometimes influences the sensitivity of produced antibodies, we also synthesized the one carbon longer hapten **11** using the similar method by condensed compound **6** with glutaric anhydride.

Scheme 2



Reagents and conditions: a. EDC·HCl, N-hydroxysuccinimide (NHS), DMF, 4°C, 12h, 62% for **12**, 68% for **13**; c. BSA, pyridine, PB

Coupling these haptens to BSA for BSA conjugates was shown as **Scheme 2**. Activation of the free acids with NHS and the resulting active esters were treated with BSA (40:1) in a mixture of pyridine and PB at 4°C overnight gave the desired BSA conjugates. These conjugates were purified as follows: dialyzed the reaction mixture against cold water for 1 day, after the addition of acetone and a small amount of NaCl, the resulting suspension was centrifuged (3000rpm) at 4°C for 15min, and then the supernatant was discarded. This procedure was repeated two times. The precipi- tate was dissolved in 20% pyridine and dialyzed against 0.9 % NaCl solution at 4°C

overnight. The conjugates were obtained as a solution in 0.9 % NaCl. Analysis determined the average number of haptens attached to BSA in the conjugates to be 28 for **14** and 30 for **15**, respectively.

In summary, different linker lengths of 6α-corticonoid haptens and their conjugates were synthesized through a more selective and produceable route. Immunizing these conjugates to rats and mouse to produce the specific antibodies for determination of cortisol is underway.

Acknowledgment

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References and Note

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6. Using strong acidic or basic conditions resulted the decomposed of compound **7**. See contents for detail.
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