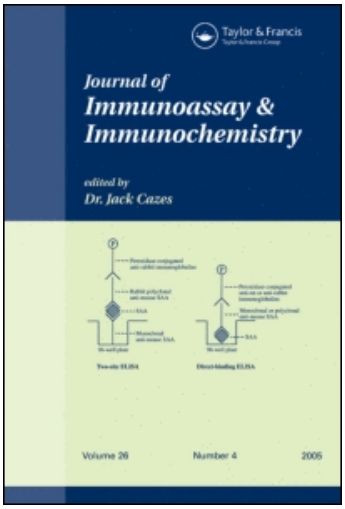

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Synthesis and Analytical Characteristics of New Progesterone Skeleton Haptens

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Synthesis and Analytical Characteristics of New Progesterone Skeleton Haptens

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Abstract: This article describes the synthesis of new conjugated progesterone-bovine serum albumin substances, used to obtain specific antisera toward this hormone and for progesterone assay kits. In general, these kits are designed to be used on the farm or at the local veterinary clinics.

Keywords: Steroid metabolism, Immunoassay, Progestogens, Haptens, Analytical characteristics

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INTRODUCTION

Progesterone is the first biologically active compound in the steroid biosynthetic pathway, formed in the corpus luteum during the estrone cycle and pregnancy.[1,2] Progesterone is found in blood, milk, and other body fluids and the cyclic changes of progesterone during the estrone cycle are reflected by the cyclic changes in the amount of progesterone present in these fluids. Progesterone is at its lowest level at the time of heat and increase during midcycle, [3] being a measure of pregnancy.

If these substances are attached to some macromolecular supports, such as proteins, they become antigenic. Such antigen semisynthetic conjugates have found a wide use, during the last decades, in uninfectious immunology, immune pharmacology, and molecular biology.[4–6]

Therefore, large teams of researchers are studying the synthesis and biological applications of antigenes and specific antibodies to biologically or pharmacologically active substances.[2]

The main uses of these substances are:

1. Identification of organic substances, with low molecular weight, in the biological liquids, with the help of an radio-immune analysis (RIA) method or related techniques.
2. Determination of the substances resulted from metabolism and in pursuit of pharmacokinetic of pharmacologic preparations.
3. Therapeutic aims, through blocking some pathogenic agents.
4. Structural study of some natural chemical products.

The problem of obtaining the progesterone conjugates has concerned researchers from hindmost decade, being elaborated more methods.[7–10] Attempting to fix the protein molecule through the ketonic groups on the 3 or 20 positions of progesterone was not satisfactory because the resultant antibodies were not specific enough. Therefore, on the basis of the fact that the ketonic group from the 3 position of progesterone is more reactive than the 20 ketonic one, it was succeeded through condensation with carboxymethyl-O-hydroxylamine.[1]

A step forward in these studies was achieved by activating the 11 position of progesterone through microbial hydroxylation with *Rhizopus nigricans* or other microorganisms, where the 11-α-hydroxyprogesterone was obtained as a main product.[11–13] Starting from this point, a team of researchers succeeded in the synthesis of a progesterone-bovine serum albumin (BSA) conjugate through the semi-ester with succinic acid, used in the preparation of anti-progesterone sera with a high degree of specificity.[14–16] The activation of the 7 position of the progesterone molecule is achieved by starting

from pregne-4,6-dien-3,20-dione and treating with mercaptopropionic acid or β-mercaptopropionic acid, in the presence of sodium ethoxide or methoxide and dioxane, which led to the 7-carboxymethyl-pregn-4-on-3,20-dione and 7-carboxyethyl-pregn-4-on-3,20-dione, respectively.

In 1972, Lindner et al.,[17] starting from 6-bromo-progesterone and thioglycolic acid obtained 6-(carboxymethylthio)-progesterone, which was then conjugated with BSA, using 1-ethyl-3-dimethylaminopropyl carbodiimide. The authors demonstrated that 28–30 hapten molecules were attached to a BSA molecule.

Taking into account that 11-α-hydroxyprogesterone is not available in our country, the aim of this research was to obtain some new progesterone skeletal haptens starting from 6-bromo-progesterone and having the general formula (I), (where $n = 1–2$ and $m = 0–1$) (Figure 1).

EXPERIMENTAL

Reagents and Materials

Progesterone and BSA were supplied by Terapia (Cluj-Napoca, Romania) and were of pharmaceutical grade. All the other reagents were analytical grade. For an NMR spectra, a Varian EM360 spectrometer was used.

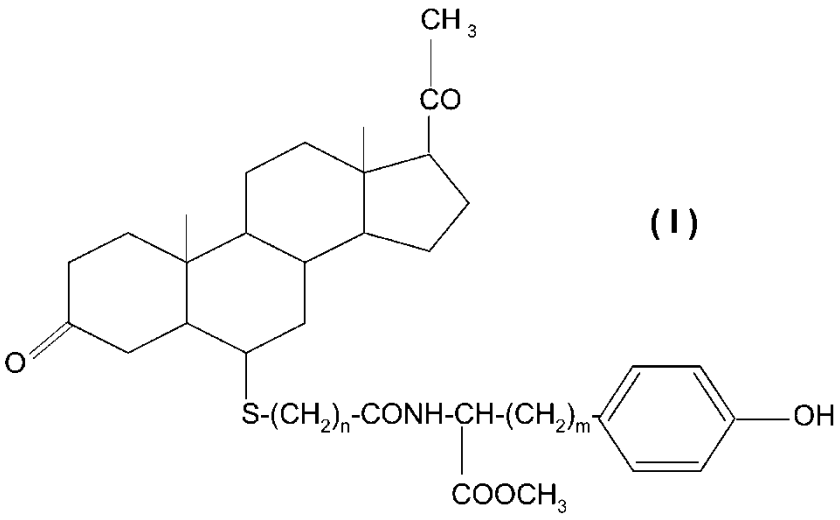
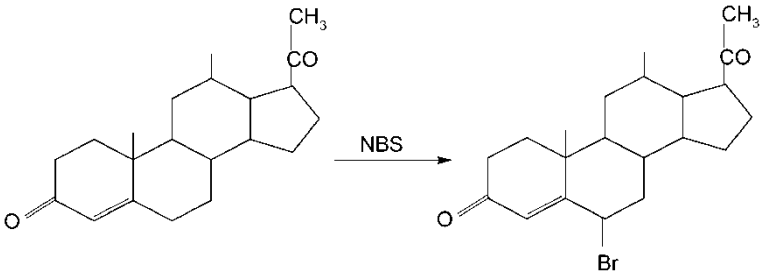


Figure 1. Chemical structure of the prepared progesterone skeleton haptens.

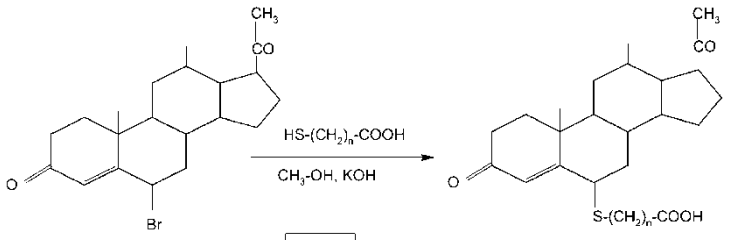
Recommended Procedure

The synthesis is based on the following steps:

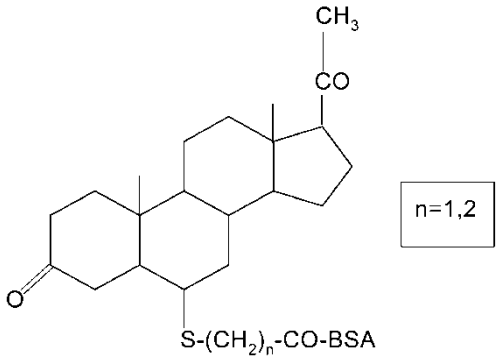
1. Preparation of 6-bromo-progesterone starting from progesterone.



2. Preparation of 6-carboxyethylthio progesterone [6COEtSPr,(n = 2)] and 6-carboxymethylthio progesterone [6COMeSPr,(n = 1)].



3. Synthesis of the BSA conjugates of 6COEtSPr, (n = 2) or 6COMeSPr, (n = 1)



For spectrum acquisition, samples were dissolved in $CDCl_3$; TMS was used as zero reference standard. Spectrum amplitude was 100/8 for

progesterone and 1000/6 for progesterone derivatives. Other parameters were: sweep time, 5; sweep width, 10; filter was 0.05 for progesterone and 0.1 for the other substances.

Synthesis of 6-Bromo Progesterone

In a 1000-mL round-bottom flask, with ascending refrigerant and a lateral tube filled with calcium chloride, add 10 g of progesterone and 250 mL of CCl_4 (dried by passing through P_2O_5 or by distillation). After dissolving the progesterone, add 6 g of N-bromo-succinimide (NBS). Then reflux for 80–90 min, with occasional stirring. In this time, the reaction mixture becomes light brown and colorless succinimide appears, floating on the surface or dispersed in the reaction mixture. The flask is cooled at room temperature (16–20°C), then filter under vacuum. The solution obtained after filtration is then concentrated under vacuum wherein a red-brown oily residue is left. If, during the concentration operation, more succinimide appears, filter it; the filtrate is then concentrated as described above. Add 10 ml of hexane and then add hot acetone, in small quantities, until a clear solution is obtained. Then, cool the solution in a refrigerator for 30 min. Crystals of 6-bromo-progesterone are filtered under vacuum. The crystals obtained are then re-crystallized from acetone or acetone-hexane 2–4 times, wherein a white-yellowish colored substance with a melting point of 140–143°C is obtained. The pure product is sensitive to light, so it should be kept in a brown glass bottle. The purity of the product can be assessed with thin layer chromatography (TLC) on silica gel plates.

Synthesis of 6-Carboxyethylthio Progesterone

To a 500-mL round-bottom flask, add 200 mL of methanol, then 2 g of KOH. Stir, under light warming (25–30°C), until the entire quantity of KOH is dissolved. Then add 5 g of 6-bromo-progesterone and, immediately, 1.6 g (1.4 mL) of β-mercaptopropionic acid under a stream of nitrogen or other inert gas (He, Ar) to remove the air. This operation will prevent the oxidation of β–mercaptopropionic acid to its disulfide. Close the flask and let it stand overnight, under casual stirring. The mixture is then refluxed for 60–80 min (if necessary, nitrogen or another inert gas can be used to avoid the oxidation of β-mercaptopropionic acid).

Place the reaction mixture into 500 mL of cold water, checking the pH to be alkaline, by adding a few drops of KOH or NaOH solution. Transfer the solution to a separatory funnel and extract with 200 mL of ethyl acetate. Repeat the extraction of the aqueous layer with another 200 mL of ethyl

acetate to eliminate the unreacted 6-bromo-progesterone and other insoluble components in alkaline aqueous solution.

The aqueous layer is then concentrated to half its volume using a rotary evaporator; acidify to pH 4–5, adding 3–4 drops of concentrated HCl (or 20% H_2SO_4). The crude 6-(carboxyethylthio)-progesterone is precipitated.

Extract this solution together with the precipitate in two stages, using 50–60 mL of ethyl acetate. Separate the organic layer and combine with the first one. The two organic phases are then concentrated almost to dryness. The solid residue is re-crystallized from ethyl acetate.

White crystals of 6COEtSPr, with the a melting point of 148–150°C, are obtained. The purity of the product can be assessed with TLC on silica gel using as elution solvent a mixture of chloroform : methanol (9 : 1, v/v) and visualizing by using iodine vapors. Generally, the acid contains traces of 6-bromo-progesterone, detected through TLC (Figure 2).

Synthesis of the Conjugate 6COEtSPr-BSA

To a 250-mL Erlenmeyer flask, add 200 mg of 6COEtSPr. Add 16 mL of dimethylformamide and 5 mL of phosphate buffer (pH 8). The reaction mixture is then treated with 200 mg of (1-cyclohexyl-3-(4-diethylamino morpholyl) cabodimide) tosylate and, after 20 minutes, add 400 mg of BSA dissolved in 20 mL of phosphate buffer (pH 8), under stirring. Place this mixture in the refrigerator for 48 hr and then purify the conjugate through gel-filtration chromatography (Sephadex G-50), followed by lyophilization.

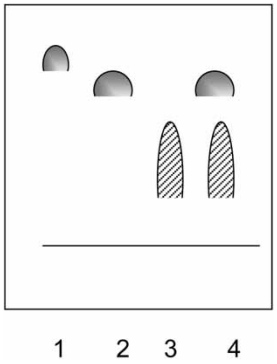


Figure 2. A TLC plate for controlling 6COEtSPr synthesis. 1) 6-Bromo-progesterone; 2) 6COEtSPr; 3) β-mercaptopropionic acid disulfide; 4) Reaction mixture. Eluting Solvent: chloroform:methanol (9 : 1 v/v); Visualizing agent: iodine vapors.

Synthesis of 6-Carboxymethylthio Progesterone

The synthesis is similar to that used for the preparation of 6COEtSPr using 2.2 mL of thioglycolic acid, 80%, instead of mercaptopropionic acid. The crystals of 6COMeSPr obtained are white and with a melting point of 148–150°C. The purity of the product can be controlled through TLC on silica gel, using as an elution solvent a mixture of chloroform: methanol

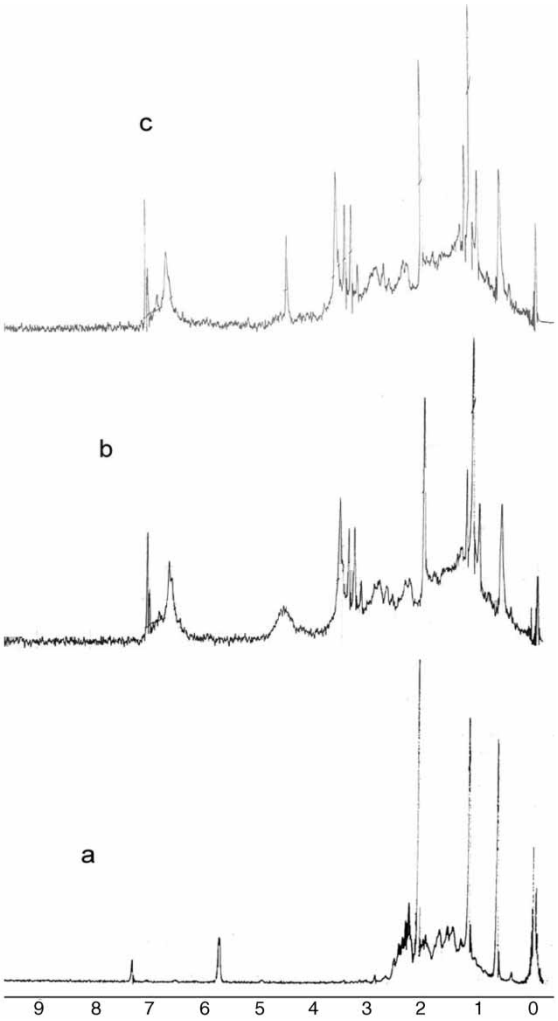


Figure 3. NMR spectra of progesterone haptens (a: progesterone, b: 6COEtSPr, *6COMeSPr*, c: 6COEtSPr-BSA).

(9 : 1, v/v) and visualizing under iodine vapors. Generally, the acid contains traces of 6-bromo-progesterone as shown by TLC.

The synthesis of BSA conjugate is similar to that used for the 6COEtSPr conjugate. The product purified through chromatography is then lyophilized and characterized through the steroid molecule number fixed onto the BSA molecule.

Analytical Characteristics

The products were characterized by melting point, physical characteristics, and TLC. We also performed NMR spectrometry of the products mentioned above, namely progesterone, 6COMeSPr, 6COEtSPr, and BSA conjugates. Some spectra are shown, comparatively, in Figure 3.

RESULTS AND DISCUSSION

The proposed method is useful for the synthesis of new conjugated progesterones from progesterone.

Attempts to replace the phosphate buffer (pH 8) with another phosphate buffer (pH 7) via modified "Dulbecco" (Pasteur Institute-Bucharest) were unsuccessful, as the latter buffer failed to produce anti-progesterone antibodies when injected into rabbits. The method described here is considered as an improved method over the method described by Linder,[18] because some modifications were introduced. For example, ether was replaced with ethyl acetate as an extraction solvent, which improved the purities of the target compounds. Also, replacing 1-ethyl-3-dimethylamino-propyl carbodiimide hydrochloride (which is unstable at the room temperature) by a more stable product, namely, the tosylate of 1-cyclohexyl-3-(3-(N-methylmorpholino)-ethyl)-carbodiimide. The purification of the conjugate was achieved using Sephadex G-50 column chromatography, instead of the dialysis operation using a phosphate buffer (pH8).

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