BBA Report

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Synthesis of 21-acryloxyprogesterone, 21-bromoacetoxyprogesterone and 11α-bromoacetoxyprogesterone for affinity labeling

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SUMMARY

Esters of 21- and 11α-hydroxyprogesterone capable of alkylating amino acids are prepared by reaction of acrylyl chloride or bromoacetyl bromide with the appropriate hydroxysteroid in dimethylformamide. Alternatively bromoacetic acid can be condensed with a steroidal alcohol in the presence of dicyclohexylcarbodiimide. Although the steroidal acrylate and bromoacetates all alkylate certain amino acids only the bromoacetates inactivate 20β-hydroxysteroid dehydrogenase by affinity labeling.

A previous report from this laboratory described the synthesis of 16α-bromoacetoxyprogesterone, which was shown to affinity label the steroid binding site of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*[1]. The present investigation was carried out to synthesize 21-acryloxyprogesterone, 21-bromoacetoxyprogesterone and 11α-bromoacetoxyprogesterone in order to extend the application of affinity labeling for characterization of macromolecular steroid binding sites. Under physiological conditions 21-acryloxyprogesterone alkylates cysteine, histidine and β-mercaptoethanol, as do the bromoacetoxyprogesterone derivatives which also alkylate methionine.

Two general procedures were employed for preparation of the steroidal esters which are summarized in Fig. 1. The appropriate acid halide was used to acylate a hydroxyprogesterone in dimethylformamide (Fig. 1, Method A) by a procedure similar to that previously reported[1]. Alternatively, bromoacetic acid was condensed with the hydroxyprogesterone by dicyclocarbodiimide in dry methylene chloride (Fig. 1, Method B). In each case, elemental analyses and spectroscopic data were consistent with the structures shown.

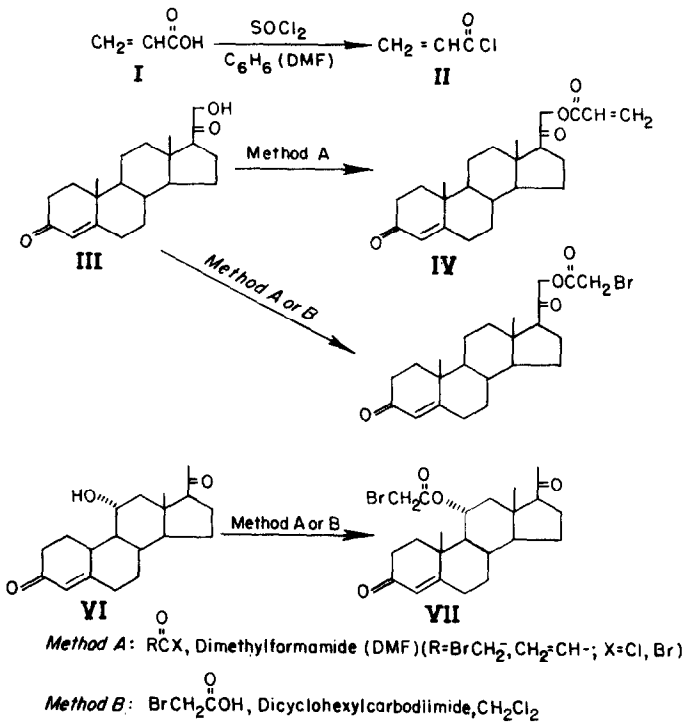


Fig. 1. Synthesis of affinity labeling progesterone derivatives.

Acrylyl chloride (II) was prepared by a modification of a procedure by Seefelder[2]. To 200 g of acrylic acid containing 20 ml of dimethylformamide were added 340 g of thionyl chloride under anhydrous conditions over a period of 8 h, while maintaining a reaction temperature of 60–70 °C. Thereafter, the mixture was heated to approximately 120 °C and the acrylyl chloride was distilled. Redistillation of the crude product gave 205 g of pure acrylyl chloride, b.p. 75–76 °C. Lit. value[2]: b.p. 74–76 °C.

Preparation of 21-acryloxyprogesterone (21-hydroxypregnen-3, 20-dione 21-acrylate, IV). Method A: To a stirred solution of 165 mg (0.5 mmole) of 21-hydroxyprogesterone in 5 ml of dimethylformamide (freshly distilled from barium oxide) was added 0.06 ml of freshly distilled acrylyl chloride. After stirring at room temperature for 2 h the reaction mixture was cooled in an ice bath and 20 ml of water were added dropwise. The white precipitated solid was collected by vacuum filtration and washed with water. Recrystallization of the solid from ethanol–water gave 160 mg (84%) of microcrystalline product, m.p. 177–180 °C decomp.; $[\alpha]_D^{25}$ +194° (chloroform); light-absorption max. in ethanol 240 nm (ϵ 16 600); infrared absorption max. in chloroform 1720 cm^{-1}, 1690 cm^{-1} and 1650 cm^{-1} characteristic of an α,β-unsaturated ester, 20-keto, and 3-keto functions, respectively; absence of 3400 cm^{-1} (characteristic of -OH). (Found: C, 75.17; H, 8.35. $C_{24}H_{32}O_4$ requires C, 74.97; H, 8.39%.)

Preparation of 11α-bromoacetoxyprogesterone (11α-hydroxy-4-pregnen-3,20-

dione 11-bromoacetate, VII). Method A: To 330 mg (1.0 mmole) of 11α-hydroxyprogesterone in 8 ml of dry dimethylformamide (freshly distilled from BaO) was added, with stirring at room temperature, 0.100 ml of bromoacetyl bromide. After stirring for 5 min, 0.080 ml of pyridine was added and stirring was continued for 3 h at room temperature. The mixture was cooled in an ice bath and 5 g of ice chips, then 150 ml of water were slowly added. A light yellow gum formed which was worked into a solid by trituration, then filtered by vacuum, washed with water, and dried. Examination of the solid by thin-layer chromatography (Eastman 1 inch X 3 inch silica gel plates with fluorescent indicator, developed with chloroform) showed it to contain a major halogen positive (Gmelin's reagent) component (R_F 0.56) and a small amount of starting material (R_F 0.25). After chromatographic separation of the major component (15 g silica gel G, elution with chloroform) and removal of the solvent, the residual oil was crystallized twice from diisopropyl ether, to give 285 mg (63%) of product, m.p. 153 °C; $[\alpha]_D^{25}$ +148° (chloroform); light absorption max. in chloroform 1730 cm^{-1} (ester), 1705 cm^{-1} (C-20 keto), 1660 cm^{-1} (C-3 keto), 680 cm^{-1} (characteristic of bromoacetate), absence of 3400 cm^{-1} band (hydroxyl). (Found: C, 61.23; H, 6.94; Br, 17.58. $C_{23}H_{31}BrO_4$ requires C, 61.19; H, 6.92; Br, 17.10%.)

Method B: To 1.00 g (3.03 mmoles) of 11α-hydroxyprogesterone in 30 ml of anhydrous methylene chloride (freshly distilled from $CaCl_2$), cooled in an ice bath, were added 4.19 g (6.04 mmoles) of bromoacetic acid in 20 ml CH_2Cl_2, 4.33 g (6.04 mmoles) of dicyclohexylcarbodiimide in 20 ml CH_2Cl_2. After 5 min of stirring, 0.50 ml of pyridine was added. A precipitate of dicyclohexylurea immediately formed. The mixture was stirred for 1 h in the cold, then for an additional hour at room temperature. After chilling the mixture in an ice bath, 1.0 ml of acetic acid was added and stirring was continued for 15 min. The reaction mixture was filtered by vacuum and the solid residue washed with methylene chloride. The combined filtrates were concentrated to dryness and 50 ml of acetone were added. Undissolved solid (dicyclohexylurea) was removed by filtration. After cooling the filtrate in an ice bath, addition of 200 ml of water precipitated 1.5 g of crude product. Two recrystallizations of the solid from diisopropyl ether gave 1.25 g of pure 11α-bromoacetoxyprogesterone, m.p. 152–154 °C. This material was identical in all respects (thin-layer chromatography, ultraviolet and infrared spectra, mixed melting point) to that obtained by Method A.

Preparation of 21-bromoacetoxyprogesterone (21-hydroxy-4-pregnen-3,20-dione 21-bromoacetate, V). Compound V was prepared from 21-hydroxyprogesterone by Method A, described above for the preparation of VII. Chromatography of the crude product was unnecessary since it could be readily purified by recrystallization from acetone-water. Hence 330 mg of 21-hydroxyprogesterone gave 320 mg of pure needles of V, which had m.p. 105–106 °C; $[\alpha]_D^{25}$ 0° (chloroform); light absorption max. in chloroform similar to VII. (Found: C, 61.30; H, 6.95; Br, 17.35. $C_{23}H_{31}BrO_4$ requires C, 61.19; H, 6.92; Br, 17.10%.)

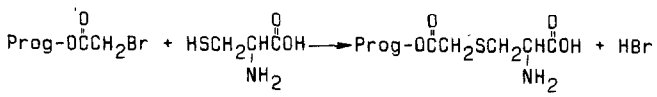
Alkylation of amino acids. Each of the above steroids (5 μmoles) was incubated with 5 μmoles of cysteine, histidine, methionine or 2-mercaptoethanol in

2.0 ml of 0.05 M phosphate buffer, pH 7.0, at 25 °C. During a 48-h period small aliquots of incubate were withdrawn and applied to 3 inch × 3 inch thin-layer chromatography plates (Eastman No. 6060 silica gel G with fluorescent indicator) and eluted with butanol–acetic acid–water (12:3:5, by vol.). Appropriate steroids and amino acids were applied to the same plates to serve as standards. After elution the dried chromatograms were visualized with ultraviolet light, then sprayed with ninhydrin reagent. In each case a new spot was detected, intermediate in mobility between the steroid and the amino acid. Since the new spot absorbed ultraviolet light and was ninhydrin positive, it was taken to be a steroid–amino acid conjugate.

A solution of 21-bromoacetoxyprogesterone (4.51 mg, 0.01 mmole) and cysteine (1.45 mg, 0.012 mmole) was incubated for 1 h at 25 °C in a mixture of 5 ml ethanol and 5 ml 0.05 M phosphate buffer at pH 7.0. Thin-layer chromatography showed the conjugation reaction to be complete after 1 h. After dilution with 10 ml of water the reaction mixture was extracted sequentially with ether, ethyl acetate and butanol. The extracts were found to contain unreacted steroid, steroid *plus* steroid–amino acid conjugate, and the steroid–amino acid conjugate, respectively. No free cysteine or other amino acid could be detected in any of the chromatographed extracts. The butanol extract was evaporated to dryness and the residue was hydrolysed with 0.1 M NaOH. After neutralization with 0.1 M HCl, thin-layer chromatography of the hydrolysate showed two spots with mobilities identical with authentic 21-hydroxyprogesterone and *S*-carboxymethyl-L-cysteine. Analysis of the hydrolysate in an amino acid analyzer confirmed the presence of *S*-carboxymethyl-L-cysteine. These results indicate that the steroid–amino acid conjugate is 21-*S*-L-cysteinylacetoxyprogesterone. Similar results were obtained with the other alkylating steroids when subjected to the above conditions. The relative rates of alkylation of amino acids were in the order: cysteine (and 2-mercaptoethanol) > histidine > methionine.

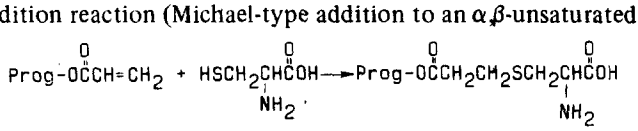
Preliminary studies with these compounds have shown that both compounds V and VII are substrates which inactivate 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* under conditions previously described[1]. Interestingly, the acrylate (IV) which alkylates cysteine and histidine has no effect on the activity of this enzyme.

Alkylation of nucleophilic amino acids by the bromoacetoxyprogesterone derivatives V and VII is expected to occur by an S_N2 displacement of the bromine atom[3],



This results in a covalent linkage of the steroid *via* an ester bridge to the amino acid.

Alkylation of amino acids by 21-acryloxyprogesterone, (IV), most likely occurs by an addition reaction (Michael-type addition to an α,β-unsaturated carbonyl)[4],



Synthesis of 21-acryloxyprogesterone, 21-bromoacetoxyprogesterone and 11α-bromoacetoxyprogesterone has provided steroids capable of alkylating amino acids. The compounds should be useful, by affinity labeling, to characterize certain steroid binding sites of high affinity present in receptor proteins of target organs.

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