Synthesis of [^{3}H]-cycloalkanoprogesterones (pregna-D′-pentaranes) and study of their interaction with a progesterone receptor

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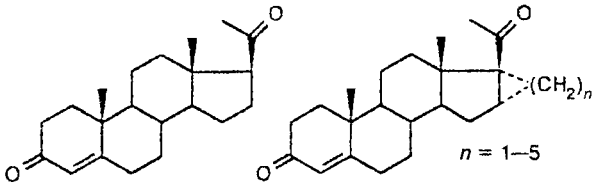
In order to search target organs and to study the mechanism of the action of steroids with separated biological functions, tritium-labeled pregna-D′-pentaranes were synthesized by an original procedure, and their interaction with a progesterone receptor was investigated. The uterine progesterone receptor was shown to be the only specific binding protein for these compounds.

Key words: steroids, pregna-D′-pentaranes, tritiation, binding with progesterone receptor.

The task of developing medicinal preparations of directed selective action set by P. Erlich 100 year ago still remains completely timely. In fact, in almost all cases, a short period after a drug has been put into practical use, side effects are discovered, and this often deprives the drug of any value.

Having synthesized a set of 16α,17α-cycloalkano-substituted progesterones in which the additional ring D′ consisted of three to seven carbon atoms,[1] we were able, first, to create a new class of highly biologically active gestagenes, pregna-D′-pentaranes,[2] and, second, to obtain for the first time analogs of hormones of this type with separated biological functions,[3] in particular, with respect to the progestagenic activity (proliferation of the uterine endometrium) and "true" gestagenic activity,[4] preservation of pregnancy of ovarioectomized animals devoid of native progesterone (Scheme 1, Table 1).



Progesterone Pregna-D′-pentaranes

This separation of biological functions should apparently be attributed to distortion of the conformation of the steroid skeleton, especially of ring D and the side chain, that occurs during fusion with highly strained three- and four-membered rings[5,6] and decreases the ability of these progesterone derivatives to react with some of its receptors. However, in fusion with a six-membered ring in a chair conformation, the geometry of the skeleton and the side chain is completely retained,[7] and access to the carbonyl group at position 20 is only slightly sterically hampered, whereas the ability of 16α,17α-cyclohexanoprogesterone (1) to bind with the uterine progesterone receptor increases owing to the increase in the hydrophobic surface of the molecule.[8] As a result, the activity of this compound is completely retained in both tests and even exceeds somewhat the activity of progesterone itself.

Having synthesized compounds with separated biological functions, we studied the biochemistry of hor-

Table 1. Biological activity of pregna-D′-pentaranes (in rabbits)[3]

Compound	Progestagenic activity relative to progesterone	Preservation of pregnancy (%)
Progesterone	1.0	60
Cyclopropano-progesterone	7.6	20
Cyclobutano-progesterone	4.6	25
Cyclohexano-progesterone	2.4	81

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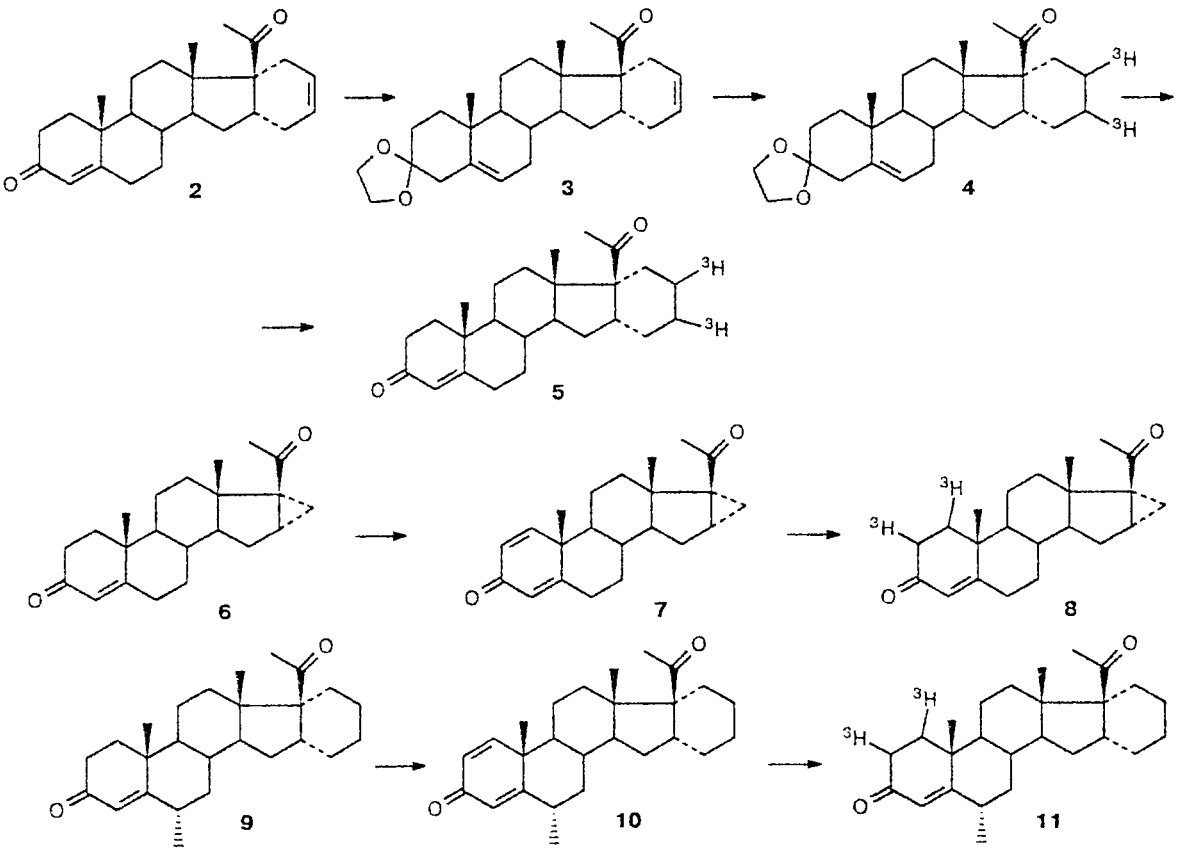
Scheme 1



mone regulation in the maintenance of pregnancy in order to identify the target organ and to elucidate the mechanism controlling this process. In this paper, we report on the development of methods for the preparation of tritium-labeled pentaranes needed for this study and on the investigation of their interaction with receptors.

To obtain reliable data on binding, it is necessary to use tritiated pentaranes with relatively high molar radioactivities of tens of curies per mmol that retain their biological activity. Therefore, at the fist stage of our work, we developed a method for the preparation of tritiated derivatives of certain pregna-D′-pentaranes (see Scheme 1), *viz.*, compounds **5**, **8**, and **11**. One of the most widely used and facile method for the introduction of tritium into biologically active compounds is the reduction of carbon—carbon multiple bonds with gaseous tritium.[9] The problem was to conserve those double bonds responsible for the hormone properties of these compounds, *i.e.*, we had to selectively hydrogenate double bonds, either present in the molecule or introduced specially at the final steps of the synthesis. The procedure of tritiation was chosen and optimized in hydrogenation.

Results and Discussion

To synthesize [^{3}H]-labeled compound **5**, the carbonyl group in position 3 of 16α,17α-cyclohex-3′-enoprogesterone (**2**) was subjected to selective ketalization,[10] which was accompanied by a shift of the conjugated double bond and afforded 3-ketal (**3**). The latter was hydrogenated in the presence of various catalysts (5% $Pd/BaSO_4$, 5% Pd/Al_2O_3, and 5% $Pd/CaCO_3$) for 0.5—3 h in dioxane or ethyl acetate. It was found that neither the catalyst nor the solvent or the reaction time have any significant effect on the outcome of hydrogenation; in most cases, the yield of the product of hydrogenation of ring D′ amounted to 55—65%. Transferring these conditions to tritiation allowed us to prepare [^{3}H]-ketal **4** with a molar radioactivity of 50.0—51.9(±1.5) Ci mmol^{-1}; hydrolysis of the latter yielded the target [^{3}H]-steroid **5** with high molar radioactivity.

To synthesize labeled compounds **8** and **11**, it was necessary to introduce an additional double bond, suitable for selective hydrogenation, into molecules **6** and **9**. The 1,2-bond could serve this purpose, because in a cross-conjugated 3-oxo-1,4-diene system, this bond has been shown[11] to be sterically less hindered than the

4,5-bond. The corresponding dienones **7** and **10** were obtained by treating the initial steroids with dichlorodicyanobenzoquinone (DDQ). According to the published data,[12—15] catalysts of homogeneous hydrogenation are the most promising for the selective reduction of a cross-conjugated system. When we used tris(triphenylphosphine)rhodium chloride, we found that already after 3 h, the reaction mixture contained ~15% completely hydrogenated steroid (^{1}H NMR). However, this duration proved to be the optimum for the reaction, because the initial compound was already consumed, the yield of 4-monoenes amounted to 50—60%, and further hydrogenation increased the proportion of perhydrogenated derivatives, which would markedly hamper chromatographic isolation of the labeled compounds. Labeled steroids **8** and **11** were synthesized by this procedure in fairly high yields (see Scheme 1).

The resulting tritium-containing steroids **5**, **8**, and **11** with molar radioactivities of 53, 47, and 43 Ci mmol^{-1}, respectively, were used subsequently for the investigation of steroid—receptor binding in relation to the progesterone receptor from a rat uterus. The main goal of this stage of investigation was to find out whether the progesterone receptor from this organ is the only protein of the supernatant fraction of the homogenate that specifically binds pentaranes **5** and **8** and which of the above-mentioned biological effects of these compounds can be explained by their interaction with this receptor. The interaction of 16α,17α-cycloalkanoprogesterones with the progesterone receptor and with other rat uterine proteins was studied by cross inhibitory analysis based on the displacement of [^{3}H]-ligands from protein complexes by non-labeled steroids. The values of relative competitive activity (RCA) were calculated from the ratio of the concentrations of non-labeled compounds resulting in 50% inhibition of the specific binding (*i.e.*, high-affinity binding suppressed by an excess of a non-labeled competitor) of the [^{3}H]-ligand. Progesterone was used as the reference compound; its activity was taken as 1. The results are presented in Table 2.

The results of the analysis of the competitive binding of [^{3}H]-pentaranes by rat uterine proteins indicate that 16α,17α-cyclopropano- and 16α,17α-cyclohexanoprogesterones are fairly efficient analogs of the natural hormone in the interaction with its receptor. Both analogs taken in excess completely displace [^{3}H]-progesterone from its specific complexes with proteins. However, [^{3}H]-16α,17α-cyclopropanoprogesterone (**8**) and [^{3}H]-16α,17α-cyclohexanoprogesterone (**5**) bind specifically only to the progesterone receptor, since this binding is equally suppressed by an excess of the corresponding non-labeled compound and by excess progesterone. The order of variation of the RCA values obtained for various [^{3}H]-ligands (see Table 2) proves that the progesterone binding sites are identical with those for its cycloalkane derivatives.

Thus, our study permits the conclusion that the progesterone receptor in the soluble fraction of the homogenate of rat uterus is the only protein able to bind specifically pentaranes **1** and **6** with rather close parameters of competitive binding, and, hence, it is responsible for the progestagenic activity exhibited by these compounds. The "true" gestagenic action is apparently accomplished in another target organ.

Table 2. Relative competitive activities (RCA) of 16α,17α-cycloalkano-derivatives of progesterone based on the displacement of [^{3}H]-ligands from complexes with a progesterone receptor from the soluble fraction of the uterus of pregnant rats

Labeled ligand	Non-labeled competitor	RCA
[^{3}H]-Progesterone	**6**	1.02
	1	0.79
[^{3}H]-16α,17α-Cyclopropanoprogesterone (**8**)	**6**	0.99
	1	0.57
[^{3}H]-16α,17α-Cyclohexanoprogesterone (**5**)	**6**	1.57
	1	1.0

Note. The activity of progesterone was taken as 1.0.

Experimental

Melting points were determined using a Boetius hot-stage microapparatus. ^{1}H NMR spectra were obtained on a Bruker WM-250 spectrometer ($CDCl_3$). Qualitative analysis of the mixtures was performed by TLC on Silufol microplates. Elution was carried out with system *A* (hexane—acetone, 7 : 3) or *B* (hexane—ether, 3 : 2); the plates were visualized in UV light. High performance liquid chromatography (HPLC) was carried out on Lichospher (18.5 μm, a 2×60 mm column) in system *C* (85% aqueous MeOH), retention times: 2.64 (**7**), 3.30 (**10**), 5.93 (**8**), and 8.93 (**11**) min, and in system *D* (75% aqueous MeOH), retention time: 4.75 min (**7**). Preparative HPLC was carried out on Partisil ODS (3.5 μm, 4.6×250 mm) in system *E* (90% aqueous MeOH), retention times: 8.58 (**11**) and 5.39 (**10**) min. Preparative chromatography was accomplished using Kieselgel-60 (0.063—0.200 mm). The conditions for labeling were optimized using 0.1% tritium by a known procedure.[16] Biochemical studies were carried out by a procedure reported previously.[17]

3,3-Ethylenedioxy-16α,17α-cyclohex-3′-enopregn-5-en-20-one (3). A mixture of 3-oxo-Δ^5-steroid (**2**) (0.1 g),[16] ethylene glycol (1 mL), and toluene-*p*-sulfonic acid (5 mL) in anhydrous toluene (10 mL) was refluxed for 6 h with a Dean—Stark trap. Then it was diluted with 5 mL of toluene and washed with a 5% solution of $NaHCO_3$ and with water. The toluene was removed *in vacuo*, and the semicrystalline residue was crystallized from an acetone—hexane mixture to give 0.091 g (82%) of compound **3**, m.p. 189—191 °C.

[3′,4′-$^3H_2$]-3,3-Ethylenedioxy-16α,17α-cyclohexanopregn-5-en-20-one (4). A mixture of steroid **3** (20 mg), 5% Pd/$BaSO_4$ (40 mg), and anhydrous ethyl acetate (0.4 mL) was placed in a 15-mL reaction vial and stirred for 1 h in a tritium atmosphere at a pressure of 400 GPa. Excess tritium was removed by evacuation from the vial frozen with liquid nitrogen, and the catalyst was filtered off and washed with ethyl acetate (3×1 mL). To remove labile tritium, the mixture was concentrated and again dissolved in AcOEt containing 10% MeOH; this operation was repeated three times. Analysis and chromatographic purification were carried out using system *B*; this gave 15.6 mg of compound **4** with a radiochemical purity of 94—96%.

[3',4'-$^3H_2$]-16α,17α-Cyclohexanopregn-4-ene-3,20-dione (5). Toluene-*p*-sulfonic acid (3 mg) was added to a solution of ketal **4** (15 mg) in 1.5 mL of anhydrous acetone, and the mixture was allowed to stand at ~20 °C for 26 h. Then the solvent was removed *in vacuo*, the residue was dissolved in 5 mL of ether, washed with a 5% aqueous solution of $NaHCO_3$ (2×3 mL), dried with $BaSO_4$, and concentrated to give 13 mg (93%) of compound **5** with a radiochemical purity of 95—97% and a molar radioactivity of 53.2 Ci mmol^{-1}. This preparation was stored at 10—15 °C as a solution in a 2 : 1 AcOEt—MeOH mixture.

16α,17α-Cyclopropanopregna-1,4-diene-3,20-dione (7). A mixture of Δ^4-3-ketone **6** (0.76 g),[18] 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (0.53 g), and benzoic acid (300 mg) in 20 mL of anhydrous benzene was refluxed with stirring for 20 h. The precipitate that formed was filtered off and washed with benzene, the filtrate was passed through a short column with 15 g of Al_2O_3 (activity II), and the column was finally washed with 30 mL of $CHCl_3$. The combined eluates were concentrated to dryness, and the residue was chromatographed on a column with silica gel. Elution with a 4 : 1 heptane—acetone mixture gave 0.33 g (43%) of $\Delta^{1,4}$-3-ketone **7**, m.p. 146—153 °C (*cf.* Ref. 19, m.p. 153 °C).

[1,2-$^3H_2$]-16α,17α-Cyclopropanopregn-4-ene-3,20-dione (8). A mixture of compound **7** (2.0 mg) and tris(triphenylphosphine)rhodium chloride (4.0 mg) in 0.3 mL of anhydrous ethyl acetate was stirred for 3 h in a tritium atmosphere at 400 GPa. Excess tritium was removed by evacuation from the vial frozen by liquid nitrogen, and the labile tritium was removed by concentrating the reaction mixture with an AcOEt—acetone (5 : 1) mixture; this procedure was repeated three times. The residue was chromatographed on a plate (20×20 cm); elution was carried out by system *A*. The zone containing product **8** was cut out, and the compound was washed out with ethyl acetate (5×10 mL); the solution was filtered and concentrated. Compound **8** was analyzed and purified by HPLC in systems *C*—*E*. The yield of the labeled preparation amounted to 45—50%. The radiochemical purity was 97—99% and the molar radioactivity was 47.1 Ci mmol^{-1}. The labeled compound was stored as described above.

6α-Methyl-16α,17α-cyclohexanopregna-1,4-diene-3,20-dione (10). A mixture of Δ^4-3-ketone **9** (0.5 g),[20] DDQ (0.3 g), and benzoic acid (0.3 g) was refluxed in 10 mL of dry benzene with stirring for 8 h. The residue obtained after a work up similar to that described above for compound **7** was chromatographed on silica gel; the product was eluted with a heptane—acetone mixture (9 : 1) to give 0.31 g of $\Delta^{1,4}$-3-ketone **10**, m.p. 198—203 °C. ^{1}H NMR, δ: 0.73 (s, 3 H, 18-Me); 1.12 (d, 3 H, 6-Me, J = 6.5 Hz); 1.25 (s, 3 H, 19-Me); 2.11 (s, 3 H, 21-Me); 6.08 (m, 1 H, HC-4, J = 1.85 Hz); 6.25 (dd, 1 H, HC-2, J = 10.1 and 1.85 Hz); 7.04 (d, 1 H, HC-1, J = 10.1 Hz).

[1,2-$^3H_2$]-6α-Methyl-16α,17α-cyclohexanopregn-4-ene-3,20-dione (11). [^{3}H]-$\Delta^{1,4}$-3-ketone **11** was prepared from compound **10** (2.0 mg) by the procedure described above for compound **8**. Yield 50—55%. Its radiochemical purity was 96—98% and the molar radioactivity was 43.4 Ci mmol^{-1}.

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