Synthesis of 6(*E*)- and 6(*Z*)-(3-ethoxycarbonylpropyl)oximes of 16α,17α-cyclohexanopregn-4-ene-3,6,20-trione and study of their interaction with proteins of the rat uterine cytosol and blood serum

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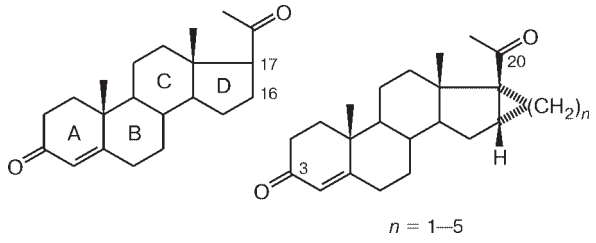
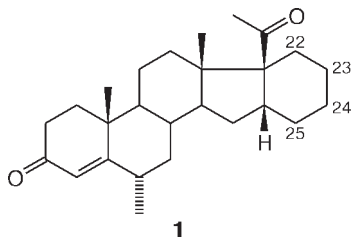
6(*E*)- and 6(*Z*)-(3-Ethoxycarbonylpropyl)- and -(3-carboxypropyl)oximes of 16α,17α-cyclohexanopregn-4-ene-3,6,20-trione were synthesized. The reactions of these ester ligands with pentarane-binding proteins of the uterine cytosol and blood serum were studied; the latter exhibits a higher affinity. The preferred binding of the oxime (*E*)-isomer relative to the (*Z*)-isomer was noted.

Key words: pentarane, progesterone, synthesis, oxime, configuration, binding, protein, receptor.

16α,17α-Pentacyclic derivatives of progesterone (pregna-D′-pentaranes)[1] are synthetic progestins promising from the standpoint of practical application. First, many of them, unlike the natural hormone progesterone, retain the hormonal activity upon oral administration. Second, some pregna-D′-pentaranes exhibit the properties of partial agonists/antagonists as they selectively reproduce some progesterone effects and suppress other effects.[2,3] Study of the interaction of these compounds with the progesterone receptor (PR) showed that the kinetic parameters of their binding are comparable with analogous parameters of progesterone itself and that they are not always correlated with the type of biological activity of the pentarane.[4—7] Owing to the use of tritium-labeled pregna-D′-pentaranes, an additional protein, other than PR, with an unknown function that binds specifically pentaranes and is possibly related to the action of these steroids was found in the rat and human uterine cytosol.[7,8]. Yet another pregna-D′-pentarane-specific protein was detected in the rat and human blood sera.[9] The two proteins differ from each other in the combination of properties.[9] The protein detected in blood serum might ensure the high progestational activity of pentaranes *in vivo* by retaining them for long in the blood stream. A pentarane having affinity to these additional proteins is 6α-methyl-1′,2′,3′,4′,5′,6′-hexahydrobenzo[16α,17α]pregn-4-ene-3,20-dione (6α-methyl-16α,17α-cyclohexanoprogesterone) (**1**).



Progesterone Pregna-D′-pentaranes



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In order to purify these uterine cytosol and blood serum pentarane-specific proteins for the subsequent elucidation of their nature, we studied approaches to the synthesis of pentarane derivatives, namely, steroid ligands that can be immobilized on a substrate. Steroids of this type are expected to retain, most likely, both polar functions needed for binding at positions 3 and 20 of the molecule and to contain a chain of several carbon atoms with a terminal functional group suitable for covalent bonding to the substrate. It is known that substituted oximes of various classes of steroids have been used, for example, to prepare steroid haptens.[10] We suggested that

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a 3,20-diketopentarane having such a chain at position 6 of the steroid skeleton could serve as a ligand. Synthesis of the first steroid ligands in the pentarane series for affinity chromatography and study of their interaction with proteins from the rat uterine cytosol and blood serum are the subject of this paper.

Results and Discussion

The 5,6-epoxide **2** described in our previous publication[11] was chosen as the starting compound for the synthesis of the 6-substituted ligand (Scheme 1). Epoxide opening resulted in diol **3**, Jones oxidation of the 6-hydroxy group in which yielded 6-ketopentarane **4**. Elimination of the 5α-hydroxy group (**4** → **5**) and hydrolysis of the 3-*O*-acetate formed yielded 4,5-didehydro-3β-hydroxy-6,20-dioxopentarane (**6**). The reaction of **6** with *O*-(3-ethoxycarbonylpropyl)hydroxylamine[12] and oxidation of the 3β-hydroxy-6-oxime mixture thus formed with pyridinium dichromate (PDC) furnished a mixture of desired 3,20-dioxo 6-oximes **7a**,**b** in 3 : 1 ratio (^{1}H NMR data). Preparative TLC of this mixture gave rise to individual compounds **7a** and **7b**.

Similarly, the reaction of 3β-hydroxy-6,20-dioxopentarane **6** with *O*-(3-carboxypropyl)hydroxylamine[12] followed by oxidation of the resulting 3β-hydroxy-6-oxime (not shown in Scheme 1) afforded a mixture of 16α,17α-cyclohexanopregn-4-ene-3,6,20-trione 6(*E*,*Z*)-(3-carboxypropyl)oximes; chromatographic resolution of the mixture followed by crystallization furnished pure (*E*)-isomer **7c**. This product will be used subsequently to prepare an affine sorbent with immobilized pentarane ligands.

Table 1. ^{1}H NMR chemical shifts (δ) of oximes **7a**—**c**

H atom	**7a**	**7b**	**7c**
1	2.07; 1.79	2.07; 1.79	2.06; 1.78
2	2.45; 2.40	2.52; 2.38	2.50; 2.45
4	6.16	6.06	6.16
7	3.30; 1.54	2.53; 1.86	3.30; 1.55
8	1.71	1.73	1.71
9	1.19	1.18	1.19
11	1.70; 1.50	1.66; 1.50	1.70; 1.50
12	1.72; 1.67	1.72; 1.67	1.72; 1.69
14	1.78	1.78	1.78
15	1.62; 1.47	1.59; 1.39	1.62; 1.47
16	3.02	3.02	3.02
18	0.73	0.73	0.73
19	1.15	1.10	1.15
21	2.15	2.15	2.15
22	2.02; 1.55	2.02; 1.55	2.02; 1.56
23	1.64; 0.90	1.64; 0.90	1.65; 0.91
24	1.47; 1.20	1.47; 1.20	1.47; 1.20
25	1.59; 1.51	1.59; 1.51	1.60; 1.52
1′	4.19	4.04	4.19
2′	2.02	1.96	2.03
3′	2.40	2.34	2.47
5′	4.13	4.13	—
6′	1.27	1.27	—

Table 2. Data of ^{13}C NMR spectra (δ) of oximes **7a**—**c**

C atom	**7a**	**7b**	**7c**
1	34.9	34.9	34.8
2	33.8	33.5	33.7
3	199.0	199.0	199.2
4	123.2	127.1	123.2
5	160.1	155.3	160.2
6	155.3	153.0	155.5
7	30.3	38.8	30.4
8	33.0	36.3	32.9
9	51.4	53.6	51.3
10	38.8	39.8	38.7
11	20.5	20.5	20.4
12	31.6	31.6	31.5
13	46.8	46.8	46.8
14	50.4	50.0	50.4
15	29.6	29.7	29.6
16	34.0	34.0	33.9
17	63.9	63.9	63.9
18	15.8	15.8	15.7
19	16.6	16.3	16.6
20	212.1	212.1	212.4
21	27.9	27.9	28.0
22	27.1	27.1	27.1
23	22.2	22.2	22.3
24	21.0	21.0	21.0
25	27.3	27.3	27.3
1′	73.5	72.9	73.2
2′	24.6	24.4	24.2
3′	30.8	30.7	30.4
4′	173.1	173.1	178.2
5′	60.3	60.3	—
6′	14.2	14.2	—

The structures of compounds **3**—**7** and the *E*- or *Z*-configurations of oximes **7a**, **7b**, and **7c** follow from the data of physicochemical analysis. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) were interpreted by combined analysis of 2D homonuclear COSY, TOCSY, and NOESY spectra and heteronuclear HSQC and HMBC spectra. The HMBC spectra were used to assign the signals of nonprotonated carbon atoms.

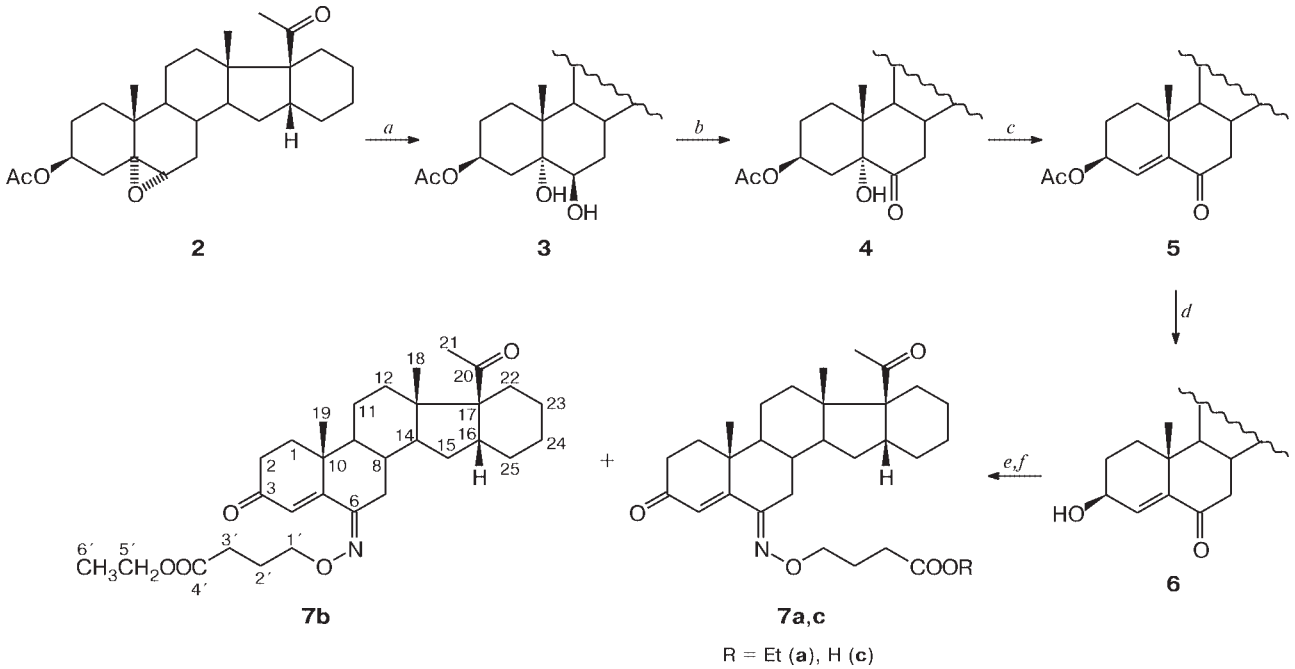
The NOESY spectra did not reveal any spatial contacts of protons at C(1′)—C(3′) with the protons of the steroid skeleton in 6-(3-carboxypropyl)oxime **7c** and in ester **7a**. In the case of 6-(3-ethoxycarbonylpropyl)oxime **7b**, the NOESY spectrum exhibited correlation peaks of the H(1′) and H(3′) protons with H(4). Thus this compound was identified as *Z*-isomer, while oxime **7a**, was identified as *E*-isomer. Since the chemical shifts of the

protons and carbon atoms in the vicinity of the C(6) atom in oximes **7a** and **7c** were identical, the substituent at C(6) in oxime **7c** was concluded to have *E*-configuration, as that in **7a**. In addition, the ^{1}H NMR spectra of *E*-isomers **7a** and **7c** exhibit a characteristic signal of the equatorial proton at C(7) as a doublet of doublets with δ 3.30 (J = 15.9 and 4.5 Hz). In the ^{1}H NMR spectrum of isomer **7b**, a similar signal was found at δ 2.55. This low-field shift of the signal of the proton at C(7) in isomers **7a** and **7c** can be attributed, most likely, to the influence of the closely located N—O bond of the $=NO(CH_2)_3CO_2R$ fragment; this confirms the validity of ascribing *E*-configuration to the substituents at C(6) in these compounds. The difference (equal to 2—8 ppm) between the chemical shifts of the C(7), C(8), and C(9) atoms in the carbon spectra of *E*- and *Z*-oximes also deserves attention.

The presence of the $=NO(CH_2)_3CO_2R$ fragment (R = Et or H) in molecules **7a**—**c** is also indicated by mass spectra. The spectra of all three compounds contain a major peak with m/z 380, which corresponds to detachment of the $O(CH_2)_3CO_2Et$ (or, correspondingly, $O(CH_2)_3CO_2H$) fragment from the molecular ions of these compounds.

In order to evaluate the suitability of steroids **7a** and **7b** as ligands in affinity chromatography for the isolation of proteins, we studied their ability to bind to proteins from the rat uterine cytosol and blood serum using competitive analysis. Examples of curves for the displacement of the [^{3}H]ligands from complexes with the uterus PR, a pregna-D´-pentarane-specific protein from rat uterus, and with a protein from rat blood serum are presented in Fig. 1, *a*, *b*, and *c*, respectively. The average values for the equilibrium dissociation constant (K_d) and the relative binding affinity (RBA) based on the results of two experiments are presented in Table 3. It can be seen that oximes **7a** and **7b** poorly react with PR, the affinity of (*Z*)-isomer **7b** to the receptor being almost an order of magnitude lower than that of (*E*)-isomer **7a**. The pregna-D´-pentarane-specific uterine protein exhibits a higher (by a factor of approximately 4—20) affinity to oximes **7a** and **7b** than PR. The preference for the (*E*)-isomer is less pronounced for this protein than in the case of PR. Of the proteins studied, the blood serum protein shows the highest affinity to the ligands (the K_d is 3—12 times lower than in the case of the pregna-D´-pentarane-specific protein from the uterus). The preference of the serum protein for (*E*)-isomer **7a** over (*Z*)-isomer **7b** is the same as in the case of PR. The more efficient binding of (*E*)-**7a** with respect to (*Z*)-isomer **7b** is, apparently, due to the fact that in the (*E*)-isomer, the conjugated ketone in ring A is more spatially accessible for the reaction with the protein molecule.

Scheme 1



Reagents and conditions: (*a*) $HClO_4$/THF, 20 °C; (*b*) CrO_3—H_2SO_4—H_2O/acetone, 20 °C; (*c*) $SOCl_2$/Py, 0 °C, 1 h; (*d*) KOH/MeOH; (*e*) $EtO_2C(CH_2)_3ONH_2 \cdot HCl$, Py, EtOH, 60 °C, 7 h or $HO_2C(CH_2)_3ONH_2 \cdot HCl$, Py, 60 °C, 5 h; (*f*) PDC/Py, 20 °C, 1.5 h.

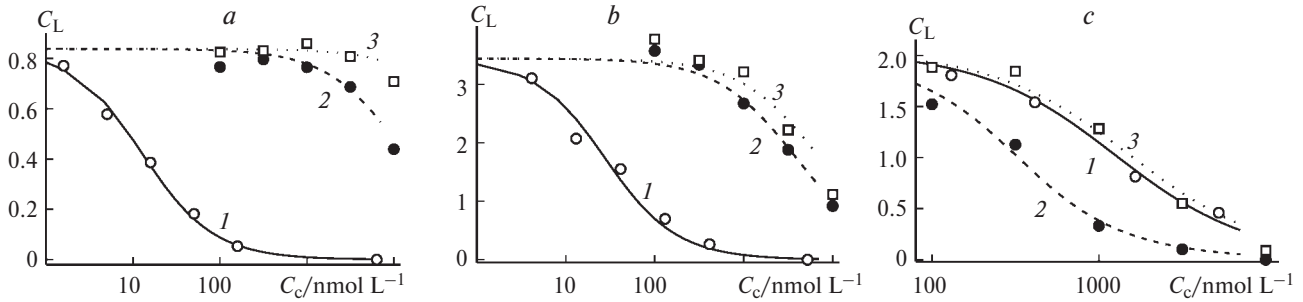


Fig. 1. Competitive analysis of interaction of (*E*)-isomer **7a** and (*Z*)-isomer **7b** with progesterone receptor (PR) (*a*), pregna-D´-pentarane-specific rat uterine protein (*b*), and rat blood serum protein (*c*): (*1*) [^{3}H]progesterone (3.0 nmol L^{-1}) (*a*); [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone (9.4 nmol L^{-1}) (*b*); [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone (10.5 nmol L^{-1}) (*c*), (*2*) (*E*)-isomer **7a**, (*3*) (*Z*)-isomer **7b**. C_L is the concentration of the bound [^{3}H] ligand, C_c is the concentration of the competitor.

The obtained results provide grounds to expect that (*E*)-isomer **7a** could be used for isolation of the pregna-D´-pentarane-binding rat serum protein by immobilization of the carboxy analog on an insoluble support with subsequent affinity chromatography.

Experimental

Melting points were determined on a Koeffler hot stage (Boetius). 1D and 2D NMR spectra were recorded on a Bruker DRX-500 instrument for solutions in $CDCl_3$ at 30 °C. The residual signal of $CHCl_3$ (δ 7.27) was used as the standard in the ^{1}H NMR spectra, and the signal of $CDCl_3$ (δ 77.0) was the reference in the ^{13}C NMR spectra. Two-dimensional spectra were recorded using standard Bruker programs. When running the NOESY spectra, the mixing time was 0.9 s. Mass spectra were obtained on a Kratos MS 30 instrument with direct sample injection into the ion source at 150—200 °C. Analytical and preparative TLC were carried out on Silica gel 60 F_{254} plates (Merck) in hexane—acetone and hexane—ether solvent systems. The spots were visualized by a 1% solution of $CeSO_4$ in 10% aqueous H_2SO_4 with subsequent heating; in the case of preparative TLC, visualization was performed by UV light. Preparative separation was done by column chromatography on Kieselgel 60 Merck silica gel (0.063—0.100 μm) at a compound : sorbent ratio of 1 : 40. The specific rotation was measured on a JASCO DIP-360 polarimeter in $CHCl_3$ at 22 °C. Dried and distilled solvents were used in reactions.

Table 3. Equilibrium parameters of the interaction of (*E*)-oxime **7a** and (*Z*)-oxime **7b** with rat proteins

Protein	[^{3}H]Ligand	Unlabeled competitor	K_d /nmol L^{-1}	RBA
Progesterone receptor	Progesterone	Progesterone	4.4	1
		(*E*)-**7a**	6390	0.00067
		(*Z*)-**7b**	46500	0.00010
Pregna-D´-pentarane-specific uterine protein	**1**	**1**	7.2	1
		(*E*)-**7a**	1520	0.0046
		(*Z*)-**7b**	2255	0.0032
Blood serum protein	**1**	**1**	570	1
		(*E*)-**7a**	130	4.2
		(*Z*)-**7b**	815	0.65

The usual workup of organic extracts implies washing with water until the washings becomes neutral, drying with $MgSO_4$, and evaporation of the solvent *in vacuo*.

3β-Acetoxy-5α,6β-dihydroxy-1´,2´,3´,4´,5´,6´-hexahydrobenzo[16α,17α]pregnan-20-one (3β-acetoxy-5α,6β-dihydroxy-16α,17α-cyclohexanopregnan-20-one) (3). A 65% solution of $HClO_4$ (0.25 mL) was added with stirring to a solution of epoxide **2** (5 g, 11.7 mmol) [11] in 170 mL of THF, and the mixture was stirred for 30 min at 20 °C and neutralized with a 5% aqueous solution of $NaHCO_3$. Tetrahydrofuran was removed *in vacuo* and the residue was dissolved in 100 mL of $CHCl_3$. The usual workup gave 4.98 g (quantitative yield) of triol monoacetate **3**. The analytical sample had m.p. 272—274 °C (from an acetone—hexane mixture). ^{1}H NMR, δ: 0.70, 1.19, 2.03, 2.14 (all s, 3 H each, C(18)Me, C(19)Me, C(3)OAc, C(21)Me); 2.96 (m, 1 H, C(16)H); 5.16 (m, 1 H, C(3)H). MS, *m/z* (I_{rel} (%)): 446 $[M]^+$ (4), 368 $[M - AcOH - H_2O]^+$ (5), 307 $[M - AcOH - 2H_2O - Ac]^+$ (32).

3β-Acetoxy-5α-hydroxy-16α,17α-cyclohexanopregnane-6,20-dione (4). Compound **3** (4.90 g, 11 mmol) in 150 mL of acetone was oxidized by 5 mL of the Jones reagent (prepared by dissolving 26.72 g of CrO_3 in 23 mL of concentrated H_2SO_4 and adding H_2O to make 100 mL) at 20 °C (TLC monitoring). The reaction mixture was poured into water and extracted with $CHCl_3$ (2×50 mL). The residue obtained after the usual workup was chromatographed. Gradient elution with an acetone—heptane mixture (8 : 92 → 12 : 88) gave 3.27 g (67%) of 5-hydroxy ketone **4**, m.p. 248—250 °C (from an acetone—hexane mixture), $[\alpha]_D$ −44 (*c* 0.5). ^{1}H NMR, δ: 0.66, 0.82, 2.01, 2.14 (all s, 3 H each, C(18)Me, C(19)Me, C(3)OAc, C(21)Me); 2.80 (t, 1 H, $C(7)H_a$, J = 15 Hz); 2.96 (m, 1 H, C(16)H); 5.05 (m, 1 H, C(3)H). MS, *m/z* (I_{rel} (%)): 444 $[M]^+$ (12), 384 $[M - AcOH]^+$ (9), 366 $[M - AcOH - H_2O]^+$ (12), 323 $[M - AcOH - H_2O - Ac]^+$ (73).

3β-Acetoxy-16α,17α-cyclohexanopregn-4-ene-6,20-dione (5). Thionyl chloride (0.3 mL, 4.11 mmol) was added at 0 °C to a solution of diol monoacetate **4** (0.75 g, 1.69 mmol) in 9 mL of anhydrous Py, and the mixture was stirred for 1 h at 0 °C. Then the reaction mixture was poured in 100 mL of H_2O and extracted with EtOAc (3×25 mL). The combined organic extracts were washed with 10% HCl (25 mL) and with brine. The residue obtained after the usual workup was recrystallized from MeOH to give 0.5 g (69%) of enedione **5**, m.p. 184—186 °C (from MeOH). ^{1}H NMR, δ: 0.70, 1.02, 2.07, 2.14 (all s, 3 H each, C(18)Me, C(19)Me, C(3)OAc, C(21)Me); 2.98 (m, 1 H, C(16)H); 5.32 (m, 1 H, C(3)H), 6.08 (br.s, 1 H, C(4)H).

3β-Hydroxy-16α,17α-cyclohexanopregn-4-ene-6,20-dione (6). A suspension of compound **5** (0.48 g, 1.125 mmol) and 1 mL of 1 *M* aqueous KOH in 25 mL of MeOH was stirred for 45 min at 20 °C and poured into 100 mL of ice-water. The precipitated powder was filtered off, washed with water, and dried in air to give 0.43 g (~100%) of hydroxy ketone **6**. The analytical sample had m.p. 199—202 °C (from an acetone—hexane mixture), $[\alpha]_D$ −14 (*c* 1.0). ^{1}H NMR, δ: 0.70, 1.02, 2.15 (all s, 3 H each, C(18)Me, C(19)Me, C(21)Me); 2.52 (dd, 1 H, C(7)H_e, ^2J = 13 Hz; ^3J < 2 Hz); 2.98 (m, 1 H, C(16)H); 4.23 (m, 1 H, C(3)H); 6.15 (br.s, 1 H, H(4)). MS, *m/z* (I_{rel} (%)): 384 $[M]^+$ (8), 341 $[M - Ac]^+$ (6).

6(*E*)- and 6(*Z*)-(3-Ethoxycarbonylpropoxy)imino-16α,17α-cyclohexanopregn-4-ene-3,20-diones (7a and 7b). *O*-(3-Ethoxycarbonylpropyl)hydroxylamine hydrochloride (0.036 g, 0.2 mmol)[12] and 0.8 mL of 2.2% ethanolic solution of Py were added to a solution of steroid **6** (0.07 g, 0.18 mmol) in 5 mL of EtOH, and the reaction mixture was stirred for 7 h at 55—60 °C. Then the mixture was poured into water and extracted with CH_2Cl_2 (8×10 mL). The residue obtained after removal of the solvent (0.093 g) was dissolved in 2 mL of Py, PDC (0.1 g) was added, the mixture was stirred for 1 h at 20 °C, EtOAc (15 mL) was added, and the precipitate that formed was filtered off and washed with EtOAc (3×3 mL). The filtrate was washed with 10% HCl, a solution of $NaHCO_3$, and brine. The residue (0.09 g) was partially purified by filtration through a column with silica gel (elution with a heptane—acetone mixture, 94 : 6 → 90 : 10) and the solvent was removed to give 0.071 g of a mixture of **7a**,**b** as a light-yellow thick oil. This mixture was chromatographed on three 20×20 cm plates (hexane—ether 7 : 3, elution repeated three times) to give 0.015 g of *Z*-oxime **7b** (R_f 0.54) and 0.036 g of *E*-oxime **7a** (R_f 0.50) (colorless oil). ^{1}H and ^{13}C NMR spectra are given in Tables 1 and 2. MS, **7a**, *m/z* (I_{rel} (%): 511 $[M]^+$ (4), 380 $[M - O(CH_2)_3CO_2C_2H_5]^+$ (36). MS **7b**, *m/z* (I_{rel} (%): 511 $[M]^+$ (5.5), 380 $[M - O(CH_2)_3CO_2C_2H_5]^+$ (48).

(6*E*)-(3-Carboxypropoxy)imino-16α,17α-cyclohexanopregn-4-ene-3,20-dione (7c). *O*-(3-Carboxypropyl)hydroxylamine hydrochloride (0.73 g, 5.2 mmol)[12] was added to a solution of steroid **6** (1 g, 2.6 mmol) in 15 mL of Py, and the reaction mixture was stirred for 5 h at 55—60 °C. The mixture was evaporated with toluene (2×20 mL), and then ether (30 mL) and water (15 mL) were added to the residue. The ethereal layer was separated and the aqueous layer was extracted with ether (3×15 mL). The combined extracts were washed with 10% HCl and then with water until the medium was neutral. The usual workup gave a colorless crystalline residue (1.2 g), which was dissolved in 30 mL of Py. Pyridinium dichromate (1.3 g) was added, and the mixture was stirred for 1 h at 20 °C. The residue (foam) obtained after the workup described above for the preparation of oximes **7a**,**b** was chromatographed on a column with SiO_2. Elution with a heptane—acetone—MeOH mixture (81 : 17 : 2 → 78 : 20 : 2) gave 0.95 g of a mixture of (*E*,*Z*)-oximes as a crystalline material. Recrystallization from an ether—hexane mixture gave 0.64 g of (*E*)-**7c** with m.p. 175—178 °C (from ether—hexane), $[\alpha]_D$ −153 (*c* 1.32). The data of ^{1}H and ^{13}C NMR spectra are given in Tables 1 and 2. MS, *m/z* (I_{rel} (%)): 483 $[M]^+$ (15), 380 $[M - O(CH_2)_3CO_2H]^+$ (82).

An additional 0.19 g of an 1 : 1 crystalline mixture of (*E*,*Z*)-isomers was isolated from the mother liquor.

Biochemical experiments. [1,2,6,7-^{3}H]Progesterone with a specific radioactivity of 86 Ci mmol^{-1} (St.-Petersburg) and 6α-methyl[1,2-^{3}H]-16α,17α-cyclohexanoprogesterone (43 Ci mmol^{-1}) that we synthesized [8] were used. Sexually mature female rats weighing 200—250 g were employed. The blood collected during decapitation was allowed to stand for 1 h at ~20 °C, cooled to 0—4 °C, and, after 1 h, centrifuged at 3000 *g* for 10 min. The serum was stored at −20 °C for up to 2 months. Prior to use, the unfrozen serum was diluted fourfold with a buffer solution (10 *mM* Tris-HCl, 10 *mM* KCl, 0.5 *mM* phenylmethanesulfonyl fluoride, 1 *mM* dithiothreitol, a 30% (v/v) solution of glycerol,[9] pH 7.5 at 25 °C). The uteri taken from two or three animals were combined, crushed, and homogenized in a fivefold volume of the buffer solution using a glass homogenizer. The supernatant fraction (cytosol) obtained after centrifuging the homogenate at 50000 *g* for 1 h was used immediately. All operations were carried out at 0—4 °C. The interaction of the compounds under study with blood serum and uterine cytosol proteins was analyzed as described previously.[7] To this end, the [^{3}H]steroid (about 6 MBq, 10 μL), the unlabeled competitor (0—10 μmol L^{-1}, 90 μL), and a solution of the protein (100 μL) were added successively to test tubes. In the case of uterine cytosol proteins, the incubation system contained additionally 3 μmol L^{-1} of hydrocortisone (for blocking the glucocorticoid receptors). When uterine cytosol proteins were used in combination with [^{3}H]6α-methyl-16α,17α-cyclohexanoprogesterone, 123 nmol L^{-1} of progesterone was added (to block progesterone receptors). After incubation for 20 h, the unbound ligand was removed by adsorption on dextran-coated activated charcoal for 5 min; the charcoal was subsequently precipitated by centrifuging. An aliquot portion (250 μL) was taken from the supernatant fraction of the protein-bound ligand, and the radioactivity content was measured. The amount of the specifically bound [^{3}H]ligand was found as the difference between the bound radioactivity in the absence and in the presence of excess (5—6 μmol L^{-1}) of the same ligand without a label.[7] The equilibrium dissociation constants (K_d) were determined. The relative binding affinity of compounds was calculated from the ratio of K_d values for the compound alike to the [^{3}H]ligand and the compound under study. Two series of measurements were carried out in parallel. Each experiment was done twice.

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