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Caged Progesterone: A New Tool for Studying Rapid Nongenomic Actions of Progesterone

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Abstract: Ketalization of the biomolecule progesterone with (6-bromo-7-hydroxycoumarin-4-yl)ethane-1,2-diol gives the photolabile progesterone derivatives **3** and **4**. These compounds display dramatically reduced bioactivity and release progesterone upon irradiation with UV/vis or IR light. In particular, **4** can be used to perform concentration-jump experiments with high temporal and spatial resolution that allows one to study elegantly the mechanisms of rapid nongenomic cellular events evoked by progesterone. The usefulness of **4** was demonstrated by measurement of changes in swimming behavior of single human sperm caused by progesterone-induced Ca^{2+} influx in the sperm flagellum.

Introduction

Besides its classical, slow mode of action, that is, the activation of transcription, the steroid hormone progesterone **1** shows rapid, nongenomic effects that are probably generated at the cell membrane. Nongenomic progesterone responses have been identified in a variety of cells such as sperm,[1] oocytes,[2] lens epithelial cells,[3] granulosa cells,[4] T cells,[5] neurons,[6] erythrocytes, and platelets.[7] The nature of the nongenomic progesterone receptors is uncertain,[8] and their characterization is a matter both of interest and of great importance.

The nongenomic actions of **1** are thought to occur on a timescale of seconds or even subseconds, and for studying signal transduction pathways of **1**, the release of the biomolecule from so-called caged compounds is advantageous. Caged compounds are photolabile inactive derivatives of biological molecules.[9] The active substance is rapidly released by a photochemical reaction. Caged compounds allow the elucidation of complex cellular processes, the study of behavioral responses, and their resolution in time and space.

Here, we report the synthesis and photochemistry of the caged progesterones **3** and **4** and their usefulness in studies of the rapid progesterone-mediated response in human sperm. The effects of **1** in human sperm are characterized by Ca^{2+} influx or mobilization of Ca^{2+} from intracellular stores.[10] Moreover, **1** has been shown to cause changes in the swimming behavior of mammalian sperm.[11] Caged compounds of **1** have not yet been described, and, to our knowledge, this report is the first that uses photoactivation of caged carbonyl compounds in a biological system. Several photoremovable keto-protecting groups have been described in the literature.[12,13] We selected for caging of **1** the (6-bromo-7-hydroxycoumarin-4-yl)ethane-1,2-diol (Bhc-

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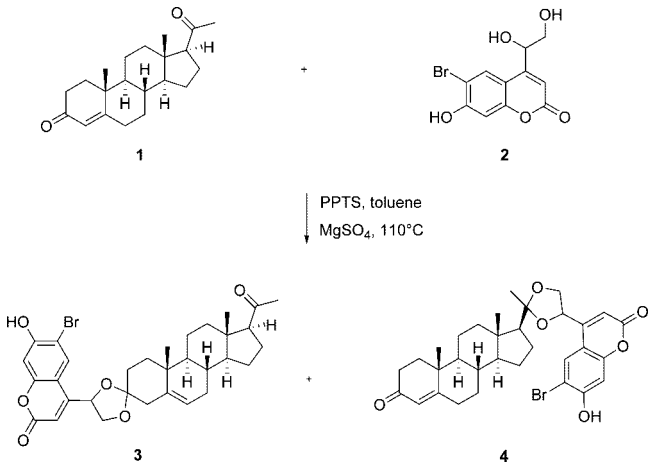
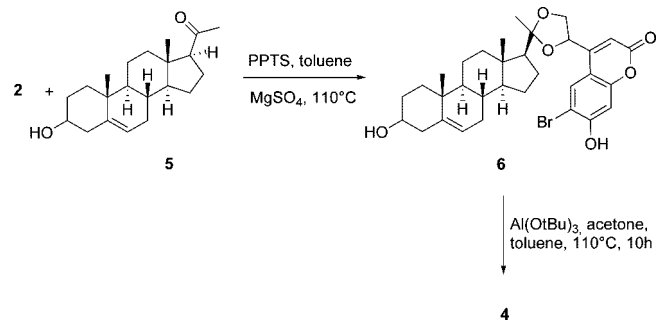
Scheme 1. Synthesis of the Caged Progesterones **3** and **4**



Scheme 2. Alternative Synthesis of **4**



ED) agent **2**,[12] which is based on the 6-bromo-7-hydroxycoumarinylmethyl protecting group.[14]

Results and Discussion

Synthesis and Photochemical Properties. In the first synthetic route (Scheme 1), **1** was ketalized with **2** (0.5 equiv) by refluxing in toluene in the presence of pyridinium *p*-toluenesulfonate (PPTS) and $MgSO_4$. The reaction resulted in a mixture of the positional isomers **3** and **4**; that is, **1** was derivatized at positions 3 and 20, respectively. The concomitant rearrangement of the double bond to the C-5,6 position during ketal formation of **3** has been previously described[15] and was here deduced, both from the ^{13}C NMR signals for C4, C5, and C6 and from the ^{1}H NMR chemical shift values for H-4 and H-6. Double ketalization at the 3 and 20 positions was not observed. The mixture of the isomers of **3** and **4** was purified by preparative reverse-phase HPLC. The overall yield was about 30%, and the ratio of **3** to **4** was approximately 4:1. Separation of the positional isomers was achieved by using flash chromatography. The yields were 17% for **3** and 3% for **4**. Each of the caged progesterones **3** and **4** consists of four diastereomers (**3a**−**d** and **4a**−**d**). Separation of two pure diastereomers and a mixture of two diastereomers of **3** and **4** was accomplished by an additional reverse-phase HPLC run. The product ratio **3a**,**b**:**3c**:**3d** was 2:2:1, and that of **4a**,**b**:**4c**:**4d** was 6:1:3. The structures of **3** and **4** were confirmed by spectroscopic methods. The position of the ketal formation was identified comparing IR, ^{1}H NMR, and ^{13}C NMR data of the diastereomer mixtures **3a**−**d** and **4a**−**d**. Characteristics for the assignment are the existence or nonexistence of the IR stretching vibration band of the C3 C═O group at about 1645 cm^{-1} (the C20 and the coumarin C═O bands appear as overlapping signals), the high field ^{1}H NMR chemical shifts of H-2 and H-4 of the isomers of **3** to $\delta = 0.90$, $\delta = 1.73$, and $\delta = 2.42$ ppm or those of the hydrogen atoms at C21 and C17 of the isomers of **4** to $\delta = 1.38$ and $\delta = 2.06$ ppm by ketal formation, and the appearance of ^{13}C NMR carbonyl carbon signals at $\delta = 198.0$ ppm (diastereomers of **3**) or $\delta = 208.5$ and 211.7 ppm (diastereomers of **4**), respectively. The structure of **4** was additionally verified by independent synthesis (see Scheme 2). All diastereomers of **4** displayed at 225 nm more pronounced UV bands as compared to **3** (see Supporting Information, Figure 1).

Because of the advantages of **4** as compared to **3** (see below), we improved the preparation of **4** adapting chemistry analogous to that described in ref 16. The synthesis is outlined in Scheme 2 and involves ketalization of the commercially available pregnenolone **5** with **2** in the presence of PPTS and $MgSO_4$ to give the ketal **6** (10% yield upon HPLC purification), followed by Oppenauer oxidation of **6** to the desired caged progesterone **4** (30% yield upon HPLC purification). **6** and **4** consist of mixtures of diastereomers. Separation of the diasteromers **4a**−**d** was achieved by RP-HPLC. The product ratio **4a**,**b**:**4c**:**4d** was almost identical to that found in the synthesis route described in Scheme 1. (See Supporting Information for preparative details.) The overall yield of the two-step procedure for **4** is similar to that of the synthetic route described in Scheme 1, but the product purification is easier.

The diastereomeric mixtures of **3** and **4** were investigated in the dark for their resistance to spontaneous hydrolysis under physiological conditions. No appreciable decomposition was observed after 24 h in pH 7.2 HEPES buffer (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid). The solubility of **3** and **4** in HEPES buffer at pH 7.2 is low (about 8 μM), but sufficient for physiological studies.

At physiological pH, the Bhc substituent of caged progesterones **3** and **4** exists as phenolate. The ionized forms show intensive S_0-S_1 absorption at 374 nm, which allows activation of the caged compounds between 330 and 420 nm. Irradiation of **3a**−**d** and **4a**−**d** with 365 nm light in acetonitrile/HEPES buffer at pH 7.2 liberates progesterone and the phenolate form of the diol **2**. The extent of progesterone release as measured by HPLC was about 30% for both caged compounds. However, although the peaks of compounds **3** and **4** almost completely disappeared, no other progesterone derivatives could be detected. This observation is consistent with results obtained with other Bhc-ED acetal/ketal derivatives.[12]

We assume that the photocleavage proceeds in analogy to the conversion of coumarinylmethyl esters via an S_N1 mechanism.[17] As shown in Scheme 3 for compound **4**, progesterone formation should proceed via the zwitterion **7** as intermediate.[12]

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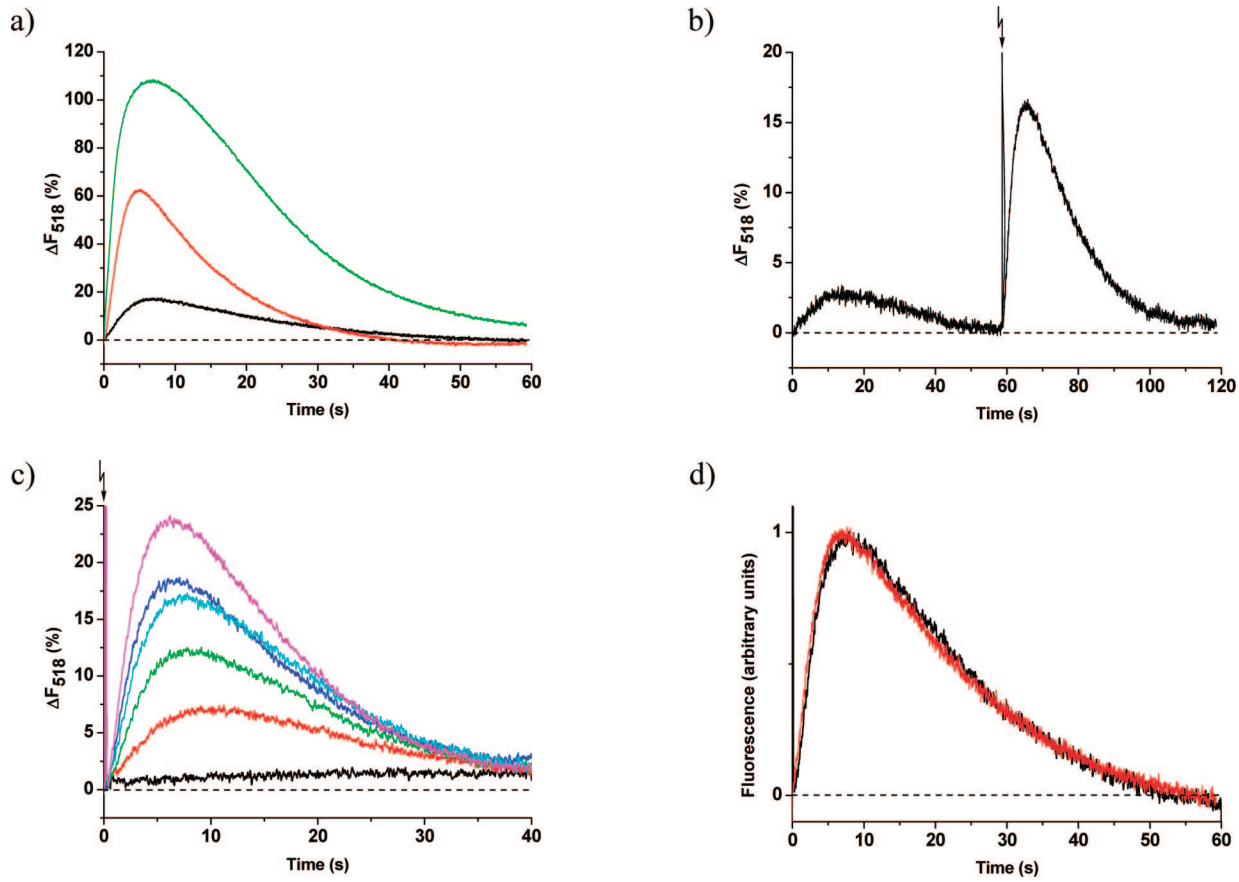
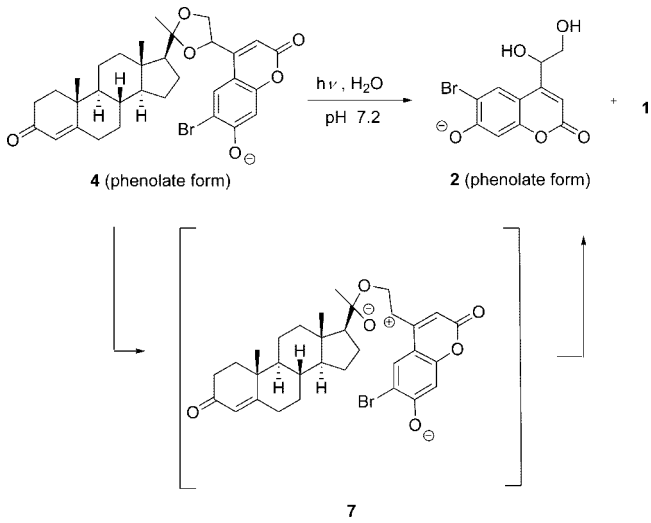


Figure 1. Changes in Ca^{2+} concentration in sperm detected by ΔF_{518} of Fluo-4. (a) Progesterone-induced Ca^{2+} signals upon stimulation of sperm at $t = 0$; 15 nM (black), 50 nM (red), and 1.25 μM **1** (green); each trace represents the average of two or three recordings. The control (mixing sperm with HTF^{++} buffer) was subtracted from the progesterone-induced signals. (b) Signal evoked by the residual activity of 5 μM **4** ($t = 0$) and then by photolysis of **4** ($t =$ 60 s); vertical line indicates flash artifact. (c) Light titration − Sperm were stimulated with 7.5 μM **4**. **1** was released by flashes of UV light of different intensities. Ca^{2+} signals evoked by 25% flash (red), 50% flash (green), 100% flash (blue), two 50% flashes separated by 50 ms (cyan), and two 100% flashes separated by 50 ms (pink). In a control experiment, photodamage due to UV light was tested by flashing only the Fluo-4 loaded sperm (black). $t = 0$ coincides with the time of flash, that is, after the Ca^{2+} signal evoked by the residual activity of **4** had returned to the basal level. (d) Comparison of the waveform of Ca^{2+} signals induced by **1** and photolysis of **4**: signal induced by 10 nM **1** (red) and by 100% flash (black); $t = 0$ denotes the time of flash and the time of mixing, respectively. The signals are normalized and superimposed on each other.

Scheme 3. Photolysis of **4**



Time-resolved fluorescence measurements upon single-pulse excitation (0.5 ns half-width, 337 nm) of **3** and **4** yield fluorescence lifetimes τ very similar to that of diol **2**; see Table 1. This is not unexpected because (i) fluorescence is emitted from the same Bhc-chromophore, and (ii) the additional photocleavage of **3** and **4** occurs with only relatively small quantum yields ϕ_{chem}. The primary reaction to the intermediate **7** is still very fast. Inserting the ϕ_{chem} value and the fluorescence quantum yields ϕ_f of **4** and **2** in the kinetic scheme developed for the coumarinylmethyl esters[17,18] yields a rate constant of $(1.2 \pm 0.5) \times 10^8$ s^{-1} for the heterolytic bond cleavage of **4**. However, we note that the experiments do not allow conclusions regarding the rate of the secondary reaction, the release of progesterone **1**.

Table 1. Photophysical and Photochemical Properties of Diastereomeric Mixtures of the Caged Progesterones **3** and **4** and of the Diol **2** in Acetonitrile/HEPES-KCl Buffer (40:60), pH 7.2

compound	λ_{abs}^{max} (nm)	ε^{max} (M^{-1} cm^{-1})	ϕ_{chem}	λ_f^{max} (nm)	ϕ_f	τ (ns)
3	374	13 800	0.002	474	0.52	4.9
4	374	14 600	0.004	473	0.38	4.7
2	375	16 100		470	0.58	4.6

Table 1 lists photophysical and photochemical data of compounds **2**, **3**, and **4**. The size of the photochemical quantum yields is small for the caged progesterones but acceptable for application, because the high absorptivities ensure sufficient

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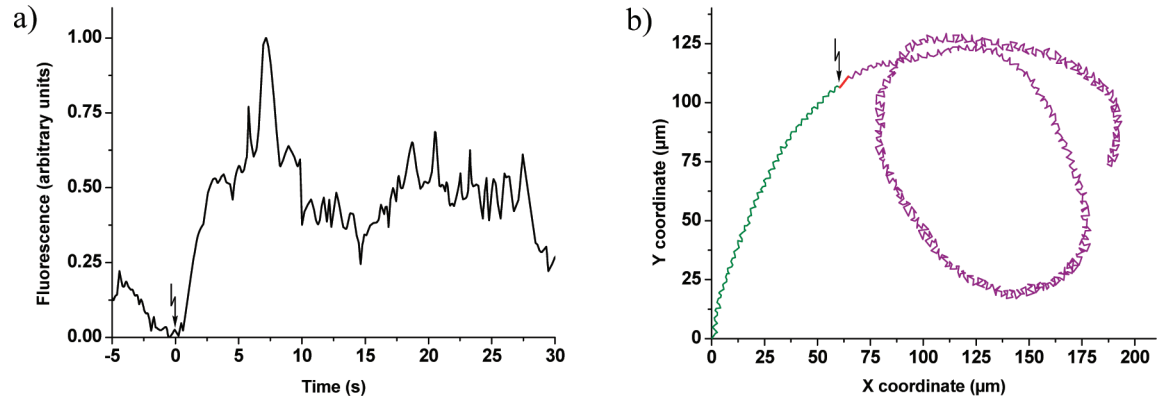

Figure 2. Changes in $[Ca^{2+}]_i$ and swimming behavior of single cells. (a) Fluorescence signal from the flagellum of a swimming cell. The sample was irradiated at $t = 0$ s. The fluorescence has been normalized. (b) Trajectory of the head before (green) and after (violet) the UV flash (red).

photosensitivity. The sensitivity of compound **4** is higher than that of isomer **3**. Fluorescence quantum yields, ϕ_f, are relatively high for caged compounds due to the large value of ϕ_f of **2** and the small efficiency of chemical deactivation of singlet excited **3** and **4** by heterolytic bond cleavage. The Bhc-ED-caged progesterones **3** and **4** are also sensitive to multiphoton excitation; that is, UV absorption can be replaced by the simultaneous absorption of infrared (IR) photons. IR light gives significantly deeper tissue penetration than UV/vis light. Exposure of **4** to femtosecond pulses of a mode-locked Ti:sapphire laser at 755 nm caused significant release of **1**. The diastereomers of **4** decompose about 5.4 times less efficiently than does (*N*-6-bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl-L-glutamic acid (Bhc-Glu).[14]

Stimulation of Human Sperm. The suitability of the caged compounds for the study of progesterone's rapid nongenomic action was tested with human sperm. Rapid mixing of sperm and **1** in a stopped-flow apparatus resulted in a transient increase of intracellular Ca^{2+} concentration, measured with the Ca^{2+} indicator Fluo-4 (Figure 1). The amplitude of the Ca^{2+} signal was graded with the concentration of **1** in the nanomolar range (Figure 1a). Caged progesterone **4** (mixture of the diastereomers **4a**−**4d**) itself was approximately 500-fold less efficient to evoke a Ca^{2+} influx as compared to **1**. Stimulation of sperm with 5 μM **4** evoked a Ca^{2+} signal similar to that evoked by ~10 nM **1** (Figure 1b). The low solubility of **4** prevented one from recording a dose−response relation. The caged progesterone **3** was less suitable than **4**. Its residual efficacy was higher and the photosensitivity was lower (data not shown) as compared to that of compound **4**.

After recovery from the weak stimulation by **4**, a flash of UV light stimulated an additional Ca^{2+} signal (Figure 1b) that was proportional to light intensity (Figure 1c). The waveforms of the Ca^{2+} signals produced by **1** and by photolyzed **4** are similar (Figure 1d). By comparing Ca^{2+} signals evoked by light and by **1**, we estimated that ~0.2% of **4** became photolyzed. The Ca^{2+} responses evoked by progesterone and by photolysis of **4** occurred rapidly with almost no delay. This result strongly suggests that progesterone binds on the extracellular site of a receptor that even may be a Ca^{2+}-permeable channel itself, or any other protein involved in Ca^{2+} transport.

Compound **4** was used to record the flash-induced Ca^{2+} increase in the flagellum of single freely moving sperm (Figure 2a). At higher Ca^{2+} levels, the beating of the flagellum became more vigorous. As a consequence, the lateral head movements were larger and the curvature of the trajectory increased (Figure 2b). Thus, the motility of human sperm depends on the intracellular Ca^{2+} concentration. Whether these changes in swimming behavior are involved in chemotaxis requires further experiments.

Conclusions

We have developed for the first time caged progesterone derivatives. Compound **4** is stable, exhibits sufficient photosensitivity at long-wavelength irradiation, and allows rapid progesterone concentration jumps upon one- and multiphoton excitation under physiological conditions. The usefulness of **4** was demonstrated by measurement of progesterone-induced Ca^{2+} influx in human sperm and changes in swimming behavior of single sperm. The results show that caged progesterone opens up new ways to study rapid nongenomic progesterone effects in sperm and other cell types.

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Supporting Information Available: Full experimental details, UV, ^1H, and ^{13}C NMR spectra, full characterization of all new compounds described herein, full experimental details of the photochemical studies, sperm preparation, and biological measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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