A Mechanism-Based Fluorogenic Probe for the Cytochrome P-450 Cholesterol Side Chain Cleavage Enzyme

Daniel J. Simpson, Clifford J. Unkefer,† Thomas W. Whaley, and Babetta L. Marrone*

Life Sciences Division, Mail Stop M888, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and Isotope and Nuclear Chemistry Division, Mail Stop C345, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

Received March 5, 1991

The rate-limiting step of steroid biosynthesis is the enzymatic conversion of cholesterol to pregnenolone by cytochrome P-450$_{scc}$ (side chain cleavage) located in the inner mitochondrial membrane of all steroid producing cells. We report here the synthesis and application of a fluorogenic probe which is a cholene-based steroid with a fluorogenic moiety (resorufin) strategically located at the site of side chain cleavage. Synthesis of the probe required four steps starting from 3β-acetoxy-22,23-bisnor-5-cholenic acid and resorufin. Reaction of the probe with P-450$_{scc}$ yields pregnenolone and the highly fluorescent resorufin, thus providing a sensitive fluorescent signal representative of enzyme activity. The fluorescence quantum yield of this probe is approximately 40-fold lower (ϕ = 0.006) than resorufin (ϕ = 0.23) and is essentially nonfluorescent at wavelengths used to excite resorufin. The utility of the probe is demonstrated biochemically by incubation with mitochondria known to contain the P-450$_{scc}$ enzyme, and its specificity for this enzyme is shown by regulation of the enzyme activity with inhibitors and through the use of a nonspecific substrate.

Introduction

Our interest in the biosynthesis of steroid hormones and its corresponding signal transduction pathway led us to develop a fluorogenic probe for the cytochrome P-450$_{scc}$ (side chain cleavage) enzyme. This enzyme, located in the inner mitochondrial membrane, is responsible for the initial rate-limiting step of steroid biosynthesis, the conversion of cholesterol to pregnenolone. The regulation[1] and activity[2] of the enzyme are thought to be controlled by various trophic stimuli through complex second messenger signal transduction pathways and are commonly studied with radiolabel techniques.[3] Thus a fluorescent probe that is specific for the side chain cleavage enzyme used in conjunction with techniques such as fluorescence imaging microscopy and/or flow cytometry could provide a sensitive method to study enzyme activity at the single cell level and could potentially be used in tandem with probes for other physiological processes involved in signal transduction. However, while a fluorescence approach such as this offers high sensitivity and excellent cellular spatial resolution, it is ultimately limited by the availability of probes for specific cellular events.

We report here the design, synthesis, and biochemical demonstration of a mechanism-based fluorogenic substrate for cytochrome P-450$_{scc}$ that avoids the use of radiolabeled materials and provides "real-time" information about enzyme activity in mitochondria isolated from steroid producing cells. The substrate, 22-((3-oxophenoxazin-7-yl)-oxy)-5-cholen-3β-ol (1, Scheme I), is a cholene-based steroid in which the side chain at the C-22 position has been replaced with a resorufin moiety. Specificity of this substrate for the P-450$_{scc}$ enzyme is achieved by incorporating known structural and stereochemical considerations to the cholene-ring for enzyme recognition. Generation of a fluorescent signal is obtained by release of the resorufin moiety by the side chain cleavage enzyme.

The mechanics of P-450$_{scc}$ enzyme–substrate binding and side-chain cleavage have been described.[4] Once a substrate (such as cholesterol) is recognized and positioned into the enzyme active site, side-chain cleavage proceeds in three oxidative steps: (1) hydroxylation of C-22 at the *pro-R* methylene position; (2) hydroxylation of the adjacent 20α-methine position to give a vicinal diol intermediate; and (3) oxidative cleavage of the diol to give pregnenolone and a side-chain fragment. In order to bind to the enzyme, a cholene-like substrate should contain a 3β-hydroxy group and unsaturation in the 5–6 bond and have cholesterol stereochemistry at the C-17 and C-20α positions. Somewhat surprisingly, the structural requirements of the side chain beyond the C-22 position are less stringent, and a number of different side chain substituted cholene-based steroids[5] have been shown to react with the enzyme.

Resorufin (7-hydroxy-3*H*-phenoxazin-3-one) was chosen as a fluorogenic reporter because of its advantageous spectral properties. Covalently conjugated through its oxy anion, resorufin shows a large increase in absorbance energy (approximately 110 nm), a drop in absorbance extinction (by approximately $^1/_3$) relative to the anion (pK_a $\sim$ 5), and a 40-fold (vide infra) difference in fluorescence quantum yield. These features combine to make a resorufin conjugate a potentially sensitive fluorogenic probe. Resorufin has been used as a fluorescent probe for mixed-function oxidase enzymes,[6] and Burke et al.[7] have found that a series of resorufin ethyl, pentyl, and benzyl ethers, based on their distinctive substrate and reaction specificity, can be used to differentiate certain microsomal cytochrome P-450 isozymes. Presumably, metabolism of a resorufin ether proceeds by hydroxylation at the C-1 position of the ether side chain, to yield a resorufin hemiacetal, which undergoes rearrangement (hydrolytic) to the highly

† Isotope and Nuclear Chemistry Division.

(1) (a) Voutilainen, R.; Tapanainen, J.; Chung, B.; Matteson, K. J.; Miller, W. L. *J. Clin. Endocrinol. Metab.* **1986**, *63*, 202–207. (b) Zlotkin, T.; Farkash, Y.; Orly, J. *Endocrinology* **1986**, *119*, 2809–2832.

(2) (a) Hall, P. F. *Rec. Prog. Horm. Res.* **1985**, *41*, 1–37. (b) Payne, A. H.; Quinn, P. G.; Sheela Rani, C. S. *Rec. Prog. Horm. Res.* **1985**, *41*, 153–197. (c) Wang, S.; Greenwald, G. S. *Biol. Reprod.* **1984**, *31*, 271–279.

(3) (a) Hochberg, R. B.; vander Hoeven, T. A.; Welch, M.; Lieberman, S. *Biochem.* **1974**, *13*, 603–608. (b) Bermudez, J. A.; Doerr, P.; Lipsett, M. B. *Steroids* **1970**, 505.

(4) (a) Lambeth, J. D. *Endocr. Res.* **1986**, *12*, 371–392. (b) Heyl, B. L.; Tyrrell, D. J.; Lambeth, J. D. *J. Biol. Chem.* **1986**, *261*, 2743–2749. (c) Lambeth, J. D.; Kitchen, S. E.; Farooqui, A. A.; Tuckey, R.; Kamin, H. *J. Biol. Chem.* **1982**, *257*, 1876–1884.

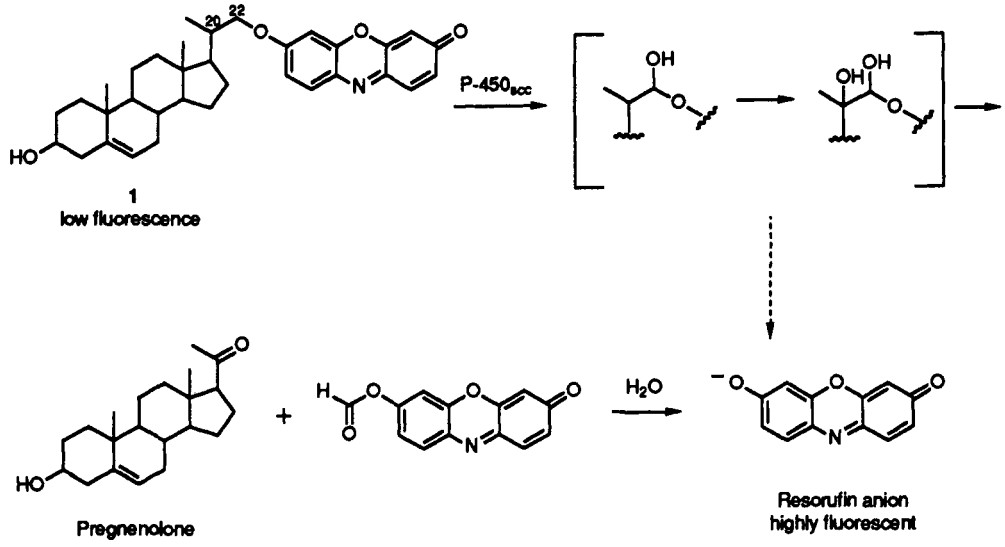
(5) Authur, J. R.; Blair, H. A. F.; Boyd, G. S.; Mason, J. I.; Suckling, K. E. *Biochem. J.* **1976**, *158*, 47–51.

(6) Prough, R. A.; Burke, M. D.; Mayer, R. T. *Methods Enzymol.* **1978**, *52c*, 372–388.

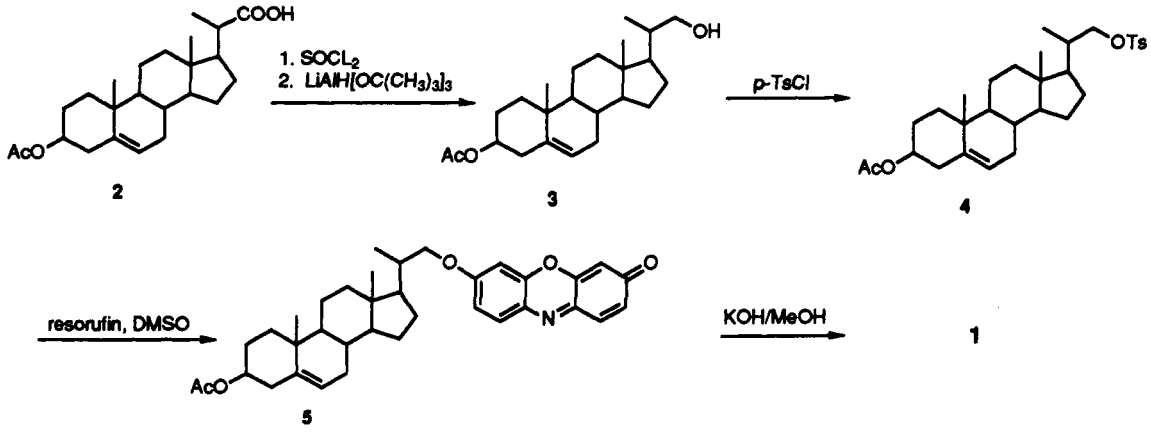
(7) (a) Burke, M. D.; Thompson, S.; Elcombe, C. E.; Halpert, J.; Haaparanta, T.; Mayer, R. T. *Biochem. Pharmacol.* **1985**, *34*, 3337–3360. (b) Lubert, R.; Mayer, R. T.; Cameron, J. W.; Nims, R. W.; Burke, M. D.; Wolff, T.; Guengerich, F. P. *Arch. Biochem. Biophys.* **1985**, *238*, 43–48.

0022-3263/91/1956-5391$02.50/0

Scheme I



Scheme II

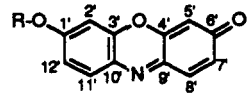


fluorescent resorufin anion. The fluorescence signal generated by the formation of resorufin is a quantitative indicator of enzyme activity. We reasoned that hydroxylation at the C-22 position of 1 by cytochrome P-450$_{scc}$ would give the C-22 hemiacetal of 1. This intermediate may either rearrange to give resorufin directly or follow a sequential mechanism as described above to give resorufin subsequent to its release from the enzyme. The overall process we expect to observe is shown in Scheme I.

Results and Discussion

A. Cytochrome P-450$_{scc}$ Fluorogenic Substrate. 22-((3-Oxophenoxazin-7-yl)oxy)-5-cholen-3β-ol (1) was synthesized from 3β-acetoxy-22,23-bisnor-5-cholenic acid (2), which has the structural and stereochemical requirements[4] for enzyme recognition and reactivity, and resorufin (Na^+ salt) in four steps (Scheme II). The conversion of the tosyl derivative 4 to the resorufin conjugate 5 (step III) was only 10% efficient. However, recovery of unreacted 4 in yields of 40–60% facilitated the recycling of 4 to increase the effective yield of 5. Among reactions with chloride, iodide, tosylate, and triflate steroids (step II), we found the tosyl moiety best suited for displacement in this synthesis.

The structures of 5 and 1 were confirmed by mass spectrometry and ^1H NMR spectroscopy, which could be assigned largely on the basis of expected chemical shifts and proton–proton coupling. ^1H NMR spectra of both 5 and 1 contained a typical steroid proton envelope from 0.75 to 2.5 ppm with distinctive methyl singlets at 0.74 and 1.0 ppm assigned to the C-18 and C-19 methyl groups and an acetyl methyl at 2.0 ppm (compound 5). The C-21 methyl group appeared as a three proton doublet at 1.14 ppm coupled to the C-20 hydrogen, which in turn was coupled to the C-22 methylene group at 3.75 and 4.0 ppm; the C-22 methylene group appeared as an AB portion of an ABX splitting pattern. The protons of the resorufin ring system were resolved into two sets of three protons, which appeared as either a doublet or a doublet of doublets consistent with the predicted splitting. Assignment of the resorufin protons was obtained from a comparison of the observed chemical shifts with the spectrum obtained for resorufin acetate (6). The protons of the resorufin ring containing the acetoxy group were shifted downfield with respect to the ring protons of 5 and 1 containing ether substituents. No shift was observed in the dienone ring of acetylresorufin relative to 5 or 1. These shifts can be used to assign the protons for the resorufin rings of 5 and 1 by inference.



6 R = Ac
7 R = Et

B. Spectral Characterization. The sensitivity of this substrate as a fluorogenic probe is derived from the dif-

Table I. Absorption and Fluorescence Properties

	absorbance		fluorescence	
compound	abs max, nm	ϵ, M^{-1} cm^{-1}	em max, nm	ϕ
1[a]	463	20600	562	0.006
resorufin ethyl ether[a]	463	20700	563	0.005
resorufin[b]	572	60000	586	0.23
fluorescein[b]	490	61400	514	0.85[c]

[a] Measured in methanol. [b] Measured in 0.001 N NaOH. [c] Reference.

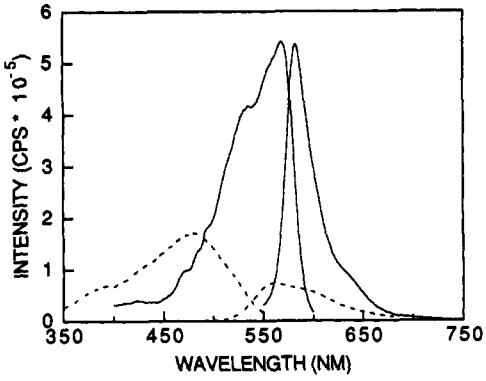


Figure 1. Fluorescence excitation and emission spectra that help demonstrate the fluorogenic nature of 1. Intensity, measured in counts per second (cps), is absolute for these spectra. The dashed lines represent a 1 μM solution of 1 in methanol the excitation collected at an emission wavelength of 560 nm and the emission spectrum with excitation at 460 nm. The solid line for comparison are the excitation and emission spectra for resorufin at 1 nM in methanol. The respective emission and excitation wavelengths used here are 610 and 530 nm. All spectra were obtained at a bandpasses of 1-nm excitation and emission.

ference in absorbance energy, in emission energy, and in quantum efficiency of resorufin relative to the substrate. The absorption and emission energy, and relative quantum yield of compound 1 are given in Table I. The values reported for 1 and resorufin ethyl ether (7) were determined in methanol due to the low solubility of 1 in water. The fluorescence excitation and emission spectra measured in methanol are given in Figure 1 for compound 1 at 1 μM and for resorufin at 1 nM. The shifted spectral properties and 40-fold quantum yield difference of 1 relative to resorufin suggest that small amounts of resorufin should be detectable in the presence of high concentrations of 1. Experimentally, we could detect resorufin in a solution of 1 down to the limit defined by our spectrofluorometer (25 pM, as configured for biochemical studies) with no interference from 1 up to concentrations (1–2 μM) routinely used in biochemical studies.

C. Biochemical Characterization. To determine if 1 was metabolized by the side chain cleavage enzyme to pregnenolone and resorufin, mitochondria known to contain P-450$_{scc}$ were isolated[8] from a clonal steroidogenic Leydig tumor cell (MA-10) line[9] and incubated with compound 1. Mitochondrial incubations were monitored for increased fluorescence. Typically, substrate dissolved in 95% ethanol was diluted into a buffered suspension to give a final concentration of approximately 1 μM, and the emission spectrum was measured immediately to establish background fluorescence, which consisted largely of scatter from the mitochondria and a small signal from the substrate when excited at 530 nm (10-nm bandpass). This

(8) (a) Asem, E. K.; Hertelendy, F. *Biol. Reprod.* **1985**, *32*, 257–268. (b) Toaff, M. E.; Strauss, J. F., III *J. Biol. Chem.* **1979**, *254*, 3977–3982.
(9) Ascoli, M. *Endocrinology* **1981**, *108*, 88–95.

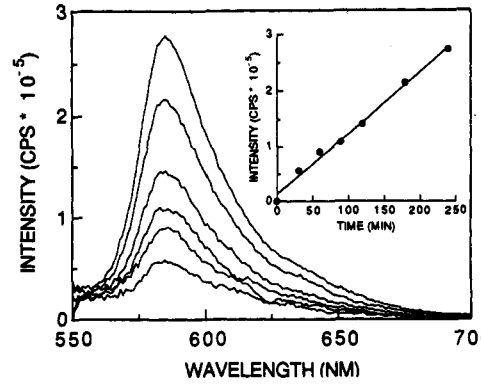


Figure 2. Fluorescence emission spectrum (530-nm excitation) obtained from MA-10 mitochondria treated with 1 (1 μM) in buffer measured at 0.5, 1, 2, 3, and 4 h. Bandpasses used to acquire these data were 10-nm excitation and 8-nm emission. The insert in this figure shows the change in fluorescence intensity at 586 nm as a function of time.

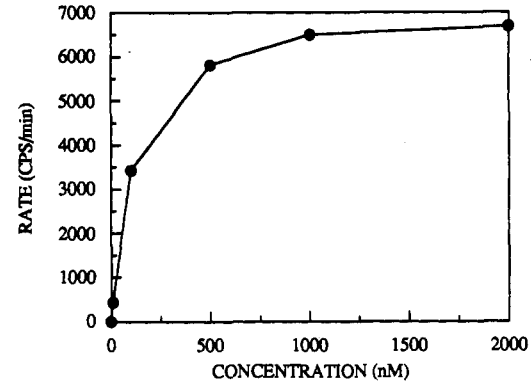


Figure 3. Mitochondrial metabolism of 1 expressed as a reaction rate in units of signal intensity at 586 nm per unit time is shown for five initial substrate concentrations. The rates used here were determined from a linear regression (see Figure 2 insert) of fluorescence intensity (cps) versus time over a 3-h experiment.

excitation wavelength was chosen because the small (14 nm) Stokes shift of resorufin and the highly scattering nature of the mitochondria made excitation near the absorbance maximum of resorufin (572 nm) difficult. This instrumental configuration allowed detection of resorufin in the pM concentration range (25 pM lower limit) and for spectral acquisition of fluorescence emission from 550 to 700 nm (8-nm bandpass).

As shown in Figure 2, mitochondria treated with 1 produced a time-dependent increase in fluorescence with line shape and emission maximum (586 nm) consistent with the emission spectrum of resorufin. This increase in fluorescence emission at a single wavelength, shown in the insert for 586 nm, was linear over the course of a 4-h experiment. The contribution of substrate fluorescence was canceled by subtracting the time zero spectrum from the later time points. Because the quantum yield of 1 was small changes in the concentration of 1 over the course of an experiment were not detected. Thus, the spectral changes observed reflect only the enzyme-catalyzed release of resorufin from 1. Using standards of resorufin in buffer to determine the concentration of resorufin released from 1 in the mitochondrial preparation, the rate of mitochondrial-catalyzed reaction (Figure 2) was determined to be approximately 3.5 pM min^{-1}. As a control mitochondria from a nonsteroidogenic line of Chinese Hamster Ovary cells (CHO) was also incubated with 1 in an experiment analogous to the MA-10 mitochondria incubation. As expected no resorufin fluorescence was observed with CHO

mitochondria, suggesting that the P-450$_{scc}$ enzyme is necessary for the metabolism of 1.

The dependence of the rate of reaction on the initial concentration of 1 with MA-10 mitochondria is shown in Figure 3. The rates are determined from the linear regime of intensity increase (586 nm) with time for 3-h incubations of the mitochondria with increasing concentrations of 1. At low concentration the rate of the reaction was dependent on the initial substrate concentration (up to 100 nM). Consistent with the Michaelis–Menton saturation kinetics, at higher concentration of 1, the rate of the reaction became independent of substrate concentration. The maximum velocity of the reaction was obtained at substrate concentrations greater than 1 μM.

To further demonstrate the specificity of the side chain cleavage enzyme for compound 1, we examined the metabolism of resorufin ethyl ether (7) by steroidogenic mitochondria. If the release of resorufin from 1 were the result of a nonspecific process one might expect resorufin ethyl ether to produce fluorescence with a comparable rate. The fluorescence emission increase observed with resorufin ethyl ether was not indicative of free resorufin, but instead displayed a line shape similar to that of resorufin ethyl ether itself, suggesting a slow increase in substrate concentration perhaps due to dissolution into the mitochondrial matrix. Pronounced resorufin-like fluorescence was only apparent after 4 h. The rate of resorufin derived from the reaction of resorufin ethyl ether was below detectable limits (approximately 0.4 pM min^{-1}) at concentrations of resorufin ethyl ether up to 10 μM. The apparent 10-fold or greater difference in rate suggests that P-450$_{scc}$ demonstrates a pronounced preference for the cholene ring system of 1 over the ethyl side chain of resorufin ethyl ether.

Two pharmacological inhibitors of steroidogenesis, aminoglutethimide[10] and ketoconazole,[11] which are known to act at the cytochrome P-450$_{scc}$ level, inhibited the production of resorufin fluorescence when added at high concentration (10 mM). However, we found that both aminoglutethimide and ketoconazole quench resorufin fluorescence to an extent of approximately 80% and 5%, respectively, when applied at these concentrations. To adjust for this quenching effect, resorufin standards were measured in the presence of each inhibitor (10 mM) in buffer over the concentration range in which resorufin was expected in these experiments. At the maximum inhibitor concentrations obtainable (10 mM aminoglutethimide or 1 mM ketoconazole) the rate of resorufin production (2 pM min^{-1} for aminoglutethimide and 1 pM min^{-1} for ketoconazole) was considerably less than that obtained in the absence of inhibitor (3.5 pM min^{-1}). Complete inhibition was not observed with either compound perhaps due to the insolubility of these inhibitors in the buffer at 1–10 mM.

In all incubations, attempts to measure pregnenolone by standard radioimmunoassay techniques were made. In steroidogenic mitochondria (MA-10), pregnenolone levels increased with time and were partially inhibited by both aminoglutethimide and ketoconazole. However, the high levels of pregnenolone produced by conversion of endogenous cholesterol precluded us from making a quantitative comparison of pregnenolone and resorufin derived from the reaction of 1 with steroidogenic mitochondria. Pregnenolone levels in samples treated with 1 were indistinguishable from levels obtained in the absence of 1, typically in the range of 10–1000 nM over the course of a 4-h experiment and highly variable depending on the mitochondrial preparation. The amount of pregnenolone found due solely to conversion of 1 by the side chain cleavage enzyme was estimated to be in the range of 100–1000 pM by inference from resorufin fluorescence.

Conclusion

We have synthesized a mechanism-based fluorogenic substrate for the rate-limiting step of steroid biosynthesis, the conversion of cholesterol to pregnenolone by the enzyme cytochrome P-450$_{scc}$. The probe is essentially nonfluorescent until it is metabolized by the P-450$_{scc}$ enzyme. This process was demonstrated by following the fluorescence emission of the reaction of 1 with mitochondria containing the P-450$_{scc}$ enzyme. In the absence of mitochondria or in mitochondria which do not express P-450$_{scc}$ enzyme (from CHO cells), no significant spectral changes indicative of the metabolism of 1 were observed. Selectivity by the P-450$_{scc}$ enzyme for this substrate was demonstrated by comparing the rate at which resorufin is produced from 1 to the rate observed for incubations with resorufin ethyl ether (7). That the enzymatic conversion of 1 to resorufin is derived from the reaction of 1 with the target enzyme is further suggested by the observed inhibition of resorufin fluorescence when MA-10 mitochondria are incubated with 1 in the presence of the cytochrome P-450 specific inhibitors, ketoconazole and aminoglutethimide.

The apparent K_m (0.2 μM) for the reaction of 1 with steroidogenic mitochondria was determined from a double-reciprocal plot of Figure 3 data. Because the mitochondrial preparation may contain endogenous cholesterol which is a competitive substrate for the enzyme this K_m represents a maximal value. The actual K_m is expected to be lower than the observed 0.2 μM value. This constant is also in reasonable agreement with the K_m (0.5–0.9 μM) reported[8a] for the reaction of 25-hydroxycholesterol with steroidogenic granulosa cell mitochondria determined from measurement of pregnenolone by radioimmunoassay (RIA). Furthermore, the detection limit of 25 pM resorufin is well within the sensitivity limit observed for RIA measurement (10–100 pM pregnenolone) of P-450$_{scc}$ enzyme activity in the MA-10 mitochondria. Thus, the approach presented here offers the same sensitivity afforded by RIA with the advantage of continuous, real-time measurement of enzyme activity.

Our motivation for developing a fluorescent probe specific for the P-450$_{scc}$ enzyme arose from our interest in studying regulation of the activity of this enzyme in single steroidogenic cells by flow and fluorescence imaging cytometry. The utilization of this probe demonstrated by its measurement in mitochondrial preparations indicates that 1 is capable of passing through a lipid bilayer. This suggests that 1 could potentially be applied to whole cells and that in such application cellular and/or subsequent mitochondrial uptake would not be a limiting factor. In preliminary experiments[12] we have observed resorufin-like fluorescence spectral increases when whole viable MA-10 cells were treated with 1. We conclude that 1 offers the potential to examine the role of trophic stimuli in steroid biosynthesis at the single cell level and is a sensitive al-

(10) Inaba, T.; Wiest, W. G.; Niswender, G. D. *Steroids* **1980**, *34*, 665–675.

(11) (a) Sheets, J. J.; Mason, J. I. *Drug Metab. Disp.* **1984**, *12*, 603–606. (b) Loose, D. S.; Kan, P. B.; Hirst, M. A.; Marcus, R. A.; Feldman, D. *J. Clin. Invest.* **1983**, *71*, 1495–1499. (c) Feldman, D. *Endocrine Rev.* **1986**, *7*, 409–420. (d) Oates, J. A.; Wood, A. J. J. *N. Engl. J. Med.* **1987**, *317*, 4812–4818.

(12) Marrone, B. L.; Simpson, D. J.; Yoshida, T. M.; Unkefer, C. J.; Whaley, T. W.; Buican, T. N. *Endocrinology* **1991**, *128*, 2654–2656.

ternative to radiolabel methods presently used to study the P-450$_{scc}$ enzyme.

Experimental Section

A. Optical Measurements. All measurements were made in air-saturated solutions at carefully controlled temperatures. Solvent measurements, characterization, and quantum yield were made in HPLC grade solvents and used without further purification. Absorbance measurements were made in a 1-cm quartz cell on a spectrophotometer at 2-nm spectral resolution. Fluorescence characterization and quantum yield measurements were made on a spectrofluorometer fitted with a thermostated cell holder for precise temperature control and coupled to an 286-compatible computer for data collection and processing. For quantum measurements excitation and emission bandpasses were 1 nm in all cases. Samples were thermostated at 25 °C. All spectra were background subtracted and corrected for instrument responses by using manufacturer supplied corrections. Quantum yields (ϕ) were calculated with respect to fluorescein disodium[13] (Eastman Kodak Co.) by the equation:

$$\phi = \frac{F_s A_f \eta_s^2 \phi_f}{A_s F_f \eta_0^2} \quad (1)$$

where the subscript s refers to the sample, f refers to fluorescein, ϕ is quantum yield, A is absorbance at the excitation wavelength, F is the corrected, integrated fluorescence, η is refractive index, η_0 refractive index of water.

B. Synthesis. Melting points, which are uncorrected, were measured on a capillary melting point apparatus. Proton and ^{13}C NMR spectra were obtained at either 200, 250, or 300 MHz; chemical shifts are reported relative to $CHCl_3$ at 7.240 ppm for ^1H and 77.0 ppm for ^{13}C. The numbering system used to assign the protons of the resorufin rings of 1 and 5 is defined in structures 6 and 7. Mass spectra (MS) were obtained by thermal desorption from a copper-tip probe, in either electron impact (EI) of chemical ionization (CI) at the Woods Hole Oceanographic Institution (Woods Hole, MA). Elemental analysis were obtained on a CHN Elemental Analyzer housed in INC division (LANL).

The phrase "dried, and evaporated" refers to drying with $MgSO_4$, followed by evaporation on a Buchi rotary evaporator under reduced pressure. Reactions were monitored by thin-layer chromatography (TLC) using Baker-flex Silica Gel IB-F precoated (250-μm thickness) plastic-backed strips. Preparative TLC was performed on 20 × 20 cm TLC plates of 1-mm thickness coated with Baker silica gel 60. Column chromatography was carried out with Sargent-Welch 60–200-mesh silica gel or E. Merck 230–400-mesh grade 60 silica gel for flash column separation under positive N_2 pressure.

3β-Acetoxy-5-cholen-22-ol (3). 3β-Acetoxy-22,23-bisnor-5-cholenic acid (2, 1.0 g, Steraloids, Inc. Wilton, NH) was dissolved in methylene chloride (5 mL) and cooled to 0–5 °C with an ice bath. Thionyl chloride (900 mg) was added dropwise via a syringe to the clear solution. The solution was stoppered, allowed to warm to room temperature, and stirred for 20 h. This mixture was diluted with methylene chloride, extracted with aqueous NaCl, and dried and evaporated to a white solid. No purification was attempted; the ^{13}C NMR suggested a high degree of purity with a prominent peak at 176 ppm (COCl) not present in the starting material.

This crude acid chloride was dissolved in methylene chloride (5 mL) and cooled to −65 °C (dry ice/acetone). $LiAlH[OC(CH_3)_3]_3$ in THF (1.0 M, 2.3 mL) was added under Ar to the acid chloride over 20 min. The reaction was kept at −65 °C for 1 h after addition then allowed to warm to room temperature. Excess hydride reagent was destroyed by the slow dropwise addition of water. The mixture was extracted with methylene chloride, washed extensively with 1.0 N HCl and aqueous NaCl, dried, and evaporated. Compound 3 was purified by column chromatography, eluting with 1% methanol/methylene chloride and obtained as a solid from methylene chloride/n-hexane. Yield: 640 mg. Mp: 154–155 °C. Anal. Calcd for $C_{24}H_{38}O_3$: C, 76.95; H, 10.23. Found: C, 76.93; H, 10.46. MS (EI): m/z 314 ($M^+ - CH_3CO_2H$, 30). ^1H NMR (250 MHz, $CDCl_3$): δ 5.34 (1 H, d, 6-H), 4.6 (1 H, m, 3α-H), 3.6 and 3.3 (2 H, dd, 22-CH_2), 2.3 (2 H, d, 4-CH_2), 2.01 (3 H, s, 3-OAc), 1.03 (3 H, d, 21-Me, J = 6.6 Hz), 1.00 (3 H, s, 19-Me), 0.68 (3 H, s, 18-Me), 2.0–0.9 (19 H, cholene ring methylene and methine H's).

3β-Acetoxy-22-O-(p-toluenesulfonyl)-5-cholen-22-ol (4). Compound 3 (200 mg) was dissolved in dry pyridine (1 mL) in a small reacta-vial, and p-toluenesulfonyl chloride (175 mg) was added. The vial was capped and stirred for 20 h. This mixture was diluted with ether, washed with 0.1 N HCl (3 × 50 mL), dried, and evaporated to a colorless oil. The product was recovered as a crystalline white solid from ether/n-hexane. Yield: 185 mg. Mp: 108–110 °C. Anal. Calcd for $C_{31}H_{44}O_5S$: C, 70.41; H, 8.39. Found: C, 70.54; H, 8.33. MS (EI): m/z 468 ($M^+ - CH_3CO_2H$, 100). ^1H NMR (250 MHz, $CDCl_3$): δ 7.75 and 7.3 (4 H, each d, phenyl H's), 5.35 (1 H, d, 6-H), 4.6 (1 H, m, 3α-H), 3.95 and 3.75 (2 H, dd, 22-CH_2), 2.43 (3 H, s, p-tosyl-Me), 2.31 (2 H, d, 4-CH_2), 2.01 (3 H, s, 3-OAc), 0.98 (3 H, d, 21-Me), 0.98 (3 H, s, 19-Me), 0.68 (3 H, s, 18-Me), 2.0–0.9 (19 H, cholene ring methylene and methine H's).

3β-Acetoxy-22-((3-oxophenoxazin-7-yl)oxy)-5-cholene (5). Steroid 4 (240 mg) was suspended in DMSO (3 mL) and combined with resorufin (200 mg). This mixture was stirred under Ar at 55 °C for 10 days. The DMSO was removed by lyophylization (10 h, 10^{-5} Torr). The resulting solid was suspended in methylene chloride and filtered to remove unreacted resorufin and give a bright orange solution. Thin-layer chromatography showed one orange spot more polar than the tosylate precursor seen after development with phosphomolybdic acid. The desired product was purified by flash chromatography (eluting with 3% tetrahydrofuran/methylene chloride) and obtained as small orange needles from methylene chloride/n-hexane. Yield: compound 5, 22 mg; unreacted 4, 140 mg. Mp: 252–255 °C dec. Anal. Calcd for $C_{36}H_{43}O_5N{\cdot}H_2O$: C, 73.59; H, 7.66; N, 2.39. Found: C, 73.78; H, 7.51; N, 2.32. MS (CI) m/z 570 [(M^+ + 1), 100], 598 [(M^+ + 29), 20], 510 [(M^+ + 1) − CH_3CO_2H, 40]. ^1H NMR (250 MHz, $CDCl_3$): δ 7.67 (1 H, d, 11′-H, $J_{11'-12'}$ = 9.1 Hz), 7.40 (1 H, d, 8′-H, $J_{7'-8'}$ = 10 Hz), 6.92 (1 H, dd, 12′-H, $J_{11'-12'}$ = 9.0 and $J_{2'-12'}$ = 2.4 Hz), 6.81 (1 H, dd, 7′-H, $J_{7'-8'}$ = 9.8 and $J_{5'-7'}$ = 1.9 Hz), 6.79 (1 H, d, 2′-H, $J_{2'-12'}$ = 2.3 Hz), 6.3 (1 H, d, 5′-H, $J_{5'-7'}$ = 1.9 Hz), 5.35 (1 H, d, 6-H), 4.6 (1 H, m, 3α-H), 4.0 and 3.77 (2 H, dd, 22-CH_2), 2.30 (2 H, d, 4-CH_2), 2.02 (3 H, s, 3-OAc), 1.14 (3 H, d, 21-Me, J = 6.5 Hz), 1.01 (3 H, s, 19-Me), 0.73 (3 H, s, 18-Me), 2.0–0.9 (19 H, cholene ring methylene and methine H's).

22-((3-Oxophenoxazin-7-yl)oxy)-5-cholen-3β-ol (1). The acetate 5 (15 mg) was suspended in 5% KOH/methanol (2 mL), containing approximately 1% water, and refluxed for 30 min while Ar was bubbled through the solution. After cooling methylene chloride was added and this mixture was washed with 1 M HCl and water, dried, and evaporated. Thin-layer chromatography showed two spots, one minor spot (R_f 0.5) which corresponded to 5 and the more polar product (R_f 0.2) 1. Because of the small amount of material, purification was achieved by preparative TLC, eluting with 3% methanol/methylene chloride, and the product recovered was obtained as an orange solid from methylene chloride/n-hexane. Yield: 7 mg. Mp: >230 °C dec. Anal. Calcd for $C_{34}H_{41}O_4N{\cdot}^1/_2H_2O$: C, 76.12; H, 7.65; N, 2.61. Found: C, 76.19; H, 7.81; N, 2.41. MS (CI): m/z 528 [(M^+ + 1), 100], 556 [(M^+ + 29), 20], 510 [(M^+ + 1) − H_2O, 30]. ^1H NMR (250 MHz, $CDCl_3$): δ 7.67 (1 H, d, 11′-H, $J_{11'-12'}$ = 8.9 Hz), 7.40 (1 H, d, 8′-H, $J_{7'-8'}$ = 9.8 Hz); 6.91 (1 H, dd, 12′-H, $J_{11'-12'}$ = 8.9 and $J_{2'-12'}$ = 2.5 Hz), 6.80 (1 H, dd, 7′-H, $J_{7'-8'}$ = 9.9 and $J_{5'-7'}$ = 1.9 Hz), 6.77 (1 H, d, 2′-H, $J_{2'-12'}$ = 2.6 Hz), 6.30 (1 H, d, 5′-H, $J_{5'-7'}$ = 1.9 Hz), 5.35 (1 H, d, 6-H), 4.0 and 3.78 (2 H, dd, 22-CH_2), 3.5 (1 H, m, 3α-H), 2.26 (2 H, d, 4-CH_2), 1.14 (3 H, d, 21-Me, J = 6.6 Hz), 1.00 (3 H, s, 19-Me), 0.74 (3 H, s, 18-Me), 2.0–0.9 (19 H, cholene ring methylene and methine H's).

C. Biochemical Studies. Cells were grown and isolated according to previously published procedure.[12] Mitochondria was prepared from MA-10 (or CHO) cells by a procedure described[14] by Toaff et al. with some modification. The MA-10 cells were

(13) (a) Habashy, M. M.; Antonious, M. S.; El-sayed, B. A.; Abdel-Mottaleb, M. S. A. *J. Lumin.* **1986**, *36*, 173. (b) Munkholm, C.; Parkinson, D.-R.; Walt, D. R. *J. Am. Chem. Soc.* **1990**, *112*, 2608–2612.

(14) Toaff, M. E.; Strauss, J. F., III; Flickinger, G. L.; Shattil, S. J. *J. Biol. Chem.* **1979**, *254*, 3977–3982.

homogenized at 4 °C in 5–7 volumes of medium containing sucrose (250 mM), EDTA (1 mM), Tris (25 mM), pH 7.4, and BSA (10 mg/mL). The homogenate was centrifuged at 600g for 10 min, and the resulting supernatant was centrifuged at 15000g for 10 min to sediment mitochondria. Mitochondria were suspended in a buffer of sucrose (200 mM), $MgCl_2$ (5 mM), KCl (20 mM), EDTA (0.2 mM), Tris (25 mM), BSA (1 mg/mL), and Na_2HPO_4 (10 mM), pH 7.4. Aliquots (1 mL) containing mitochondrial protein (between 100 and 1000 μg/mL) were combined (100:1) with sodium isocitrate (10 mM) and inhibitor (10:1) when relevant and preincubated at 37 °C for 15 min prior to addition of 1. The substrate was added from a stock solution (100 μM in methoxyethanol) to give a final concentration of 1–2 μM with a final solvent concentration of 1–2%. To establish the background (substrate) fluorescence the emission spectrum of the mitochondrial suspension was taken immediately following addition of 1 to the suspension. Between measurements the mitochondria were kept at 37 °C in a shaking water bath. Control samples which did not contain 1 were treated with 1–2% methoxyethanol. Inhibitors were added from stock solutions (10 and 100 mM) in water (pH 4, HCl). Some cloudiness was evident with ketoconazole when diluted into the mitochondrial incubate.

Acknowledgment. We gratefully acknowledge Dr. Mario Ascoli, University of Iowa, College of Medicine, Iowa City, IA, for generously providing MA-10 cells, Dr. Robert A. Tobey of LANL for providing CHO cells, and D. Ehler (INC division, LANL) for obtaining elemental analysis data. We also thank Dr. Neil V. Blough and Carl G. Johnson of the Woods Hole Oceanographic Ins., Woods Hole, MA, for collecting mass spectral data. This work was supported by NIH grant R01-HD22195 to B.L.M. and by funds from the Los Alamos National Laboratory Research and Development (I.S.R.D.) program and the National Flow Cytometry Resource P41-RR01315.

Registry No. 1, 135212-24-7; 2, 1474-14-2; 3, 10162-98-8; 4, 120664-97-3; 5, 135075-43-3; resorufin, 635-78-9; cytochrome P-450, 9035-51-2; steroid 17–20 desmolase, 9044-50-2.

Interconversion and Phosphoester Hydrolysis of 2′,5′- and 3′,5′-Dinucleoside Monophosphates: Kinetics and Mechanisms

Pia Järvinen, Mikko Oivanen, and Harri Lönnberg*

Department of Chemistry, University of Turku, SF-20500 Turku, Finland

Received March 5, 1991 (Revised Manuscript Received May 23, 1991)

First-order rate constants for the interconversion and hydrolytic cleavage of several 2′,5′- and 3′,5′-dinucleoside monophosphates (UpU, UpA, ApU, ApA) have been determined over an acidity range from $H_0 = -0.2$ to $H_- = 12.4$ at 363.2 K. Both reactions proceed at comparable rates at pH < 2 and are of first order with respect to hydronium ion at pH < pK_a of the phosphate moiety (0.7) and second-order under less acidic conditions (pH 1–2). With dinucleoside monophosphates derived from adenosine, acid-catalyzed depurination of the starting material competes with the phosphate migration and phosphoester hydrolysis at pH < 3. The migration rates extrapolated to zero buffer concentration become pH-independent at pH > 4. Under these conditions the migration is considerably faster than the phosphoester hydrolysis, which exhibits acid catalysis at pH < 5 and base catalysis under more basic conditions. By contrast, hydrolysis of the 5′-phosphoester bond is the only reaction detected in alkaline solutions (pH > 8). The reaction is first order with respect to hydroxide ion at $[OH^-] < 0.01$ mol dm^{-3} and approaches zero-order dependence at higher alkalinities, where the unesterified 2′- or 3′-hydroxyl group becomes ionized. The mechanisms of different partial reactions, and the effects of base moiety structure (purine vs pyrimidine) on their rates are discussed. The data are compared to the known reaction kinetics of monoalkyl esters of adenosine 2′- and 3′-monophosphates.

Introduction

Internucleosidic 3′,5′-phosphodiester bonds play a central role in chemistry and biochemistry of nucleic acids. In particular, their hydrolytic reactions have been the subject of considerable interest as a model system for the action of ribonucleases. Breslow and co-workers have shown that uridylyl(3′,5′)uridine[1,2] and adenylyl(3′,5′)-adenosine[3] undergo under neutral or mildly acidic condition a buffer-catalyzed isomerization to the corresponding 2′,5′-dinucleoside monophosphates and a buffer-catalyzed hydrolysis to form a nucleoside cyclic 2′,3′-monophosphate with cleavage of the 5′-linked nucleoside. Both reactions have been shown to proceed through the same phosphorane intermediate obtained by nucleophilic attack of the neighboring 2′/3′-hydroxyl group on the tetracoordinated phosphorus atom.[2,3] Formation of this intermediate exhibits a specific-acid/general-base catalysis, its breakdown to isomeric dinucleoside monophosphates a general-acid catalysis, and cleavage of the 5′-linked nucleoside a specific-base/general-acid catalysis.

We have recently studied the buffer-independent isomerization and phosphoester hydrolysis of the monomethyl and monoisopropyl esters of adenosine 2′- and 3′-monophosphates over a wide acidity range ($H_0 = -0.2$ to pH 11).[4] The alkylphosphate migration and hydrolysis to cyclic 2′,3′-monophosphate and alcohol proceed at comparable rates under acidic conditions. At neutral pH a pH-independent phosphate migration prevails, in striking contrast to the buffer-catalyzed reactions investigated by Breslow.[1,2] By contrast, in alkaline solutions only phosphoester hydrolysis was observed to take place, consistent with earlier studies on hydrolysis of dinucleoside monophosphates.[5] In all likelihood both migration and hydrolysis proceed through a common phosphorane intermediate. However, in aqueous alkali, where no phosphate migration could be

(1) Corcoran, R.; LaBelle, M.; Czarnik, A. W.; Breslow, R. *Anal. Biochem.* **1985**, *144*, 563.
(2) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473.
(3) Breslow, R.; Huang, D.-L. *J. Am. Chem. Soc.* **1990**, *112*, 9621.
(4) Oivanen, M.; Schnell, R.; Pfleiderer, W.; Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 3623.
(5) Koike, T.; Inoue, Y. *Chem. Lett.* **1972**, 569.

0022-3263/91/1956-5396$02.50/0