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4'-Hydroxystyryldiazines: Synthesis and Fluorescence Properties

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Abstract: In order to investigate the effect that additional nitrogen substitution in the 4'-hydroxystyryl*pyridine* system would have on the photophysical properties of this molecule, we have synthesized a series of four isomeric 4'-hydroxystyryl*diazines*: the *2,4-diaza* (**3e**), *2,5-diaza* (**3f**), *2,3-diaza* (**3g**), and *3,4-diaza* (**3h**) isomers [the positions of the nitrogens relative to the site of styryl attachment are given as italicized numbers], and we have studied their UV absorbance and fluorescence emission in a variety of solvents and under different pH conditions. These compounds are prepared readily by the condensation of the appropriate methyldiazine with 4-methoxybenzaldehyde; deprotection with boron trifluoride-dimethylsulfide complex results in partial rehydration of the styrene double bond, but dehydration is readily effected by treatment with acid. The UV spectra of compounds **3e-h** in ethanol show under neutral conditions a long wavelength absorbance at 330-350 nm, which shifts sharply to the red in both acid and base (390-420 nm). The most marked shift is seen with the 2,4- and 3,4-diaza isomers (**3e** and **3h**). These four styryl diazines also show long wavelength fluorescence that is highly solvatochromic and sensitive to pH. Emission in acetonitrile is at 400-435 nm, but shifts in acid or base to 500-560 nm with the 2,4- and 3,4-diaza isomers (**3e** and **3h**) and to 590-640 nm with the 2,5- and 2,3-diaza isomers (**3f** and **3g**). The high environmental and pH sensitivity of these 4-hydroxystyryldiazine fluorophores makes them potentially useful as probes for biological systems.

INTRODUCTION

Fluorescent molecules that interact with various proteins have been widely used as probes in many different areas of biological research,[1] including the localization of specific proteins in cell organelles[2] and the characterization of cellular binding sites,[3] providing thereby a means for quantitation and spacial characterization. Of particular interest in the development of such probes are fluorophores exhibiting high environmental sensitivity[3,4] that results in substantial changes in emission intensity and/or wavelength in media of different polarity and/or pH. The binding or covalent attachment of such an environmentally sensitive fluorescent compound to a biological molecule can provide valuable structural and chemical information as a result of the spectral changes that are observed, either upon the interaction of the fluorophore with the target molecule or when a subsequent change of the environment occurs.

In the course of our ongoing investigation concerning the development of a fluorescent-based method for the assay of the quantity and distribution of estrogen receptors in breast cancer cells—an indicator of the

patient response to hormonal therapy[5]—we have explored several different approaches,[6-8] including the development of phenanthrene-based fluorescent probes which are derived from photocyclization-oxidation of stilbene systems (photofluorogenic agents).[8] The latter compounds proved to be promising in terms of their fluorescent and biological properties, and we were intrigued at the possibility of their further development by incorporating more environmentally sensitive fluorescent moieties within their molecular skeleton. Since the design of such fluorescent probes must include a donor-acceptor system,[9] and the presence of a phenol (donor) is a prerequisite for good binding affinity with the estrogen receptor,[10] our earlier work concerned the incorporation of various acceptor moieties.[11-12] We found that the substitution of one phenyl ring of stilbene with a pyridine (acceptor) significantly affected the photophysical and photochemical behavior of these systems, because of the involvement of the (n,π*) state.[13,14] Thus, compounds such as 4-hydroxystyrylpyridines and hydroxyazaphenanthrenes were found to possess photophysical properties, in terms of emission maximum and environmental sensitivity (solvatochromism), suitable for biological applications.[12]

In order to examine the effect of an additional nitrogen atom substitution on the fluorescence behavior of these systems, we substituted pyrazine, pyridazine or pyrimidine rings for the phenyl ring of the basic 4'-hydroxy stilbene system and studied the photophysical properties of these new chromophores. These new analogs are models for the further design and development of more sophisticated fluorescent probes for the estrogen receptor.[7a,9a]

RESULTS AND DISCUSSION

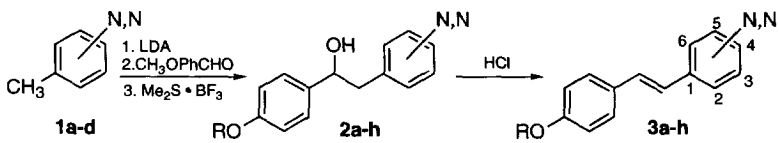
Synthesis

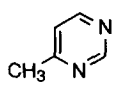
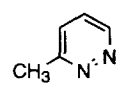
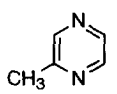
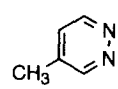
Synthetic methods leading to several *trans*-styryldiazines have been reported previously,[15] but the yields have been moderate to poor. In this report, however, we have prepared the four isomeric 4'-substituted styryldiazines (**3a-h**) in excellent yields, using the synthetic sequence outlined in Scheme I. More specifically, regardless of the substantial differences in the acidity of the methyl group of the isomeric substrates **1a-d**,[16] the anion generated by treatment of the appropriate methyldiazine with lithium diisopropylamide in THF at -50 °C underwent a smooth reaction with 4-methoxybenzaldehyde. In the case of the more acidic 4-methylpyrimidine (**1a**) and 2-methylpyrazine (**1b**), the reaction was complete within 3 and 5 h, respectively, in almost quantitative yields, whereas reaction of their isomeric pyridazines (**1c** and **1d**) required 10 h, and gave 68% and 66% yields, respectively.

Deprotection of the 4'-methoxystyryldiazines by treatment with boron trifluoride-dimethylsulfide complex[17] caused their simultaneous partial rehydration to compounds **2e-h**. Formation of these products can be rationalized on the basis of high electrophilic reactivity of the double bond because of its conjugation with the diazine ring. Thus, we preferred first to deprotect the adducts **2a-d** and then by an acid-catalyzed dehydration to obtain the desired *trans* 4'-hydroxystyryldiazines (**3e-h**). It is noticeable, however, that during this synthetic sequence no trace of *cis* isomers was observed, and all final products have vicinal olefinic coupling constants in the range 16-16.3 Hz, indicative of their *trans*conformation.

Ultraviolet Absorbance Spectra

The ultraviolet spectra for the 4'-methoxy- and the 4'-hydroxy-substituted styryldiazines were measured in tetrahydrofuran, acetonitrile, ethanol and water, under neutral, acidic (0.1 N HCl) and basic (0.1 N KOH) conditions. The most characteristic feature of these compounds is that in all cases they absorb at 5-45 nm longer wavelength than their corresponding monoazo derivatives (styrylpyridines, e.g., **2-styp**). Selected spectral data for the 4'-hydroxy substituted compounds are summarized in Table I, while their spectra in ethanol are shown in Figure I.



Substrate	R-	Compound	Isomer *	Substrate	R-	Compound	Isomer *
	CH_3	a	*2,4-diaza*		CH_3	c	*2,3-diaza*
	H	e			H	g	
	CH_3	b	*2,5-diaza*		CH_3	d	*3,4-diaza*
		f				h	

*The positions of the nitrogens relative to the site of styryl attachment are given as italicized numbers.

SCHEME I. Synthesis of Styryldiazines **3a-h**.

Under neutral conditions in ethanol (Table I, Fig. I solid lines), the styryldiazines **3e, 3f** and **3h** [*2,4-*, *2,5-*, and *3,4-diaza* substitution patterns] showed similar spectra, having a long wavelength band with a maximum at *ca.* 350 nm, while the 3-styryl-1,2-diazine **(3g)** [*2,3-diaza* substitution] absorbed at 20 nm shorter wavelength. Comparison of the absorption bands of styryldiazines in different solvents (Table I) indicates that like the **2-styp**, they have only limited solvatochromicity in absorbance under neutral conditions.

In acidic medium (0.1 N HCl, dashed lines), 4-styryl-1,3-diazine **(3e)** and 4-styryl-1,2-diazine **(3h)** [the *2,4-* and *3,4-diaza* isomers] showed a new, more intense, longer wavelength band (416 and 424 nm, respectively), resulting in a red shift of *ca.* 70 nm, while 2-styryl-1,4-diazine **(3f)** [*2,5-diaza*] retained the absorption at 346 nm but showed a new band at 414 nm (65 nm red shift). A further increase in acidity (2 N HCl, dashed-double dotted line) resulted in an increase in the intensity of this new band, with a corresponding decrease in the orignal band. As the absorbance of the other three isomers was unaffected by further acidification, this indicates that the basicity of the pyrazine nitrogens is lower compared to those of the other somers. A red shift was also observed for the isomer **3g** [*2,3-diaza*] in acid (392 nm, red shift of 62 nm). On the other hand, in base (dash-dotted lines), all isomers showed a new, rather intense longer wavelength band, with pyrazine derivative **3g** having this band at the shortest wavelength (388 nm).

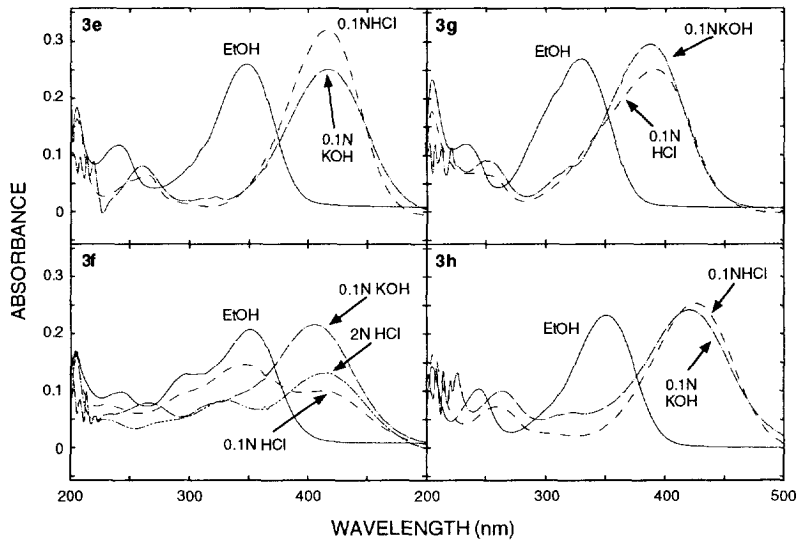


FIGURE I. UV-Visible spectra of 4'-hydroxystyryldiazine, **3e-h**. Spectra were recorded in $1x10^{-5}$ M ethanolic solutions, alone or with 0.1N HCl or 0.1N KOH.

This alteration in the absorption maxima of the hydroxy-substituted styryldiazines with solvent acidity can be explained by considering the species in the ground state present under neutral, acidic or basic conditions (Scheme II), where the phenolate anion (**A**) in base and the diazinium cations (**C**) in acid have longer wavlength absorptions than their corresponding species in neutral solution (**N**). It is noticeable that in all cases the long wavelength band in base undergoes a distinct blue shift as the solvent is changed from non-protic to protic (THF to EtOH). On the other hand, under neutral or acidic conditions, the band appears at longest wavelength and maximum intensity in ethanol. The effect of solvent polarity on the position of the absorbance band (absorbance solvatochromicity) in these systems is complex, as it depends on the relative stability of the species **N**, **A**, and **C** (cf. Scheme II) in the ground and Frank-Condon excited states, as a function of solvent and acidity.

The 4'-methoxy-substituted styryldiazines (**3a-d**) under neutral and acidic conditions showed absorbance spectra very similar to those of the corresponding hydroxy-substituted ones (data not shown), with their absorbance maxima at shorter wavelength (2-15 nm) and of larger relative intensity. Unlike the hydroxy compounds, however, the methoxy analogs, as expected, do not undergo a spectral shift under basic conditions. Their absorbance is also less solvent sensitive.

TABLE I. Long wavelength absorbance maxima for 4'-hydroxystyryldiazines

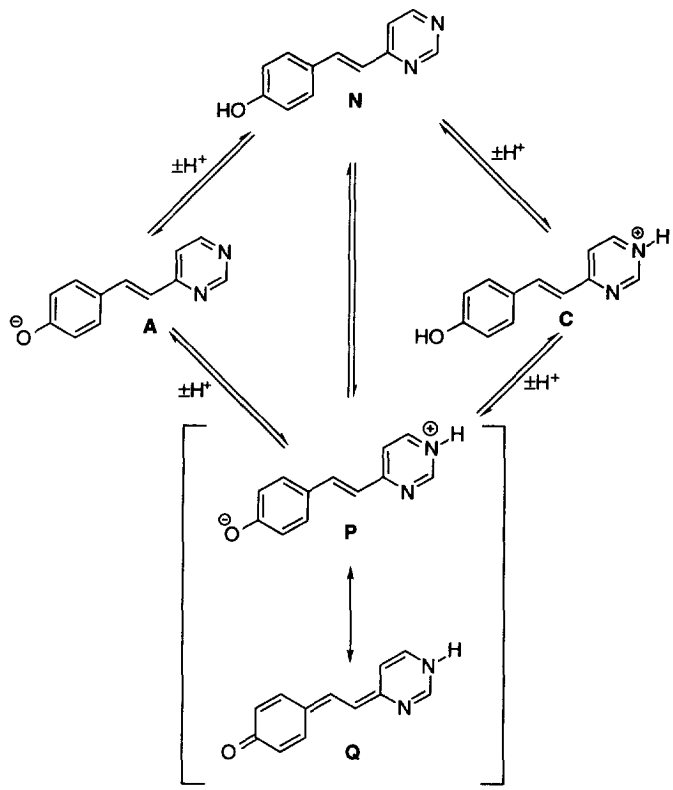
		λ_{abs}^{max} (ε)			
Compound No.	Condition[a]	THF	CH_3CN	EtOH	H_2O
3e	neutral	340 (24,800)	334 (33,200)	348 (26,100)	338 (23,500)
[*2,4-diaza*]	acid	402 (39,200)	404 (40,100)	416 (32,100)	396 (31,500)
	base	b	428 (36,900)	416 (25,200)	390 (29,100)
3f	neutral	350 (10,900)	342 (15,400)	350 (20,800)	344 (18,300)
[*2,5-diaza*]	acid	348 (13,500) 414 (8,800)	332 (17,000) 398 (19,900)	346 (14,600) 414 (9,900)	342 (14,200) 402 (10,200)
	2N HCl	345 (8,600) 414 (13,800)		345 (8,400) 414 (13,200)	344 (7,000) 402 (13,200)
	base	b	420 (21,600)	406 (21,600)	384 (22,600)
3g	neutral	326 (20,800)	320 (28,500)	330 (27,600)	322 (18,500)
[*2,3-diaza*]	acid	372 (23,800)	378 (20,200)	392 (25,100)	370 (17,800)
	base	b	400 (33,800)	388 (29,600)	366 (23,900)
3h	neutral	338 (26,800)	334 (27,700)	350 (23,300)	348 (20,300) 400[c] (11,800)
[*3,4-diaza*]					
	acid	408 (27,800)	412 (30,900)	424 (25,400)	402 (26,700)
	base	b	430 (33,600)	420 (24,300)	390 (26,400)
2-styp[d]	neutral	e	326 (23,700)	330 (23,000)	332 (18,500)
	acid	e	370 (27,800)	384 (25,200)	360 (25,300)
	base	e	384 (28,600)	376 (32,900)	358 (24,900)

[a] Acid = 0.1 N HCl, Base = 0.1 N KOH
[b] Not soluble
[c] Shoulder
[d] 4'-Hydroxy-2-styrylpyridine, data from ref. 12
[e] Not available

Fluorescence Spectra

The fluorescence spectra of the methoxy- and hydroxy-substituted styryldiazines were measured in tetrahydrofuran, acetonitrile, ethanol and water, under neutral, acidic (0.1 N HCl) or basic (0.1 N KOH) conditions. The spectra in acetonitrile are given in Figure II; data on emission maxima and relative intensities are summarized in Table II.

A few generalizations can be made about the fluorescence emission behavior of styryldiazines: Emission maxima and intensities in all cases show strong dependence upon solvent and acidity. Thus, under neutral conditions, there is a strong red shift in the emission maxima for all molecules as solvent polarity increases from tetrahydrofuran to acetonitrile to ethanol to water (normal fluorescence solvatochromism); there is also a pronounced red shift upon going from neutral to acidic or basic conditions, the magnitude of which is greater in acetonitrile and tetrahydrofuran. As a consequence, fluorescence solvatochromism, which is strong under neutral conditions, is relatively weak in acid or base.



SCHEME II. Species involved in absorbance and fluorescence of styryldiazines (exemplified by compound **3e**).

As we have noted before with the styrylpyridines,[12] interpretation of the fluorescence properties of ionizable molecules such as the styryldiazines is complicated by the fact that proton transfer in the excited state can lead to the formation of not only the anion (**A**) and cation (**C**) form, but also the polar/quinoid (**P/Q**) form (cf., Scheme II).[18-21] Because in the excited state, the acidity of the phenol and the basicity of the pyridine or diazine are both increased,[22] this polar/quinoid form will be formed under conditions when proton transfer is sufficiently rapid to occur during the lifetime of the excited state. Since emission from the polar/quinoid form is at longer wavelength than from the other three forms, its presence can explain the very long wavelength emissions that are observed in the hydroxystyrylpyridine and diazine systems. Partial formation of the polar/quinoid form, with persistence of some of the other forms, can account for dual fluorescence emission.[12]

The behavior of each isomer is distinctly different from the others. Thus, the 4-styryl-1,3-diazines (**3a, 3e** [*2,4-diaza*]) under neutral conditions, showed moderate solvatochromism, with the emission of the hydroxy compound appearing as two broad bands, while the emission bands in acid (ca. 504-518 nm) and in base

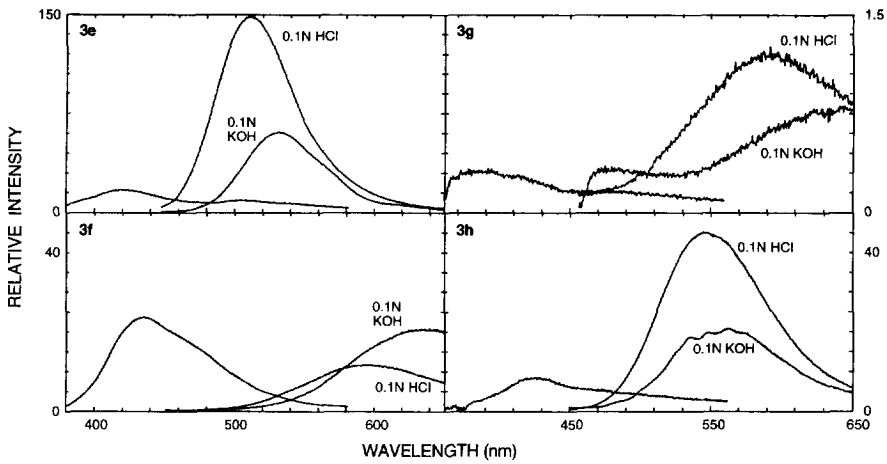


FIGURE II. Fluorescence emission spectra of 4'-hydroxystyrylpyridine, **3e-h.** Spectra were recorded in $5x10^{-6}$ M acetonitrile solution, alone or with 0.1 N HCl or KOH. Excitation was at the major long wavelength band (320-430 nm; cf. Table I and Fig. I).

(ca. 527-533 nm) show low solvatochromism. It is also noticeable that in both acid and base, the relative intensity of emissions of this isomer was higher than that of the other isomers. In contrast, the 2-styryl-1,4-diazines (**3b, 3f** [*2,5-diaza*]) and 3-styryl-1,2-diazines (**3c, 3g** [*2,3-diaza*]) under neutral conditions showed much greater solvatochromism, while in acid and base their emission maximum was shifted to considerably longer wavelength. Their emission intensities, however, especially those of compounds **3c** and **3g,** were considerably lower compared to those of the other isomers. Finally, the 4-styryl-1,2-diazines (**3d, 3h** [*3,4-diaza*]) are the isomers that demonstrate the most favorable fluorescence properties in terms of wavelength and intensity, since in acid and base both their intensity and emission maxima are considerably red shifted (70-240 nm). This makes them attractive candidates as biological fluorescent probes, since under these conditions their emission is at longer wavelength than most cellular autofluorescence.

The emission spectra of the methoxy-substituted styryldiazines in water (data not shown) differ considerably from those of hydroxy-substituted analogs. Most notable is the reduced magnitude of the shift to longer wavelength in base, as expected, since these systems cannot deprotonate; the shift in acidic medium is also reduced, but to a lesser degree. The methoxy analogs generally gave higher intensities in protic medium than their corresponding hydroxy analogs, suggesting that the reduced intensity of the latter in water resulted from prototropic quenching of the excited state. Generally, under neutral and acidic conditions, the methoxy-substituted styryldiazines emit at wavelengths about 10-30 nm shorter than the corresponding hydroxy compounds, with comparable or somewhat greater intensities, particularly in ethanol and water, where they are less subject to quenching.

TABLE II. Fluorescence solovatochromism of 4'-hydroxystyryldiazines

		λ_{em}^{max} (relative intensity)[b]			
Compound No.	Condition[a]	THF	CH_3CN	EtOH	H_2O
3e	neutral	414 (2.1) 496 (0.7)	419 (1.7) 507 (0.9)	428 (3) 493 (1.3)	437 (4.1)
[*2,4-diaza*]		540[c] (0.4)	540[c] (0.6)	535[c] (0.7)	
	acid	504 (140)	509 (150)	527 (100)	518 (19)
	base	d	530 (61)	527 (72)	533 (17)
3f	neutral	424 (31)	435 (23)	491 (90)	533 (2.5)
[*2,5-diaza*]	acid	583 (7)	593 (18)	522 (1.2) 585 (1.5)	608 (0.2)
	base	d	633 (17)	600 (7.2)	
3g	neutral	e	392 (0.4) 474 (0.8)	438 (0.9)	e
[*2,3-diaza*]	acid	587 (1)	591 (1.3)	558 (1.2)	e
	base	d	472 (0.3) 640 (0.6)	480 (1.2) 630 (0.4)	e
3h	neutral	484 (0.5)	425 (0.9)	460 (6)	494 (10)
[*3,4-diaza*]	acid	530 (130)	544 (45)	542 (55)	658 (4.6)
	base	d	562 (21)	560 (40)	593 (3.5)
2-styp[f]	neutral	g	388 (0.5)	401 (0.5)	434 (0.5) 501[c] (0.4)
	acid	g	506 (2.5)	511 (3.2)	511 (1.1)
	base	g	525 (19)	510 (4.6)	516 (2.2)

[a] Acid = 0.1 N HCl, Base = 0.1 N KOH. Excitation was always at the major long wavelength band (cf., Table I)
[b] Numbers in parentheses represent the relative intensity of emission ($\times 10^4$ cps) at λ_{em}^{max}
[c] Shoulder
[d] Not soluble
[e] Very weak
[f] 4'-Hydroxy-2-styrylpyridine, data from ref. 12
[g] Not available

CONCLUSION

We have described the synthesis of four isomeric 4'-hydroxystyryldiazines and the preliminary investigation of their optical spectroscopic properties Their absorbance and fluorescence properties are better than that of the corresponding monoazo derivatives (4'-hydroxystyrylpyridines) and demonstrate strong dependence on solvent polarity and pH, which is related in part to the relative orientation of the ionizable functions on the molecules. Even though all the systems examined show potential for utilization as spectroscopic probes in biological systems, 4-styryl-1,2-diazines (**3d, 3h**) appear to be the best, and their incorporation into receptor binding probes and further spectroscopic studies are currently underway.

EXPERIMENTAL

General Aspects

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were recorded on General Electric QE 300 (300 MHz) spectrometer in the indicated solvents. Chemical shifts are reported in parts per million downfield from tetramethylsilane as internal standard (δ scale). Mass spectral data were obtained on a Varian MAT CH-5 mass spectrometer (electron impact at 70 eV). High-resolution mass spectra were obtained on a Varian 731 high-resolution mass spectrometer. Data are presented in the form *m/z* (intensity relative to base peak 100). Reaction progress was followed by analytical thin-layer chromatography (TLC), performed with 0.25-mm silica gel plates with fluorescent indicator UV_{254} (Merck). All column chromatography was done by the flash chromatography technique using 32-63 μm silica gel packing (Merck).[23] Microanalytical data were provided by the Microanalytical Service Laboratory of the University of Illinois.

Materials

All starting materials were purchased from Aldrich. Tetrahydrofuran (THF) was distilled from sodium-benzophenone immediately prior to use. Diisopropylamine and methylene chloride were distilled over CaH_2 prior to use. High purity solvents for UV-vis and fluorescence measurements were obtained from either Aldrich or Burdick and Jackson Laboratories and used without further purification.

Methyldiazine Condensations (Method A)

To a stirred solution of lithium diisopropylamine (LDA) (1.1 equiv.) in dry THF at -40 °C and N_2 atmosphere, 1 equiv. of methyl diazine was added dropwise. After being stirred for 50 min, the temperature was allowed to rise to -20 °C, and a solution of 4-methoxybenzaldehyde (1 equiv.) was added dropwise. The reaction was allowed to warm to the room temperature and was then stirred until TLC analysis indicated that the reaction was complete. A saturated solution of NH_4Cl was added to hydrolyze the lithium salt, and the product was extracted twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and evaporated to dryness to give a yellow solid which was decolorized by washing with cold diethyl ether. Recrystallization from methylene chloride/petroleum ether furnished the analytically pure product as white needles.

(R,S) Ethanol, 1-(4-methoxyphenyl)-2-(4-pyrimidinyl) (**2a**). Prepared from 4-methyl pyrimidine by method A with a 3 h reaction (yield 94%); **mp** 109-110 °C; **^{1}H NMR** ($CDCl_3$) δ, *pyrimidinyl*: 9.13 (s, 1H, H_2), 8.60 (d, J = 5.1 Hz, 1H, H_6), 7.15 (d, J = 5.1 Hz, 1H, H_5); *phenyl*: 7.32 (dd, J = 8.8 and 1.8 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.88 (dd, J = 8.8 and 1.8 Hz, 2H, $H_{3'}$, $H_{5'}$); 5.15 (dd, J = 8.7 and 3.5 Hz, 1H, CH), 3.95 (br, OH), 3.80 (s, 3H, CH_3), 3.13 (m, 2H, CH_2); **MS** *m/z*: 231 (M^+ + 1, 1.4), 230 (M^+, 8.7), 211 (4.2), 150 (17), 137 (48.3), 136 (20.8), 135 (40.8), 109 (21.1), 94 (100), 81 (10.2), 77 (29.5), 39 (14.8); **Anal**. Calcd for $C_{13}H_{14}N_2O_2$: C, 67.81; H, 6.13; N, 12.17. Found: C, 67.96; H, 6.14; N, 12.17.

(R,S) Ethanol, 1-(4-methoxyphenyl)-2-(2-pyrazinyl) **(2b)**. Prepared from 2-methylpyrazine by method A with a 5 h reaction (yield 98%); **mp** 93-94 °C; **^{1}H NMR** ($CDCl_3$) δ, *pyrazinyl*: 8.47 (d, J = 2.2 Hz, 1H, H_4), 8.42 (d, J = 2.2 Hz, 1H, H_5), 8.39 (s, 1H, H_3); *phenyl*: 7.30 (d, J = 8.8 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.87 (s, J = 8.8 Hz, 2H, $H_{3'}$, $H_{5'}$); 5.11 (dd, J = 8.4 and 3.9 Hz, 1H, CH), 3.98 (br, OH), 3.79 (s, 3H, CH_3), 3.14 (m, 2H, CH_2); **MS** *m/z*: 230 (M^+, 5.5), 211 (4.2), 150 (11.4), 137 (64.8), 135 (15.1), 109 (24.6), 94 (100), 77 (23.3), 66 (9.2), 39 (14.5); **Anal.** Calcd for $C_{13}H_{14}N_2O_2$: C, 67.81; H, 6.13; N, 12.17. Found, C, 67.85; H, 6.16; N, 12.16.

(R,S) Ethanol, 1-(4-methoxyphenyl)-2-(3-pyridazinyl) **(2c).** Prepared from 3-methylpyridazine by method A with a 20 h reaction. The product was isolated by flash chromatography (ethyl acetate-hexane, 4:1 vol/vol) as a yellowish oil and no special attempt was made to crystallize it (yield 68%); **^{1}H NMR** ($CDCl_3$) δ, *pyridazinyl and phenyl*: 8.95 (dd, J = 4.9 and 1.3 Hz, 1H, H_6), 7.37-7.28 (m, 4H, H_4, H_5, $H_{2'}$, $H_{6'}$), 6.83 (d, J = 8.8 Hz, 2H, $H_{3'}$, $H_{5'}$); 5.17 (m, 1H, CH), 4.46 (br, OH), 3.76 (s, 3H, CH_3), 3.24 (m, 2H, CH_2); **MS** *m/z*: 231 (M^+ + 1, 1.3), 230 (M^+, 8.9), 211 (5.6), 150 (10.2), 137 (21.6), 136 (24.4), 135 (62.8), 109 (16.4), 94 (100), 84 (23.2), 81 (26.4), 77 (32.7), 65 (23.8), 39 (34); **HRMS** Calcd for $C_{13}H_{14}N_2O_2$: 230.1055. Found 230.1061.

(R,S) Ethanol, 1-(4-methoxyphenyl)-2-(4-pyridazinyl) **(2d)**. Prepared from 4-methylpyridazine by method A with a 10 h reaction. The product was isolated by flash chromatography (ethyl acetate-hexane, 9:1 vol/vol) as a white solid (yield 66%); **mp** 112-113 °C; **^{1}H NMR** (CD_3COCD_3) δ, *pyridazinyl:* 9.01 (m, 2H, H_3, H_6), 7.43 (dd, J = 5.1 and 2.3 Hz, 1H, H_5); *phenyl:* 7.27 (dd, J = 8.7 and 1.8 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.85 (dd, J = 8.7 and 1.8 Hz, 2H, $H_{3'}$, $H_{5'}$); 4.95 (dd, J = 7.1 and 4.1 Hz, 1H, CH), 4.59 (d, J = 4.1 Hz, 1H, OH), 3.77 (s, 3H, CH_3), 3.02 (m, 2H, CH_2); **MS** *m/z*: 230 (M^+, 1.6), 212 (1.4), 137 (75.9), 135 (13.3), 109 (25.8), 94 (100), 77 (25.1), 66 (9.6), 65 (9.4), 49 (11), 39 (23). **Anal.** Calcd for $C_{13}H_{14}N_2O_2$: C, 67.81; H, 6.13; N, 12.17. Found: C, 67.81; N, 6.12; N, 12.18.

Dehydration of 1-Aryl-2-(diazenyl)ethanols (Method B)

The condensation product (5 mmol) was dissolved in 20 ml MeOH and 5 ml concentrated HCl and refluxed for 7 h. The solvent was evaporated under vacuum, and the remaining slurry was dissolved in H_2O and neutralized with a saturated solution of Na_2CO_3. The product was extracted twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4, evaporated to dryness, and the resulting solid was recrystallized from ethyl acetate/petroleum ether.

Pyrimidine, 4-[2-(4-methoxyphenyl)ethenyl]-(E) **(3a)**. Prepared by dehydration of compound **2a**, according to method B (yield 96%); **mp** 101-102 °C; **^{1}H NMR** ($CDCl_3$) δ, *pyrimidinyl:* 9.14 (s, 1H, H_2), 8.63 (d, J = 5.1 Hz, 1H, H_6), 7.28 (d, J = 5.1 Hz, 1H, H_5); *phenyl:* 7.54 (d, J = 8.9 Hz, 2H, $H_{2'}$, $H_{5'}$), 6.93 (d, J = 8.9 Hz, 2H, $H_{3'}$, $H_{5'}$); 7.84 (d, J = 16 Hz, 1H, CH), 6.91 (d, J = 16 Hz, 1H, CH), 3.84 (s, 3H, CH_3); **MS** *m/z*: 213 (M^+ + 1, 4.2), 212 (M^+, 34), 211 (100), 197 (6), 196 (5), 168 (15.7), 115 (9.2), 89 (6.8), 63 (6.4); **Anal.** Calcd for $C_{13}H_{12}N_2O$: C, 73.56; H, 5.70; N, 13.20. Found: C, 73.39; H, 5.62; N, 13.09.

Pyrazine, 2-[2-(4-methoxyphenyl)ethenyl]-(E) (**3b**). Prepared by dehydration of compound **2b**, according to method B (yield 98%); **mp** 104-105 °C; **^{1}H NMR** ($CDCl_3$) δ, *pyrazinyl*: 8.61 (d, J = 1.2 Hz, 1H, H_3), 8.51 (dd, J = 2.3 and 1.2 Hz, 1H, H_4), 8.37 (d, J = 2.3 Hz, 1H, H_5); *phenyl*: 7.54 (dd, J = 8.8 and 1.8 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.93 (dd, J = 8.8 and 1.8 Hz, 2H, $H_{3'}$, $H_{5'}$); 7.70 (d, J = 16.1 Hz, 1H, CH), 7.03 (d, J = 16.1 Hz, 1H, CH), 3.85 (s, 3H, CH_3); **MS** *m/z*: 213 (M^+ + 1, 6.8), 212 (M^+, 50.9), 211 (100), 197 (10), 169 (13.4), 168 (16.7), 115 (9.6), 89 (9.5), 63 (7.2); **Anal.** Calcd for $C_{13}H_{12}N_2O$: C, 73.56; H, 5.70; N, 13.20. Found: C, 73.64; H, 5.63; N, 13.09.

Pyridazine, 3-[2-(4-methoxyphenyl)ethenyl)]-(E) (**3c**). Prepared by dehydration of compound **2c**, according to method B (yield 95%); **mp** 123-124 °C; **^{1}H NMR** ($CDCl_3$) δ, *pyridazinyl*: 9.02 (dd, J = 5.5 and 1.5 Hz, 1H, H_6), 7.59 (dd, J = 8.6 and 1.5 Hz, 1H, H_4), 7.40 (dd, J = 8.6 and 5.5 Hz, 1H, H_5); *phenyl*: 7.54 (dd, J = 8.7 and 1.9 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.93 (dd, J = 8.7 and 1.9 Hz, 2H, $H_{3'}$, $H_{5'}$); 7.64 (d, J = 16.3 Hz, 1H, CH), 7.22 (d, J = 16.3 Hz, 1H, CH), 3.84 (s, 3H, CH_3); **MS** *m/z*: 213 (M^+ + 1, 2.3), 212 (M^+, 22), 211 (100), 197 (10.3), 168 (12.3), 115 (24.8), 89 (7.1), 63 (7.6); **Anal.** Calcd for $C_{13}H_{12}N_2O$: C, 73.56; H, 5.70; N, 13.20. Found: C, 73.86; H, 5.63; N, 13.16.

Pyridazine, 4-[2-(4-methoxyphenyl)ethenyl)]-(E) (**3d**). Prepared as pale yellow needles by dehydration of compound **2d**, according to method B (yield 96%); **mp** 92-94 °C; **^{1}H NMR** ($CDCl_3$) δ, *pyridazinyl:* 9.26 (d, J = 2.2 Hz, 1H, H_3), 9.09 (d, J = 5.1 Hz, 1H, H_6), 7.46 (dd, J = 5.1 and 2.2 Hz, 1H, H_5); *phenyl:* 7.52 (dd, J = 8.8 and 1.8 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.93 (dd, J = 8.8 and 1.8 Hz, $H_{3'}$, H_5); 7.38 (d, J = 16.3 Hz, 1H, CH), 6.83 (d, J = 16.3 Hz, 1H, CH), 3.85 (s, 3H, CH_3); **MS** *m/z:* 214 (M^++2, 1.6), 213 (M^++1, 15.8), 212 (M^+, 100), 211 (6.6), 169 (9.7), 158 (11), 141 (41.7), 135 (11.2), 115 (58.1), 89 (12.6), 65 (9.6), 63 (17.7), 39 (13.1); **Anal.** Calcd for $C_{13}H_2N_2O$: C, 73.56; H, 5.70; N, 13.20. Found: C, 73.52; H, 5.68; N, 13.19.

Deprotection of Methoxy Ethers (Method C)

To a solution of 1.3 mmol of protected ethanol or ethene products in 25 ml dry methylene chloride, cooled to 0 °C in N_2 atmosphere was added 3 ml of boron trifluoride-dimethylsulfide complex dropwise under stirring. The reaction was allowed to warm to room temperature and was stirred for 36 h. The resulting precipitate was separated by decantation and dissolved in 10 ml water. A saturated solution $NaHCO_3$ was added (pH ~7.5), and the product was extracted twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The resulting yellow solid was chromatographed (ethyl acetate-hexane, 4:1 vol/vol) to give the desired product as a white solid and a small amount of the corresponding dehydrated product.

(R,S) Ethanol, 1-(4-hydroxyphenyl)-2-(4-pyrimidinyl) (**2e**). Prepared by method C from compound **2a** (yield 88%); **mp** 163-165 °C; **^{1}H NMR** (DMSO-d_6) δ, *pyrimidinyl:* 9.06 (s, 1H, H_2), 8.61 (d, J = 5.1 Hz, 1H, H_6), 7.32 (d, J = 5.1 Hz, 1H, H_5); *phenyl*: 7.13 (d, J = 8.4 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.69 (d, J = 8.4 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.25 (br, OH), 5.25 (br, OH), 4.91 (dd, J = 8.3 and 5.2 Hz, 1H, CH), 2.98 (m, 2H, CH_2); **MS**

m/z: 217 (M^+ + 1, 1.2), 216 (M^+, 7.1), 197 (8.9), 136 (17.3), 123 (36.7), 121 (16.6), 95 (39.9), 94 (100), 81 (29.9), 77 (30.5), 67 (13.8), 65 (16.8), 39 (32); **HRMS** Calcd for $C_{12}H_{12}N_2O_2$: 216.0899. Found 216.0896.

(R,S) Ethanol, 1-(4-hydroxyphenyl)-2-(2-pyrazinyl) **(2f).** Prepared by method C from compound **2b** (yield 93%); **mp** 137-138 °C; **^{1}H NMR** (DMSO-d_6) δ, *pyrazinyl:* 8.53 (m, 1H, H_4), 8.49 (m, 2H, H_3, H_6); *phenyl:* 7.12 (d, J = 8.5 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.68 (d, J = 8.5 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.24 (s, 1H, OH), 5.22 (d, J = 4.4 Hz, 1H, OH), 4.86 (dd, J = 4.4 and 8.1 Hz, 1H, CH), 3.01 (m, 2H, CH_2); **MS** *m/z*: 217 (M^++ 1, 1), 216 (M^+, 6.6), 197 (10.8), 136 (17), 123 (55.1), 121 (9.9), 95 (33.9), 94 (100), 65 (9.5), 39 (21.5); **HRMS** Calcd for $C_{12}H_{12}N_2O_2$: 216.0899. Found 216.0900.

(R,S) Ethanol, 1-(4-hydroxyphenyl)-2-(3-pyridazinyl) **(2g).** Prepared as an off-white solid by method C from compound **2c** (yield 78%); **mp** 71-73 °C; **^{1}H NMR** (DMSO-d_6) δ *pyridazinyl:* 9.05 (d, J = 4.8 Hz, 1H, H_6), 7.48 (m, 2H, H_4, H_5); *phenyl:* 7.14 (d, J = 8.5 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.70 (d, J = 8.5 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.27 (s, 1H, OH), 5.29 (d, J = 4.6 Hz, 1H, OH), 4.88 (dd, J = 4.6, 7.6 Hz, 1H, CH), 3.17 (m, 2H, CH_2); **MS** *m/z* 216 (M^+, 2.3), 197 (16.1), 135 (20.1), 94 (100), 81 (23.1), 77 (33.8), 65 (19.1), 39 (40.8); **HRMS** Cald for $C_{12}H_{12}N_2O_2$: 216.0899. Found 216.0895.

(R,S) Ethanol, 1-(4-hydroxyphenyl)-2-(4-pyridazinyl) **(2h).** Prepared as an off white solid by method C from compound **2d** (yield 82%); **mp** 148-149 °C; **^{1}H NMR** (DMSO-d_6) δ, *pyridazinyl:* 9.05 (d, J = 4.7 Hz, 1H, H_6), 9.00 (s, 1H, H_3), 7.45 (dd, J = 4.7 and 2 Hz, 1H, H_5); *phenyl:* 7.11 (d, J = 8.5 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.69 (d, J = 8.5 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.28 (s, 1H, OH), 5.33 (d, J = 4 Hz, 1H, OH), 4.74 (m, 1H, CH), 2.89 (m, 2H, CH_2); **MS** *m/z:* 216 (M^+, 1.9), 198 (26), 169 (9.4), 123 (40.4), 122 (57.4), 121 (67.5), 115 (14.5), 94 (100), 77 (18.8), 65 (56.7), 63 (15.2), 39 (75); **HRMS** Calcd for $C_{12}H_{12}N_2O_2$: 216.0899. Found 216.0898.

Phenol, 4-[2-(4-pyrimidinyl)ethenyl]-(E) **(3e).** Prepared as a pale yellow solid from compound **2e** by method B (yield 97%); **mp** 235-237 °C (dec); **^{1}H NMR** (DMSO-d_6) δ, *pyrimidinyl and phenyl*: 9.06 (s, 1H, $H_{2'}$), 8.68 (d, J = 5 Hz, 1H, $H_{6'}$), 7.55 (m, 3H, H_5, $H_{2'}$, $H_{6'}$), 6.82 (d, J = 8.4 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.89 (br, OH), 7.85 (d, J = 16.1 Hz, 1H, CH), 7.04 (d, J = 16.1 Hz, 1H, CH); **MS** *m/z*: 199 (M^+ + 1, 3), 198 (M^+, 26.7), 197 (100), 170 (5.1), 144 (5.7), 115 (7.9), 89 (4.9), 63 (5); **Anal.** Calcd for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.14. Found: C, 72.81; H, 5.02; N, 13.85.

Phenol, 4-[2-(2-pyrazinyl)ethenyl]-(E) **(3f).** Prepared as an ivory solid from compound **2f** by method B (yield 98%); **mp** 186-188 °C; **^{1}H NMR** (DMSO-d_6) δ, *pyrazinyl:* 8.73 (s, 1H, H_3), 8.57 (d, J = 2.2 Hz, 1H, H 4), 8.43 (d, J = 2.2 Hz, 1H, H_5); *phenyl:* 7.53 (d, J = 8.5 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.82 (d, J = 8.5 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.80 (s, 1H, OH), 7.70 (d, J = 16.1 Hz, 1H, CH), 7.14 (d, J = 16.1 Hz, 1H, CH); **MS** *m/z*: 199 (M^+ + 1, 4.3), 198 (M^+, 39.4), 197 (100), 169 (5.1), 115 (7.6), 89 (5.9), 63 (5.4); **Anal**. Calcd for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.14. Found: C, 72.40; H, 5.14; N, 13.96.

Phenol, 4-[2-(3-pyridazinyl)ethenyl]-(E) :(**3g**). Prepared as an off-white solid from compound **2g** by method B (yield 98%); **mp** 238-240 °C (dec); **^{1}H NMR** (DMSO-d_6) δ, *pyridazinyl:* 9.03 (d, J = 5.2 Hz, 1H, H_6), 7.90 (d, J = 8.5 Hz, 1H, H_4), 7.62 (dd, J = 8.5, 5.2 Hz, 1H, H_5); *phenyl:* 7.54 (d, J = 8.5 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.83 (d, J = 8.5 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.78 (s, 1H, OH), 7.69 (d, J = 16.3 Hz, 1H, CH), 7.22 (d, J = 16.3 Hz, 1H, CH); **MS** *m/z:* 199 (M^+ + 1, 2.2), 198 (M^+, 21.2), 197 (100), 144 (7.7), 115 (28.6), 89 (6.8), 63 (8.5), 39 (10.7). **Anal.** Calcd for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.14. Found: C, 72.39; H, 5.09; N, 13.95.

Phenol, 4-[2-(4-pyridazinyl)ethenyl]-(E) (**3h**). Prepared as yellow needles from compound **2h** by method B (yield 97%); **mp** 217 °C (after extensive decomposition); **^{1}H NMR** (DMSO-d_6) δ, *pyridazinyl:* 9.39 (s, 1H, H_3), 9.10 (d, J = 5.2 Hz, 1H, H_6), 7.74 (d, J = 5.2 Hz, 1H, H_5); *phenyl:* 7.51 (d, J = 8.5 Hz, 2H, $H_{2'}$, $H_{6'}$), 6.82 (d, J = 8.5 Hz, 2H, $H_{3'}$, $H_{5'}$); 9.80 (br, OH), 7.64 (d, J = 16.4 Hz, 1H, CH), 6.99 (d, J = 16.4 Hz, 1H, CH); **MS** *m/z:* 200 (M^++2, 1.3), 199 (M^++1, 13.8), 198 (M^+, 100), 169 (37.4), 152 (7), 144 (16.3), 141 (29.6), 123 (13.8), 115 (64.5), 94 (25.1), 89 (13.6), 77 (18.8), 65 (20), 64 (23.5), 63 (18.9), 51 (16), 39 (25.8); **Anal.** calcd for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.14. Found: C, 72.62; H, 5.10; N, 14.11.

Recording of the UV-Vis and Fluorescence Spectra

Ultraviolet-visible (UV-vis) spectra were recorded on a Hewlett-Packard 8450A Diode Array spectrometer. Fluorescence spectra were acquired by photon counting on a Spex Fluorolog 2 Spectrophotometer (Model 111C) with Datamate microprocessor using four 1.25 mm slits. All spectra were recorded at room temperature and are corrected for phototuberesponse and by subtraction of the solvent background. Samples were prepared from stock solution (10^{-3} M) of the corresponding compounds in EtOH, giving final concentrations of 10^{-5} M and 5 x 10^{-6} M for UV-vis and fluorescence, respectively. Acidic or basic solutions were prepared by addition of an amount of conc. HCl or 6 N KOH solution in water, calculated to give a final concentration of 0.1 N.

REFERENCES AND NOTES

1. Rousseau, D.L. *Optical Techniques in Biological Research*, Academic Press: New York, 1984, chapt. 4.
2. Thrieringe, R.; Shio, H.; Han, Y.S.; Cohen, G.; Lazarow, P.B. *Mol. Cell. Biol.* **1991**, *11*, 510-522.
3. Lakowicz, J.R. *Principles of Fluorescence and Spectroscopy*, Plenum Press: New York 1983.
4. Stevens, A.C.; Frutos, R.P.; Harvey, D.F.; Brian, A.A. *Bioconjugate Chem.* **1993**, *4*, 19-24.
5. (a) Thorpe, S.M. *Acta Oncol.* **1988**, *27*, 1-19. (b) Valavaara, R.; Touminen, J.; Johansson, R. *Cancer* **1990**, *66*, 2264-2269.
6. For lead references for the fluorophore-ligand conjugates, see: (a) Fevig, T.L.; Lloyd, J.E.; Zablocki, J.A., Katzenellenbogen, J.A. *J. Med. Chem.* **1987**, 156-165. (b) Carlson, K.E.; Coppey, M.; Magdelenat, H.; Katzenellenbogen, J.A. *J. Steroid Biochem.* **1989**, 345-355. (c) Rao, B.R.; Fry, C.G.; Hunt, S.; Kuhnel, R.; Dandliker, W.B. *Cancer* **1980**, 2902-2906. (d) Van, N.T.; Raber, M.; Barrows, G.H.; Barlogie, B. *Science* **1984**, *224*, 876-879.

7. For lead references for inherently fluorescent estrogens, see: (a) Hwang, K.-J.; O'Neil, J.P.; Katzenellenbogen, J.A. *J. Org. Chem.* **1992**, *57*, 1262-1271. (b) Anstead, G.M.; Altenbach, R.J.; Wilson, S.R.; Katzenellenbogen, J.A. *J. Med. Chem.* **1988**, 1316-1326. (c) Katzenellenbogen, J.A.; Carlson, K.E.; Bindal, R.D.; Neeley, R.L.; Martin, P.M.; Magdelenat, H.P. *Anal. Biochem.* **1986**, *159*, 336-348.
8. For an approach using photofluorogenic ligands, see: (a) Bindal, R.D.; Katzenellenbogen, J.A. *J. Steroid Biochem.* **1985**, *23*, 929-937. (b) Bindal, R.D.; Katzenellenbogen, J.A. *Photochem. Photobiol.* **1986**, *45*, 121-126.
9 (a) Hwang, K.-J.; Carlson, K.E.; Anstead, G.M.; Katzenellenbogen, J.A. *Biochemistry* **1992**, *31*, 11536-11545. (b) Letard, J.-F.; Lapouyade, R.; Rettig, W. *J. Am.Chem. Soc.* **1993**, *115*, 2441-2447. (c) Saltiel, J.; Waller, A.S.; Sears, D.F. **1993**, *115*, 2453-2465. ibid.
10. J. A. Katzenellenbogen, H. J. Johnson, Jr., and H. N. Myers. *Biochemistry* **1973**, *12*, 4085-4094.
11. Scribner, A.; Haroutounian, S.A.; Katzenellenbogen, J.A. *In preparation.*
12. Haroutounian, S.A.; Katzenellenbogen, J.A. *Photochem. Photobiol.* **1988**, *47*, 503-516.
13. (a) Chakravorti, S.; Sarkar, S.K.; Mallick, P.K. *Chem. Phys. Let.* **1991**, *187*, 93-97. (b) Gregory, A.R.; Siebrand, W.; Williams, D.F. *J. Am. Chem. Soc.* **1979**, *101*, 1903-1904.
14. Bong, P.-H.; Kim, H.J.; Chae, K.H.; Shim, S.C.; Nakashima, N.; Yoshihara, K. *J. Am. Chem. Soc.* **1986**, *108*, 1006-1014.
15. Perkampus, H.-H.; Bluhm, Th. *Tetrahedron* **1972**, *28*, 2099-2110.
16. Fraser, R.R.; Mansour, T.S.; Savard, S. *J. Org. Chem.* **1985**, *50*, 3232-3234.
17. Williard, P.G.; Fryhle, C.B. *Tetrahedron Lett.* **1980**, *21*, 3731-3734.
18. Jacques, P. *J. Phys. Chem.* **1986**, *90*, 5535-5539.
19. Hagopian, S.; Singer, L.A. *J. Am. Chem. Soc.* **1983**, *105*, 6760-6761.
20. Krishnamurthy, M.; Dogra, S.K. *Photochem. Photobiol.* **1986**, *44*, 571-577.
21. Wolfbeis, O.S.; Begum, M.; Hochmuth, P. *Photochem. Photobiol.* **1986**, *44*, 551-554.
22. Ireland, J.F.; Wyatt, A.H. *Adv. Phys. Org. Chem.* **1976**, *12*, 131-221.
23. Still, W.C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923-2925.

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