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Fluorescent Molecular Probes II. The Synthesis, Spectral Properties and Use of Fluorescent Solvatochromic Dapoxyl™ Dyes

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ABSTRACT

2,5-Diphenyloxazoles that embody a dimethylamino group at position 4 of the 5-phenyl ring and a sulfonyl group at position 4 of the 2-phenyl ring were prepared as new fluorescent solvatochromic dyes. In these molecules, there is a "push–pull" electron transfer system from the 5-phenyl moiety to the 2-phenyl ring. These compounds show strong solvent-dependent fluorescence that is well correlated with the empirical solvent polarity parameter E_T (30). The solvent polarity dependence suggests that the fluorescence arises from an intramolecular charge transfer. The fluorescence-environment dependence, long emission wavelength, large extinction coefficients, high fluorescence quantum yields and large Stokes shift of the fluorophores can be used to develop ultrasensitive fluorescent molecular probes to study a variety of biological events and processes.

INTRODUCTION

In the past decades fluorescence techniques have become firmly established and widely employed tools of analytical sciences and are now routinely used in the detection, quantitation, identification and characterization of inorganic and organic compounds and of biological structures and processes (1,2). Their inherently high sensitivity and selectivity have been the major driving forces for this development. This trend has also been greatly accelerated by the recent advances in fluorescent reagents and instrumentation (1,3,4).

When compared with conventional absorbance-based techniques, fluorescence is often several orders of magnitude more sensitive and more selective. The increase in sensitivity arises primarily because the emitted radiation is measured directly and can be increased simply by increasing the incident power. Moreover, fluorescence is often considered a zero-background technique, whereas absorbance is a measurement of the difference between incident and transmitted intensities. Selectivity in fluorescence-based techniques is also much higher than in absorbance-based approaches. This can be attributed primarily to the relatively low number of fluorescent species compared with absorbing ones and the ability to employ both excitation and emission wavelengths as dual selectivity parameters (5). A few other selectivity parameters have also been successfully employed. This multi-dimensional nature of fluorescence-based techniques makes fluorometry an ideal tool for bioanalytical studies (1,5).

Fluorescent molecules whose spectra or quantum yields are sensitive to their environments are valuable in the study of heterogeneous media, organized media and biological media and many fluorescent solvatochromic dyes have been developed for these applications (6,7). Dansyl chloride has been used to make a variety of bioconjugates, whose environment-sensitive fluorescence is successfully used to follow a variety of biological processes (8). 8-Anilinonaphthalene-1-sulfonic acid (1,8-ANS)† has been used to determine the relative hydrophobicities of the binding sites in a number of proteins and to detect the protein conformational changes induced by ligand binding (9). Similarly, lanthanide ions were used as probes to determine the presence of water and its mobility at the cation-binding sites of proteins (10). Probes such as pyrene (6) and hypocrellins (11) were used extensively to study the local polarity of micelles, silica gel, zeolite and other organized media. These studies were directed to evaluate the microenvironment surrounding the fluorescent solvatochromic probes and such probes are of practical value in the development of fluorescent sensors. However, the existing fluorescent solvatochromic dyes either have short absorption and emission wavelengths (potentially causing high background due to the autofluorescence of samples), low extinction coefficients, low quantum yields or small Stokes shifts.

In this paper, we report the fluorescence properties of new solvatochromic probes, 5-(4″-dimethylaminophenyl)-2-(4′-phenyl)oxazole (Dapoxyl™) dyes, whose fluorescence maximum shifts to longer wavelengths and fluorescence quantum yield decreases with increasing solvent polarity. These dyes

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†*Abbreviations:* 1,8-ANS, 8-anilinonaphthalene-1-sulfonic acid; 2,6-ANS, 6-anilinonaphthalene-2-sulfonic acid; Dapoxyl, 5-(4″-dimethylaminophenyl)-2-(4′-phenyl)oxazole; DMSO, dimethylsulfoxide; DPO, 2,5-diphenyloxazole; EDMAB, ethyl 4-dimethylaminobenzoate; ICT, intramolecular charge transfer; m.p., melting point; Prodan, 6-propionyl-2-dimethylaminonaphthalene.

Table 1. Comparison of the solvent dependence of compounds **9–11**, **13** and **17**

Compound	λ_{max} in $CHCl_3$	λ_{max} in MeOH	$\Delta\lambda_{max}$*	λ_F in $CHCl_3$	λ_F in MeOH	$\Delta\lambda_F$†
9	306	303	3	362	361	−1
10	348	348	0	435	480	45
11	332	324	8	392	400	8
13	382	373	9	501	575	74
17	380	373	7	509	585	76

*$\Delta\lambda_{max} = \lambda_{max}$ (in $CHCl_3$) − λ_{max} (in MeOH).
†$\Delta\lambda_F = \lambda_F$ (in MeOH) − λ_F (in $CHCl_3$).

also have long emission wavelengths, high extinction coefficients, high quantum yields and large Stokes shifts. Such fluorescent compounds are obviously promising probes for certain biological applications. We also report here the synthesis and characterization of a series of reactive Dapoxyl reagents that can be conjugated to various biomolecules such as proteins, nucleic acids and carbohydrates.

MATERIALS AND METHODS

Absorption and fluorescence spectra were recorded on an Aminco SPE-5000 and an Aminco SPF-500C, respectively. The NMR spectra were obtained on a Bruker YLIV370.040. Melting points were measured on a Mel-Temp II apparatus (Laboratory Devices, Inc., Holliston, MA) and are uncorrected.

The fluorescence quantum yields were determined using quinine sulfate in 5 *M* H_2SO_4 as the reference standard (Φ_F = 0.55) (12). In most solvents the concentrations of the dyes were adjusted to obtain an absorbance of 0.25 (1 cm cell) at the peak wavelength (Tables 1 and 2) except that in hexane most of the tested oxazole dyes have only an absorbance of 0.10 (5 cm cell) due to their limited solubility in the solvent. The concentration of the reference was also adjusted to have an absorbance of 0.25 at the same excitation wavelength of the dye tested except that in the case of hexane as a solvent, a 0.10 absorbance of the reference (5 cm cell) was used instead to match the absorbance of the dyes tested at the same excitation wavelength. Under these conditions, the fluorescence quantum yield of the tested compound (Φ_F^X) in the indicated solvent was calculated from the following formula, considering that the peak area (A_R) of the emission spectrum of the reference and that of the tested dye (A_X) can be readily determined:

$$\Phi_F^X = A_X\Phi_F^R/A_R$$

where Φ_F^R and Φ_F^X are the fluorescence quantum yields of the reference and the testing dye. The measurements were done in triplicate and the estimated errors were no more than 1%. The E_T (30) values of the various solvents were obtained from Reichardt and Skwierczynski and Connors' work (13,14).

All the solvents of spectral grade and 2,5-diphenyloxazole were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. Succinimidyl 3-(2-pyridyldithio)propionate is a commercial product of Molecular Probes, Inc. (Eugene, OR). All the other oxazoles and their synthetic intermediates were prepared as described below:

*2-Benzoylamino-4′-dimethylaminoacetophenone (**3**).* 2-Amino-4′-dimethylaminoacetophenone hydrochloride (400 mg, 2.0 mmol) and benzoyl chloride (281 mg, 2.0 mmol) were suspended in anhydrous dichloromethane (100 mL) and the resulting mixture was cooled to 0°C. To the suspension, anhydrous pyridine (474 mg, 6.0 mmol) was added dropwise at 0°C with stirring. The resulting mixture was stirred for 12 h at room temperature and was then diluted with chloroform (200 mL). The resulting solution was washed with cold water (3 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a colorless solid (320 mg, yield: 57%). Melting point (m.p.) = 139–141°C. ^{1}H-NMR ($CDCl_3$): 7.95 (4H, m); 7.45 (3H, m); 7.42 (1H, b, D_2O exchangeable); 6.70 (2H, dd); 4.8 (2H, d); 3.12 (6H, s).

*2-(4′-Fluorosulfonylbenzoylamino)acetophenone (**4**).* According to the procedure of compound **3**, 2-aminoacetophenone hydrochloride (1.2 g, 6.7 mmol) was reacted with 4-fluorosulfonylbenzoyl chloride (1.0 g, 4.5 mmol) to afford a colorless solid (772 mg, yield: 55%). m.p. = 207–209°C. ^{1}H-NMR ($CDCl_3$): 8.15 (4H, m), 8.05, 7.55 (2 × 2H, dd); 7.68 (1H, m); 7.38 (1H, m, D_2O exchangeable); 5.00 (2H, d).

*2-(4′-Fluorosulfonylbenzoylamino)-4″-dimethylaminoacetophenone (**5**).* Analogous to the procedure of compound **3**, 2-amino-4′-dimethylaminoacetophenone hydrochloride (5 g, 21 mmol) was reacted with 4-fluorosulfonylbenzoyl chloride (4.5 g, 17.9 mmol) to afford a colorless solid (6.4 g, yield: 95%). m.p. = 187–189°C. ^{1}H-NMR ($CDCl_3$): 8.15 (4H, s); 7.95, 6.70 (2 × 2H, dd); 7.55 (1H, b, D_2O exchangeable); 4.85 (2H, d); 3.10 (6H, s).

*2-(4′-Fluorosulfonylphenyl)-5-(4″-dimethylaminophenyl)oxazole (**6**, Dapoxyl sulfonyl fluoride).* Compound **5** (5 g, 13.8 mmol) was suspended in concentrated sulfuric acid (20 mL) and stirred for 24 h. The resulting solution was poured into ice (200 g), and the precipitate was collected by filtration. The resulting yellow solid was washed with cold water and dried under vacuum. Recrystallization from ethyl acetate gave pure compound **6** (4.6 g, yield: 92%). m.p. = 206–208°C. ^{1}H-NMR ($CDCl_3$): 8.25, 8.07 (2 × 2H, dd); 7.48, 6.73 (2 × 2H, dd); 7.31 (1H, s); 3.02 (6H, s).

*2-Phenyl-5-(4′-dimethylaminophenyl)oxazole (**10**).* Compound **3**

Table 2. Spectral properties of compound **17** in different solvents

Solvent	Solvent polarity $E_T(30)$* (kcal/mol)	Absorption maximum (nm)	Fluorescence maximum (nm)	Stokes' shift (nm)	Fluorescence quantum yield
Hexane	31.0	367	434	67	0.91
EtOAc†	38.1	370	516	146	0.77
Chloroform	39.1	380	509	129	0.77
Acetone	42.2	373	551	178	0.64
DMSO‡	45.1	380	581	201	0.59
Acetonitrile	45.6	373	578	205	0.56
Methanol	55.4	373	580	207	0.39
4:1 Acetonitrile/water (vol/vol)	54.5	373	598	205	0.32
3:2 Acetonitrile/water (vol/vol)	55.6	373	610	237	0.19
1:1 Acetonitrile/water (vol/vol)	56.2	373	618	245	0.14
2:3 Acetonitrile/water (vol/vol)	56.8	373	625	252	0.10
1:4 Acetonitrile/water (vol/vol)	59.9	373	636	263	0.04

*The $E_T(30)$ data are from Reichardt (13) and Skwierczynski and Connors (14).
†EtOAc = ethyl acetate.
‡DMSO = dimethylsulfoxide.

(320 mg, 1.1 mmol) was suspended in $POCl_3$ (20 mL) and refluxed for 30 min. The resulting solution was poured into ice (200 g), and the precipitate was collected by filtration. The resulting solution was poured into ice (200 g), and the precipitate was collected by filtration. The resulting yellow solid was washed with cold water and dried under vacuum (250 mg, yield: 83%). m.p. = 145–147°C. ^{1}H-NMR ($CDCl_3$): 8.08, 7.60 (2 × 2H, dd); 7.45 (3H, m); 7.24 (1H, s); 6.78 (2H, m); 3.00 (6H, s).

*2-(4′-Fluorosulfonylphenyl)-5-phenyloxazole (**11**).* Compound **4** (628 mg, 1.9 mmol) was suspended in concentrated sulfuric acid (4 mL) and stirred for 24 h. The resulting solution was poured into ice (200 g), and the precipitate was collected by filtration. The resulting pale yellow solid was washed with cold water and air dried (572 mg, yield: 99%). m.p. = 148–150°C. ^{1}H-NMR ($CDCl_3$): 8.35, 8.10 (2 × 2H, dd); 7.75 (2H, d); 7.57 (1H, s); 7.49 (2H, m); 7.44 (1H, m).

*2-(4′-Butylaminosulfonylphenyl)-5-phenyloxazole (**12**).* Compound **11** (573 mg, 1.9 mmol) and butylamine (292 mg, 4.0 mmol) were dissolved in anhydrous tetrahydrofuran (10 mL). The solution was refluxed for 12 h and then concentrated *in vacuo*. The residue was poured into water (100 mL) and extracted with chloroform (2 × 50 mL). The combined chloroform layers were sequentially washed with water (3 × 100 mL), 1% HCl (3 × 50 mL) and water (3 × 100 mL), dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a yellow solid (500 mg, yield: 74.3%). m.p. = 150–151°C. ^{1}H-NMR ($CDCl_3$): 8.21, 7.96 (2 × 2H, dd); 7.62, 6.87 (2 × 2H, dd); 7.33 (1H, s); 4.45 (1H, t, D_2O exchangeable); 3.05 (6H, s); 2.90 (2H, m); 1.45 (2H, m); 1.30 (2H, t); 0.86 (3H, t).

*2-(4′-Sulfophenyl)-5-(4″-dimethylaminophenyl)oxazole, sodium salt (**14**, Dapoxyl sulfonic acid, sodium salt).* Compound **6** (4 g, 11.0 mmol) was suspended in 10% NaOH (50 mL) and refluxed for 2 h. The reaction solution was then cooled to room temperature and the precipitate was collected by filtration and washed with cold water. The material was recrystallized twice from water to afford a pale yellow solid (3.6 g, yield: 86%). m.p. > 300°C. ^{1}H-NMR [dimethylsulfoxide (DMSO)-d_6]: 8.03, 7.76 (2 × 2H, dd); 7.65, 6.78 (2 × 2H, dd); 7.54 (1H, s); 2.97 (6H, s).

*2-(4′-Chlorosulfonylphenyl)-5-(4″-dimethylaminophenyl)oxazole (**15**, Dapoxyl sulfonyl chloride).* Compound **14** (3 g, 7.8 mmol) was suspended in $POCl_3$ (20 mL) and refluxed for 1 h. The reaction solution was cooled to room temperature, poured into ice (200 g) and extracted with chloroform (3 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a pale yellow solid (2.4 g, yield: 81%). m.p. = 260°C (d). ^{1}H-NMR ($CDCl_3$): 8.29, 8.13 (2 × 2H, dd); 7.64, 6.90 (2 × 2H, dd); 7.38 (1H, s); 3.07 (6H, s).

*2-(4′-Butylaminosulfonylphenyl)-5-(4″-dimethylaminophenyl)oxazole (**13**, Dapoxyl butyl sulfonamide).* Compound **15** (0.5 g, 1.4 mmol) was dissolved in anhydrous *N,N*-dimethylformamide (15 mL). To the solution, butylamine (302 mg in 5 mL of dichloromethane, 3.0 mmol) was added dropwise. The reaction mixture was stirred for 12 h at room temperature and then concentrated *in vacuo*. The residue was poured into water (100 mL) and extracted with chloroform (2 × 50 mL). The combined chloroform layers were sequentially washed with water (3 × 100 mL), 1% HCl (3 × 50 mL) and water (3 × 100 mL), dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a yellow solid (502 mg, yield: 79%). m.p. = 193–194°C. ^{1}H-NMR ($CDCl_3$): 8.21, 7.96 (2 × 2H, dd); 7.62, 6.87 (2 × 2H, dd); 7.33 (1H, s); 4.45 (1H, t, D_2O exchangeable); 3.05 (6H, s); 2.90 (2H, m); 1.45 (2H, m); 1.60 (2H, t); 0.86 (3H, t).

*2-(4′-Hydrazinosulfonylphenyl)-5-(4″-dimethylaminophenyl)oxazole (**16**, Dapoxyl sulfonyl hydrazine).* Hydrazine monohydrate (700 mg, 14.0 mmol) was dissolved in dichloromethane (20 mL) and the resulting solution was cooled to 0°C. To this solution, compound **15** (0.5 g in 5 mL of anhydrous *N,N*-dimethylformamide, 1.4 mmol) was added dropwise. The resulting mixture was stirred for 12 h at room temperature and was then concentrated *in vacuo*. The residue was poured into water (100 mL) and extracted with chloroform (2 × 50 mL). The combined chloroform layer was washed with cold water (3 × 100 mL). The organic layer was extracted with 1% HCl (2 × 50 mL), and the combined aqueous layer was washed with ethyl acetate (3 × 100 mL). The aqueous layer was then neutralized with 10% NaOH and reextracted with chloroform (3 × 100 mL). The combined chloroform layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a yellow solid. The crude material was further purified on a silica gel column using 10:1:1 chloroform/methanol/ethyl acetate as eluant. The final product was obtained as a pale yellow solid (301 mg, yield: 61%). m.p. = 176–178°C. ^{1}H-NMR (DMSO-d_6): 8.51 (1H, t, D_2O exchangeable); 8.21, 7.93 (2 × 2H, dd); 7.67, 6.81 (2 × 2H, dd); 7.63 (1H, s); 4.20 (2H, d, D_2O exchangeable); 2.97 (6H, s).

*2-(4′-[2‴-Aminoethylaminosulfonyl]phenyl)-5-(4″-dimethylaminophenyl)oxazole (**17**, Dapoxyl 2-aminoethyl sulfonamide).* Analogous to the procedure of compound **16**, compound **15** (0.5 g, 1.4 mmol) was reacted with ethylenediamine (840 mg, 14.0 mmol) to give a pale yellow solid (248 mg, yield: 46%). m.p. = 232–234°C. ^{1}H-NMR (DMSO-d_6): 7.92, 7.67 (2 × 2H, dd); 7.44, 6.98 (2 × 2H, dd); 7.17 (1H, s); 7.10 (2H, m, D_2O exchangeable); 2.98 (2H, m); 2.84 (6H, s); 2.71 (2H, m).

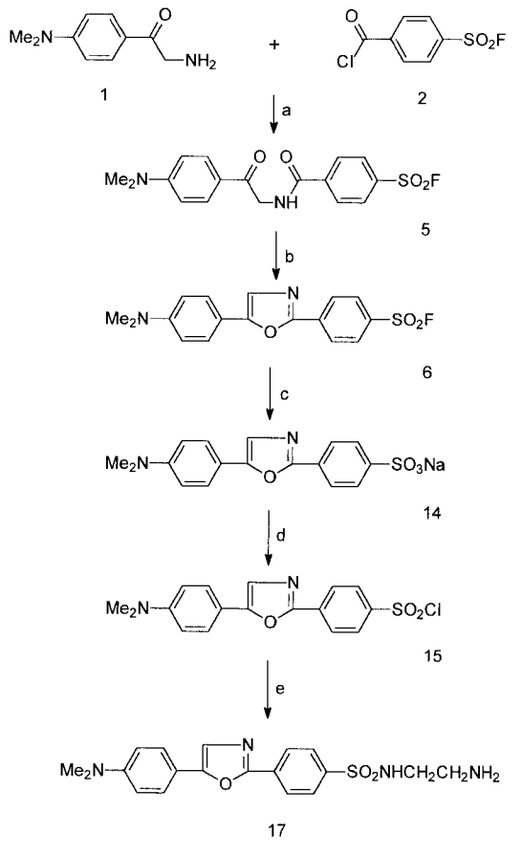
*2-(4′-[2‴-Bromoacetylaminoethylaminosulfonyl]phenyl)-5-(4″-dimethylaminophenyl)oxazole (**18**, Dapoxyl 2-bromoacetamidoethyl sulfonamide).* Compound **17** (450 mg, 1.2 mmol) and anhydrous pyridine (140 mg, 1.8 mmol) were dissolved in anhydrous tetrahydrofuran (15 mL), and the resulting solution was cooled to 0°C. To the solution, bromoacetyl bromide (353 mg in 5 mL of anhydrous dichloromethane, 1.8 mmol) was added dropwise. The resulting mixture was stirred for 2 h at room temperature and was then concentrated *in vacuo*. The residue was poured into water (100 mL) and extracted with chloroform (3 × 50 mL). The combined chloroform layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a yellow solid. The crude material was purified on a silica gel column using 10:1 chloroform/methanol as eluant. The final product was obtained as a pale yellow solid (374 mg, yield: 57%). m.p. = 197–200°C (d). ^{1}H-NMR (DMSO-d_6): 8.22, 7.93 (2 × 2H, dd); 7.65, 6.83 (2 × 2H, dd); 7.55 (1H, s); 3.82 (2H, s); 3.14 (2H, m); 2.95 (6H, s); 2.85 (2H, m).

*2-(4′-[2‴-Ethoxycarbonylethylaminosulfonyl]phenyl)-5-(4″-dimethylaminophenyl)oxazole (**19**).* β-alanine ethyl ester hydrochloride (800 mg, 5.2 mmol) and compound **15** (0.5 g, 1.4 mmol) were dissolved in anhydrous *N,N*-dimethylformamide (15 mL). To the solution, triethylamine (303 mg in 5 mL of anhydrous *N,N*-dimethylformamide, 3.0 mmol) was added dropwise. The resulting mixture was stirred for 12 h at room temperature, poured into water (100 mL) and extracted with chloroform (2 × 50 mL). The combined chloroform layers were sequentially washed with water (3 × 100 mL), 1% HCl (3 × 50 mL) and water (3 × 100 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to afford a yellow solid (590 mg, yield: 93%). ^{1}H-NMR ($CDCl_3$): 8.23, 7.96 (2 × 2H, dd); 7.67, 7.01 (2 × 2H, dd); 7.38 (1H, s); 5.31 (1H, t, D_2O exchangeable); 4.13 (2H, m); 3.24 (2H, m); 3.01 (6H, s); 2.53 (2H, t); 1.22 (3H, t).

*2-(4′-[2‴-Carboxyethylaminosulfonyl]phenyl)-5-(4″-dimethylaminophenyl)oxazole (**20**).* Compound **19** (148 mg, 0.33 mmol) was dissolved in methanol (10 mL). To the solution, 1M NaOH (1 mL, 1.0 mmol) was added dropwise. The resulting solution was stirred at room temperature for 12 h, concentrated *in vacuo*, and the residue was redissolved in water (10 mL). The aqueous layer was neutralized with 10% HCl, and the precipitate was collected by filtration, washed with cold water (10 mL) and air-dried (130 mg, yield: 94%). m.p. = 196–198°C ^{1}H-NMR (DMSO-d_6): 12.29 (1H, s, D_2O exchangeable); 8.25, 7.91 (2 × 2H, dd); 7.83 (2H, t); 7.68, 6.81 (2 × 2H, dd); 7.62 (1H, s); 2.91 (6H, s); 2.41 (2H, t).

*2-(4′-[2‴-Carboxyethylaminosulfonyl]phenyl)-5-(4″-dimethylaminophenyl)oxazole, succimidyl ester (**21**, Dapoxyl sulfonamidopropionic acid, succinimidyl ester).* Compound **28** (130 mg, 0.31 mmol) and *N*-hydroxysuccinimide (43 mg, 0.37 mmol) were dissolved in anhydrous *N,N*-dimethylformamide (5 mL). To the solution, diisopropylcarbodiimide (46 mg, 0.37 mmol) was added dropwise. The resulting solution was stirred at room temperature for 12 h, concentrated *in vacuo*, and the residue was poured into ether (20 mL). The precipitate was collected by filtration and washed with ether (100 mL). The crude solid was redissolved in *N,N*-dimethylformamide (1 mL), and the resulting solution was poured into ethyl acetate to give a pale yellow solid (146 mg, yield: 92%). m.p. = 199–201°C. ^{1}H-NMR ($CDCl_3$): 8.22, 7.98 (2 × 2H, dd); 7.66, 6.75 (2 × 2H, dd); 7.29 (1H, s); 5.42 (1H, t, D_2O exchangeable); 3.45 (2H, m); 3.05 (6H, s); 2.82 (4H, s); 2.80 (2H, t).

2-(4′-[2″-(3‴-Pyridyldithiopropionyl)aminoethyl]aminosulfonyl-



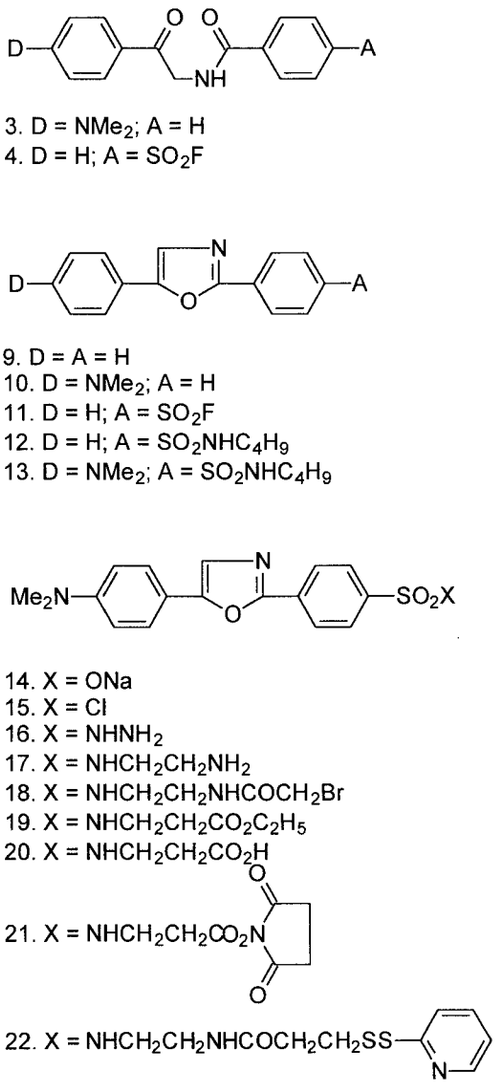
Scheme 1. Synthesis of Dapoxyl dyes. a = pyridine/dimethylformamide, room temperature, 12 h; b = sulfuric acid, room temperature, 24 h; c = 10% NaOH, reflux, 6 h; d = $POCl_3$ reflux, 30 min; e = ethylenediamine/tetrahydrofuran, room temperature, 12 h.

*phenyl)-5-(4″-dimethylaminophenyl)oxazole (**22**, Dapoxyl 2-[3-(2-pyridyldithio)propionamido]ethylsulfonamide).* Compound **17** (450 mg, 1.2 mmol) and anhydrous pyridine (140 mg, 1.8 mmol) were dissolved in anhydrous tetrahydrofuran (15 mL) and the resulting solution was cooled to 0°C. To the solution, succinimidyl 3-(2-pyridyldithio)propionate (437 mg in 5 mL of anhydrous *N,N*-dimethylformamide, 1.4 mmol) was added dropwise. The resulting mixture was stirred for 6 h at room temperature and was then concentrated *in vacuo*. The residue was poured into water (100 mL) and extracted with chloroform (3 × 50 mL). The combined chloroform layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a yellow solid. The crude material was purified on a silica gel column using 10:1 chloroform/methanol as eluant. The final product was obtained as a pale yellow solid (449 mg, yield: 86%). m.p. = 153–155°C. ^{1}H-NMR ($CDCl_3$): 8.45 (1H, m, D_2O exchangeable); 8.18, 7.91 (2 × 2H, dd); 7.59, 6.79 (2 × 2H, dd); 7.57 (2H, m); 7.23 (1H, s); 7.13 (2H, m); 5.51 (1H, t, D_2O exchangeable); 3.44 (2H, m); 3.13 (2H, m); 3.05 (2H, t); 3.03 (6H, s); 2.57 (2H, t).

RESULTS AND DISCUSSION

Synthesis

The Dapoxyl dyes were readily synthesized as outlined in Scheme 1. Aminoacetophenone **1** selectively reacts with fluorosulfonyl benzoyl chloride **2** to give amide **5** nearly quantitatively. A similar amide was previously prepared from 4-chlorosulfonyl benzoyl chloride with no yield reported (15). However, we noted that the reaction of the chlorosulfonyl benzoyl chloride with compound **1** is extremely



Scheme 2. Chemical structures of Dapoxyl dyes and their synthetic intermediates.

complex, and in our hands the desired amide was obtained in very low yield. Apparently the fluorosulfonyl group is an excellent sulfonate-protecting group for the synthesis of amide **5**, as expected. Compound **5** was readily cyclized to give oxazole **6** in concentrated sulfuric acid at room temperature. This reaction condition is much milder than the previous method (15). We found that sulfuric acid is the best dehydrating agent for the cyclization among the many other dehydrating agents that we also tried, including phosphorus oxychloride, acetic anhydride, methanesulfonic acid and dicyclohexylcarbodiimide. Sulfonyl fluoride **6** is hydrolyzed to the corresponding sulfonic acid in high yield. The sulfonic acid is readily purified by recrystallization from water and converted to sulfonyl chloride **15** in excellent yield. This sulfonyl chloride reacts with ethylenediamine to give compound **17**. Compounds **15** and **17** were used to synthesize a variety of reactive Dapoxy dyes, as listed in Scheme 2.

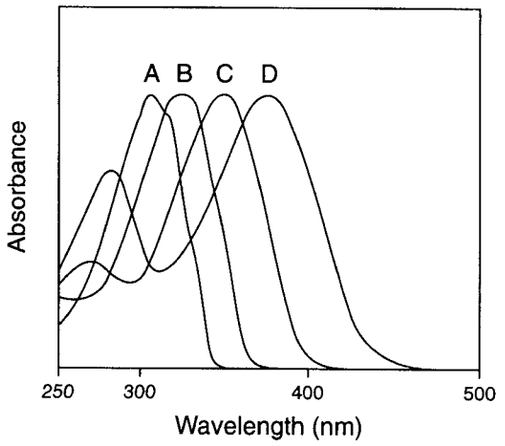


Figure 1. The absorption spectra of compounds **9–11**, and **13** in methanol. The concentrations of the compounds are 1–5 μ*M*. Spectrum A = compound **9**; spectrum B = compound **11**; spectrum C = compound **10**; spectrum D = compound **13**.

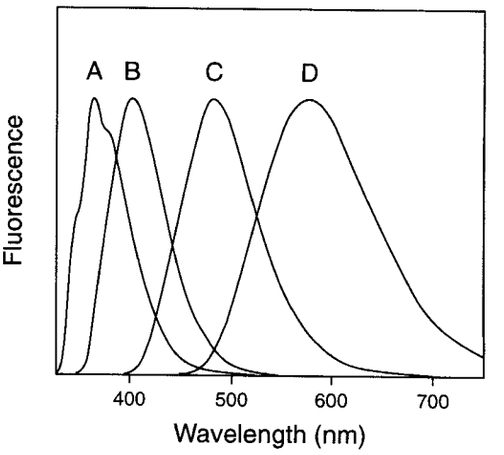


Figure 2. The fluorescence spectra of compounds **9–11**, and **13** in methanol. The concentrations of the compounds are 0.1–0.5 μ*M*. Spectrum A = compound **9**; spectrum B = compound **11**; spectrum C = compound **10**; spectrum D = compound **13**. The spectra were normalized to the same peak intensity.

Absorption spectra

As shown in Scheme 2, compounds **13** and **17** are two typical representatives of the Dapoxyl dyes. These dyes embody the strong electron-donating dimethylamino group at position 4 of the 5-phenyl ring and the strong electron-withdrawing sulfonyl group at position 4 of the 2-phenyl ring forming a "push–pull" electron transfer system from the 5-phenyl moiety to the 2-phenyl ring. For comparison, three parent 2,5-diphenyloxazoles (DPO, compounds **9–12**) were also included in our spectral studies of the Dapoxyl dyes. Compound **9** has neither an electron donor nor an electron acceptor, and compounds **10–12** have only one of each.

The absorption spectra of the Dapoxyl dyes were compared with those of the three parent DPO in Fig. 1. The absorption spectra of compounds **13** and **17** are considerably red shifted and broadened relative to those of the parent DPO as seen in Fig. 1 and Table 1. Interestingly, the Dapoxyl dyes maintain the characteristic vibronic absorption bands of the DPO nucleus only in hexane, whereas the fine resolution of absorption bands is absent in the polar solvents (Figs. 1 and 3). This implies that the observed electronic transitions of the Dapoxyl dyes might result in intramolecular charge transfer (ICT) bands involving the dimethylamino moiety and the sulfonyl group (Scheme 1).

The absorption wavelength and intensity of the Dapoxyl dyes are fairly insensitive to the solvent polarity or the hydrogen-bonding ability of the solvent (Table 1). This insensitivity indicates that the interaction of the participating ground and excited states with the solvent is such that the energy gap between the ground state and the corresponding Franck–Condon excited states responsible for the observed electronic transition is independent of the solvent polarity and hydrogen-bonding ability.

Fluorescence spectra

The fluorescence spectrum of a representative Dapoxyl dye (compound **17**) was compared with those of the three parent DPO in Fig. 2. As observed for their absorption spectra (*vide supra*), the fluorescence spectra of the Dapoxyl dyes are also considerably red shifted and broadened relative to the fluorescence spectra of the parent DPO. Additionally, the Dapoxyl dyes maintain the characteristic vibronic fluorescence bands of the DPO nucleus only in hexane, whereas the fine resolution of fluorescence bands is absent in polar solvents (Figs. 2 and 3). This further indicates the ICT properties of electronic transitions of the Dapoxyl dyes.

Unlike the absorption spectra, both the fluorescence wavelength and quantum yield of the Dapoxyl dyes change dramatically with the solvents of different polarity. For example, the fluorescence of compound **17** shows a 144 nm red shift when the solvent is changed from hexane, a nonpolar solvent, to acetonitrile, a polar and aprotic solvent. On the other hand, the characteristic vibronic bands of the Dapoxyl dyes in hexane are absent in acetonitrile, as described above. A close inspection of the emission spectrum reveals that it

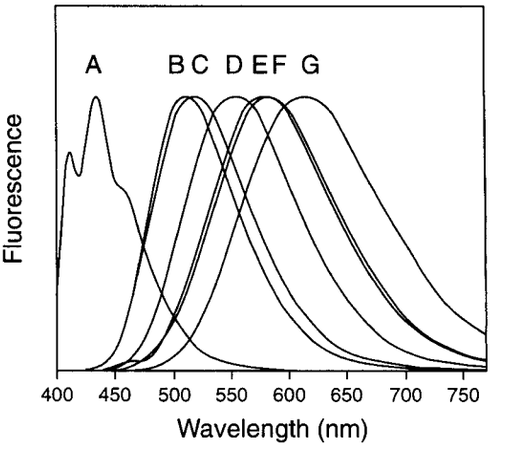


Figure 3. The fluorescence spectra of compound **17** in different solvents. The concentrations of the compound are 0.1–0.5 μ*M*; spectrum A = hexane; spectrum B = chloroform; spectrum C = ethyl acetate; spectrum D = acetone; spectrum E = acetonitrile; spectrum F = dimethylsulfoxide; spectrum G = 1:1 acetonitrile–water (vol/vol). The spectra were normalized.

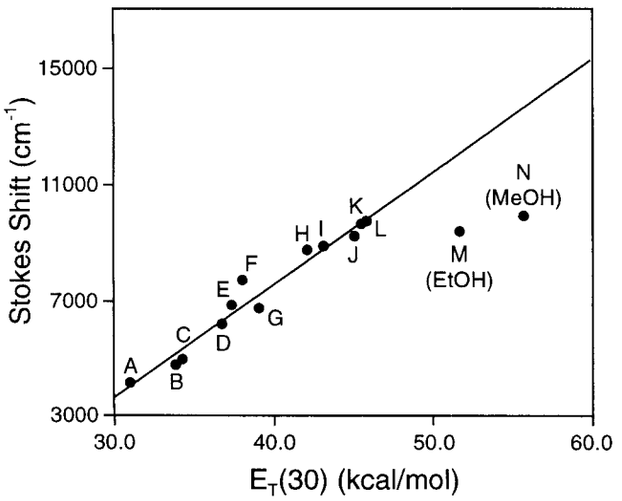


Figure 4. The plot of the Stokes' shift of compound **17** against solvent polarity parameter $E_T(30)$. A = hexane; B = toluene; C = benzene; D = chlorobenzene; E = tetrahydrofuran; F = ethyl acetate; G = chloroform; H = acetone; I = *N,N*-dimethylformamide; J = dimethylsulfoxide; K = acetonitrile; L = trichloroacetone; M = ethanol; N = methanol.

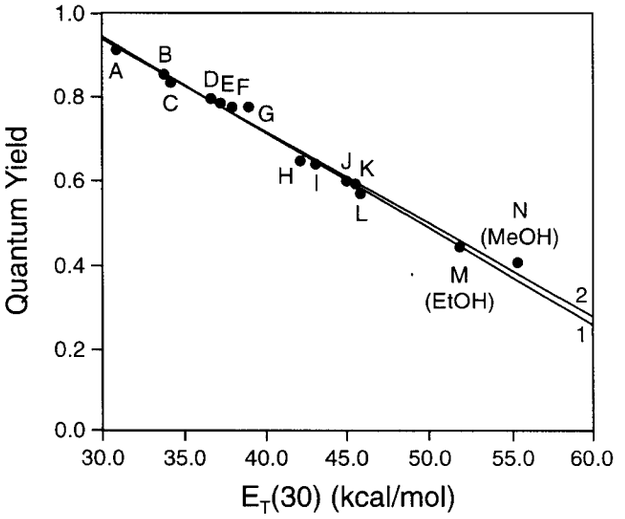


Figure 5. The plot of the fluorescence quantum yields of compound **17** against solvent polarity parameter $E_T(30)$. Curve 1 with ethanol and methanol points; curve 2 without ethanol and methanol points. A = hexane; B = toluene; C = benzene; D = chlorobenzene; E = tetrahydrofuran; F = ethyl acetate; G = chloroform; H = acetone; I = *N,N*-dimethylformamide; J = dimethylsulfoxide; K = acetonitrile; L = trichloroacetone; M = ethanol; N = methanol.

is red shifted and broadened extensively with an increase in solvent polarity. Such spectral characteristics are typical of ICT excited states (16). The marked solvent sensitivity of the Dapoxyl dyes derives from the large dipole moment developed in the excited state as a consequence of facile charge delocalization between the dimethylamino moiety and the sulfonyl group. This is also supported by the fact that similar red shifts in fluorescence spectrum are absent for compounds **9–11** (Table 1).

Solvent effects

As described above, the fluorescence properties of the Dapoxyl dyes greatly depend on solvents. Many solvent parameters have been developed to measure solvent effects. Among these parameters, the $E_T(30)$ value has proven to be a reliable measurement of solvent polarity. Thus the fluorescence maxima and Stokes shift of compound **17** were measured in different solvents with $E_T(30)$ values ranging from 31 to 60 (Table 2), and were plotted as a function of solvent polarity in Fig. 4, giving a linear fit. This is consistent with the ICT assignment (17). It was noted that the exclusion of the methanol and ethanol points results in a much better fit. This implies that there are also certain hydrogen-bonding interactions that affect the energy levels of the excited Dapoxyl dyes in addition to dipole–dipole interactions. This phenomenon has been observed for many other solvatochromic dyes (7,13). The effect of hydrogen bonding on the Stokes shifts of the Dapoxyl dyes was further supported by the fact that the deviation of the methanol and ethanol points is eliminated if another solvent parameter D_N, a measurement of hydrogen-bonding interaction (11), is incorporated into the correlation (data not shown).

The fluorescence quantum yields of compound **17** were also measured in different solvents. A plot of the fluorescence quantum yield *versus* solvent polarity for the Dapoxyl dye shows an even better linear correlation (Fig. 5) than the Stokes shift plot (Fig. 4). Interestingly, there is still a good linear correlation even if methanol and ethanol points are included (curve 1 in Fig. 5). It was found that the exclusion of the methanol and ethanol points does not make a significant difference in the plots (compare curves 1 and 2 in Fig. 5). This implies that hydrogen-bonding interaction exerts little effect on the fluorescence quantum yields of the Dapoxyl dyes. This simplified correlation between the fluorescence quantum yields of the probes and the nature of the surrounding environments should make them more useful for complex biophysical studies than other solvatochromic dyes that often have both general and special probe–environment interactions. The multiple modes of the interactions between the probes and the surrounding environments often makes the development of theoretical calculations or models quite difficult (16,17).

Ethyl 4-dimethylaminobenzoate (EDMAB) is concluded to be a classic ICT system (6,16). Thus, the solvent effects of the Dapoxyl dyes were compared with those of EDMAB under similar conditions. The comparison indicated that they have quite similar solvent effects. The fluorescence maxima of compound **17** were plotted against those of EDMAB in different solvents. There is a correlation between the Stokes shifts of the two dyes as shown in Fig. 6. This further supports the ICT fluorescence property of the Dapoxyl dyes.

It was observed that the solvent effects of the Dapoxyl dyes are much greater than those of the well-known solvatochromic fluorescent dyes such as dansyl dyes, 6-propionyl-2-dimethylaminonaphthalene (Prodan), ANS, EDMAB. *etc.* (Table 3). Such enhanced solvent effects should make these new fluorescent dyes promising probes. Additionally, the different correlation between the different fluorescence parameters of the Dapoxyl probes and the nature of the surrounding environments offers multiple choices for one to study various biological systems. For example, the correlation of the fluorescence quantum yields of the Dapoxyl dyes

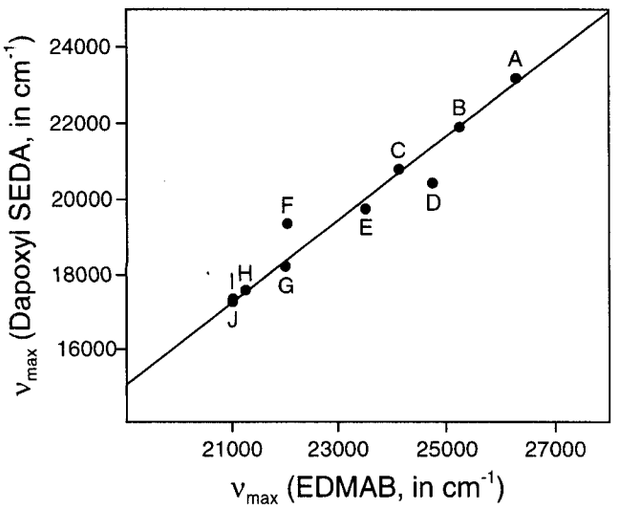


Figure 6. The plot of the fluorescence maxima of compound **17** against the ICT fluorescence maxima of EDMAB in different solvents. A = hexane; B = toluene; C = tetrahydrofuran; D = benzene; E = chlorobenzene; F = ethyl acetate; G = acetone; H = *N,N*-dimethylformamide; I = acetonitrile; J = dimethylsulfoxide.

Table 3. Comparison of the spectral properties of compound **17** with those of the other well-known solvatochromic fluorescent dyes

Dye	λ_{max} (ϵ, in MeOH)*	λ_F (nm, in MeOH)	λ_F (nm, in $CHCl_3$)	$\Delta\lambda_F$ (nm)†
Dapoxyl SEDA‡	373 (28 000)	584	509	71
Dansyl EDA	335 (4600)	526	499	27
ADMAN	360 (15 000)	499	440	59
Prodan	361 (16 000)	498	440	58
1,8-ANS	372 (7800)	480	490	−10
2,6-ANS	319 (27 000)	422	410	12
EDMAB§	312 (28 000)	520	424	96
7-Ethoxycoumarin	324 (11 000)	399	385	4

*λ_{max} is in nm and ϵ is in cm^{-1} mol^{-1}; there is generally little effect of solvents on the absorption spectra of fluorescent solvatochromic dyes.

†λ_F (in MeOH) − λ_F (in $CHCl_3$).

‡Dapoxyl SEDA = Dapoxyl sulfonyl ethylenediamine (compound **17**); Dansyl EDA = Dansyl ethylenediamine; ADMAN = 6-acetyl-2-dimethylaminonaphthalene; Prodan = 6-propionyl-2-dimethylaminonaphthalene; 1,8-ANS = 8-anilinonaphthalene-1-sulfonic acid; 2,6-ANS = 6-anilinonaphthalene-2-sulfonic acid; EDMAB = ethyl 4-dimethyaminobenzoate.

§The ICT fluorescence of EDMAB is quite weak in both polar and apolar solvents.

with a single solvent parameter $E_T(30)$ might be used to investigate complex systems considering the simpler theoretical modeling, whereas the correlation of the Stokes shifts of the Dapoxyl dyes with multiple property parameters might be chosen to study simpler systems because the correlation can also probe hydrogen-bonding interactions besides the dipole–dipole interaction. In some cases, one may use the multiple correlation to get more information.

Reactive dapoxyl dyes and their potential biological applications

Fluorescent molecules, whose spectra or quantum yields are sensitive to their environments, are widely used as reporter probes in the study of ligand binding, membrane structures and dynamics, "hydrophobic pockets" and conformational changes in proteins and other biomacromolecules and many other biological interactions. This solvent sensitivity manifests itself as an alteration of fluorescence wavelength, intensity or both. The changes are readily detectable in living cells with fluorescence ratio imaging (18).

Merocyanine-modified proteins have, for example, been used to measure the binding of Ca^{2+} to calmodulin (19) and parvalbumin (20). When calcium binds to the derivatized protein, the dye moves from an aqueous to a hydrophobic environment, with consequent changes in fluorescence (21). This dye-labeled calmodulin analog is used as an indicator of calmodulin activation in individual living cells, revealing the kinetics and spatial distribution of calmodulin activity during serum stimulation and wound healing (22). A solvatochromic coumarin-labeled phosphate-binding protein has been successfully used to measure the kinetic release of inorganic phosphate from enzymes such as phosphatases (23). The protein-bound coumarin exhibits a 5.2-fold increase in fluorescence with a 10 nm peak wavelength shift in the presence of saturating inorganic phosphate and is used to measure the rate of inorganic phosphate release from solutions of actomyosin subfragment 1 during ATPase activity. It can detect submicromolar inorganic phosphate anions in solutions and has potential for use in living cells. Richieri and coworkers (24) used a similar approach to produce fluorescent indicators for determining the concentration of specific fatty acids. An intestinal fatty acid-binding protein, I-FABP, was labeled with the solvent-sensitive fluorescent dye acrylodan. Fluorescent changes in this indicator were used to monitor concentrations of the fatty acid during activation of living cytotoxic T lymphocytes. To study actin assembly *in vitro,* Kouyama and Mihashi (25) used covalently bound pyrene to measure the assembly of globular actin molecules into actin microfilaments, while Detmers *et al.* (26) produced a similar reagent by reacting 4-chloro-7-nitrobenz-2-oxa-1,3-diazole with filamentous actin. The pyrene-labeled actin has since become the reagent of choice for dissecting the assembly and disassembly of actin *in vitro* because of its large change in fluorescence intensity upon polymerization.

As discussed above, it is apparent that solvent-sensitive fluorophores have wide applications in many fields, and this utility is greatly augmented by the availability of the covalently reactive dyes. Such probes are obviously most useful when a small perturbation causes a large change in fluorescence. As seen in Table 3, the Dapoxyl dyes have certain advantages over the existing fluorescent solvatochromic dyes, such as longer emission wavelengths, larger extinction coefficients, higher fluorescence quantum yields, bigger Stokes shifts and greater solvent sensitivities. The versatility in preparing reactive forms of the Dapoxyl dyes should make them dyes of choice in the development of a new generation of fluorescent molecular probes. Compounds **15** and **21** can be used to modify proteins or nucleic acids *via* reaction with their amino groups, while compounds **18** and **22** react efficiently and selectively with thiol groups in biomolecules. Compound **16** and **17** can be used to modify carbohydrates

or glycoproteins. Additionally, compound **18** can react with carboxylic acid groups in proteins and hydroxy groups in carbohydrates under some conditions.

CONCLUSIONS

The Dapoxyl dyes are attractive fluorophores as molecular probes because of their long emission wavelength, large extinction coefficients, high fluorescence quantum yields, large Stokes shift and great solvent sensitivity. The fluorescence changes of the dyes are well correlated with the polarity of solvent, solvent composition or the surrounding environment, *i.e.* polarity parameter $E_T(30)$. This fluorescence-environment dependence can be used to develop new fluorescent molecular probes to study a variety of biological events and processes. The development of Dapoxyl dye-based fluorescent molecular probes should be facilitated by the currently commercial availability of a versatile set of reactive Dapoxyl dyes.

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