Solid-Phase Synthesis and Modification of Thiazole Orange and Its Derivatives and their Spectral Properties

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A new solid-phase synthesis is shown to be effective in the preparation of cyanine dye Thiazole Orange (TO) and its derivatives, which can be obtained as a traceless cleavage of the Merrifield resin method. The influence of different solvents, substitutional groups, introduced to benzothiazole such as chlorine, methyl, and nitro was extensively studied. The changes of the special characteristic and fluorescence intensity of the TO derivatives were described. The phenomenon of the synergetic effect was also depicted after modifying the TO molecule with chitosan oligosaccharide (CTS) at the end of the alkyl, which can effectively improve the sensitivity of the fluorescent probe.

Introduction

With the progress of fluorescent spectra and laser light technology, fluorescent dyes are used in a variety of applications such as markers for flow cytometry studies, detection of nucleic acids and proteins, clinical diagnosis, and as photo therapeutic agents. It is entirely possible that fluorescent dyes will be used in detection of DNA sequences and the earlier detection of tumors in the future.[1]

As a new kind of fluorescence probe, Thiazole Orange (TO) and its derivatives have several special properties such as a wider spectrum scale, a higher molar absorption coefficient, a higher fluorescence quantum yield when inserted into a twin-helix of DNA, no fluorescence background in detecting of nucleic acids and proteins, and better photosensitivity and security.[2–7]

TO and its derivatives can be synthesized in the liquid phase by different routes: (1) 2-mercaptobenzothiazole reacts with lepidine derivatives to synthesize TO,[8–12] (2) 2-aminobenzothiazole instead of 2-mercaptobenzothiazole reacts with lepidine derivatives to prepare TO,[13,14] and (3) TO and its derivatives can be synthesized by 2-methylbenzothiazole reacting with lepidine derivatives.[15]

But, the reaction processes of separation and purification are often extraordinary complex in liquid-phase reaction while they are easy in solid-phase synthesis methodology which has many advantages in the preparation of some particular organic compounds.[16,17]

In this paper, TO and its derivatives were synthesized in the solid phase as shown in Scheme 1. Poly(styrene) (PS) compound (I) and its derivatives were prepared by 2-mercaptobenzothiazole attached to a Merrifield resin, then, they were reacted with methyl 4-methylbenzenesulfonate to form a polymer salt PS-compound (II), which reacted with derivatives of lepidine to obtain TO and its derivatives. The traceless cleavage of Merrifield resin was easily carried out in the solid-phase synthesis.

In the synthesis process, it is easy for TO to be modified by introducing different substitutional groups at the active positions of the molecule, which can bring a series of effects on the reaction rate and spectrum idiosyncrasy.

While the amino was attached to the end of the alkyl of TO molecule, which can be modified by the folic acid to be used for the study of identifying cancer cells as targets that have an extra folacin acceptor on the cellular surface. Significantly, its biology tissue compatibility and fluorescence intensity can be increased by connecting chitosan oligosaccharide (CTS) at the end of the alkyl of TO molecule.

Results and Discussion

In order to gain an optimal reaction result of the title PS-compounds, we studied a series of factors which effect the reaction process of synthesis TO {1}.

Effect of Conditions on Solid-Phase Synthesis Reaction I. Effect of Solvent on Reaction I. In the paper, we focused on some advantageous aspects of the solvents used in reaction I of solid-phase synthesis. The parameters of some solvents and some experimental results of refluxing for 4 h are shown in Table 1.

In reaction I, the polymer resin beads were first swelled up to make its reacting group outspread from its surface and were surrounded by the reaction solvent for creating favorable reaction condition. Simultaneously in the same system, the solvent can form a solvent shell around the anion of 2-mercaptobenzothiazole by reacting with K_2CO_3 removed a hydrogenous proton suitable for finishing the nucleophilic substitution reaction. From Table 1, we can see that both toluene and ethyl acetate as solvent had good results for the reaction and the conversion rate of Merrifield resin was 85% and 89%, respectively. On the basis of the data of the

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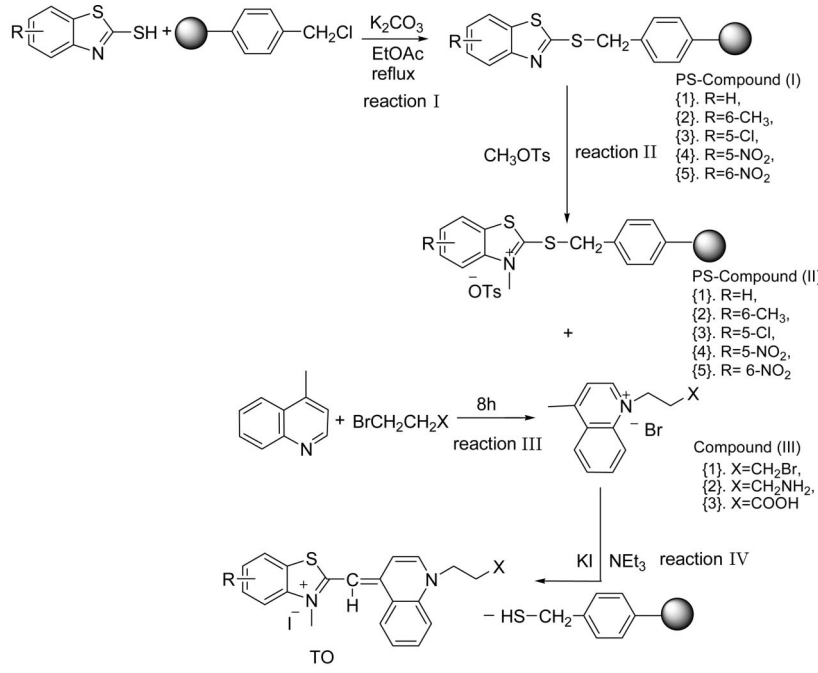
Scheme 1. Solid-Phase Synthetic Route to TO



TO{1-11}1: R=H, X=Br; 2: R=H, X=NH_2; 3: R=H, X=COOH; 4: R=6-CH_3, X=Br; 5: R=6-CH_3, X=COOH; 6: R=5-Cl, X=Br; 7: R=5-Cl, X=COOH; 8: R=5-NO_2, X=Br; 9: R=5-NO_2, X=COOH, 10: R=6-NO_2, X=Br; 11: R=6-NO_2, X=COOH

Table 1. Effect of Solvent on the Conversion Rate of Reaction I

entry	solvent	temp (°C)	dielectric constant (25 °C)	dipole moment (*D*)	conversion rate (%)
1	$PhCH_3$	110	2.40	1.33	85
2	EtOAc	77	6.02	1.89	89
3	$CHCl_3$	61	4.81	1.15	76
4	CH_3COCH_3	56	20.70	2.85	80

dielectric constant and dipole moment (*D*), acetone can adapt to the reaction better since it is easy to form a solvent shell around reaction molecule suitable for a reaction; its low boiling point, however, results in low conversion rate of the reaction.

A change was obviously not shown in the conversion rate when chloroform was used as the reaction–solvent with its higher boiling point and lower data of the dielectric constant and dipole moment. A higher conversion rate was gained in refluxing ethyl acetate for its higher data of the dielectric constant and dipole moment (*D*) and boiling point, but it is lower in toluene at 110 °C because of its low values of the dielectric constant and dipole moment (*D*). As summarized above, it can be concluded that ethyl acetate was employed in the reaction as a solvent resulting in a satisfying conversion rate.

Effect of Temperature on Reaction I. The effect of temperature on the conversion rate of reaction I for reacting for 4 h in EtOAc was also investigated. The experimental results are shown in Table 2.

As shown in Table 2, reaction temperature is an important factor that affects the rate in the reaction process obviously. A small quantity of Merrifield resin was reacted when the reaction carried through at room temperature for 4 h. When the temperature reached 55 °C, the reaction rate increased

Table 2. Effect of Temperature on the Conversion Rate of Reaction I

entry	temp (°C)	conversion rate (%)
1	16	10
2	55	80
3	reflux	89

Table 3. Effect of Reacting Time on the Conversion Rate of Reaction I (in refluxing ethyl acetate)

entry	time (h)	conversion rate (%)
1	1	43
2	2	70
3	4	89
4	6	89

and the Merrifield resin was reacted 80% after 4 h. Finally, the reaction conversion rate was 89% after being refluxed for 4 h in acetic ether.

Effects of Time on the Reaction I. As shown in Table 3, we also extended our attention to the reaction time of reaction I

As known from the reaction time, Merrifield resin was reacted 43% after 1 h, 2 h later a 70% reacted results, the conversion rate can be reached to 89% reacting for 4 h. Nevertheless the conversion rate was scarcely increased any more as time prolonging.

Effect of Substrate on Reaction I. Various substitutional groups on the substrate of 2-mercaptobenzothiazole obviously affect the reaction rate of reaction I. The conversion rates of 2-mercaptobenzothiazole substitutd by C5–Cl, C5–NO_2, C6–NO_2, and C6–CH_3 respectively reacting with Merrifield resin are shown in Table 4 in refluxing acetic ether for 2 h.

As summarized in Table 4, when Merrifield resin reacted with 5-cholo-2-mercaptobenzothiazole, the conversion rate

Table 4. Effect of Reacting Substrates on the Conversion Rate of Reaction I

entry	substrate	conversion rate (%)
1	H	70
2	6–CH_3	66
3	5–Cl	82
4	5–NO_2	38
5	6–NO_2	43

Table 5. Effect of Agent Property on Reaction II

entry	solvent	temp (°C)	dielectric (25 °C)	dipole moment (*D*)	conversion rate (%)
1	acetone	56	20.7	2.85	0
2	toluene	110	2.4	1.33	50
3	xylene	138–141			87

Table 6. Effect of Temperature on the Conversion Rate of Reaction II

entry	solvent	temp (°C)	conversion rate (%)
1	xylene	50	0
3	xylene	110–115	52
4	xylene	138–141	87

reached 82%, which was higher than reacting with 2-mercaptobenzothiazole (conversion rate was 70%). In the same way, when 2-mercaptobenzothiazol was substituted by 6-methyl-2-mercaptobenzothiazole, the conversion rate was 66% and it was 38% with 5-nitro-2-mercaptobenzothiazole as the substrate. So, the right way is that the reacting substrate modified with different substitutes obviously influences the process of the nucleophilic reaction. Similarly, it also can affect its spectrum characteristics as mentioned below.

Effect of Conditions on Solid-Phase Synthesis Reaction II. Effect of Solvent Property on the Solid-Phase Synthesis Reaction II. PS-compound (I) reacted with methyl 4-methylbenzenesulfonate in different solvents such as acetone, toluene, and xylene to form PS-compound (II). Similarly, in this reaction, solvent is also one of the important factors that can influence the reaction process. The parameters of some solvents and some experimental results in various solvents are summarized in Table 5.

As show in Table 5, the conversion rates were apparently different using different solvents. When refluxing in acetone for 4 days, the conversion rate was zero. But using toluene as the reaction solvent, PS-compound (1) was reacted 50%, and Merrifield resin could be reacted 87% when toluene was substituted by xylene. So, in the reaction, xylene is the better solvent, with which the highest conversion rate of reaction II can be achieved.

Effect of Temperature on Reaction II. In order to confirm which is the dominating factor between solvent categories and reacting temperature influencing this reaction process, experiments were carried out in xylene at different temperatures. The data of the experiments are shown in Table 6.

As summarized in Table 6, the reaction was carried out in xylene at the boiling point of acetone, toluene, and xylene, respectively. The experimental results show that while increasing the reacting temperature, the conversion rate can be increased and the highest conversion rate can be gained in refluxing xylene.

Characteristic Identity Analysis of PS-Compound (I) and PS-Compound (II). PS-compound (I) and PS-compound (II) were identified by IR spectrum and element analysis. The identified results were shown in Figure 1 and Table 7.

It is difficult to distinguish between 2-mercaptobenzothiazole derivatives and Merrifield resin because they have similar IR curves and enshroud each other. But, 1039, 1239, and 900 cm^{-1} of the curve of PS-compound (I){1}; 1304, 1241, and 990 cm^{-1} of PS-compound (I){2}; 1296, 1245, 993 cm^{-1} of PS-compound (I){3}; 1237 and 990 cm^{-1} of PS-compound (I){4}; and 1265 and 1001 cm^{-1} of PS-compound (I){5} are new spectrum peaks to the IR curve of Merrifield resin. We can also find 1337 and 1327 cm^{-1} in the curves of PS-compound (I){4} and PS-compound (I){5}, respectively, which may be the skeletal vibrate for Ar–NO_2 of compound (I) and 1331 cm^{-1} in the curves of PS-compound (II){5}. By associating IR with the element analysis data of PS-compound (I) and PS-compound (II), we can realize that 2-mercaptobenzothiazole derivatives were banded to Merrifield resin.

Study of the Fluorescence Spectrum Properties. The calculation formula of fluorescence quantum yield as below:

$$\Phi_{F_1} = \Phi_{F_2} \frac{F_1 A_2}{F_2 A_1}$$

In the formula, F_1 and F_2 represent the measured data of fluorescence integral strength of the sample and standard material of rhodamine B, respectively; A_1 and A_2 represent the absorbance of the sample and standard material of rhodamine B (in EtOH, at 480 nm, $\Phi = 0.97$) under the fluorescence excitation wavelength, respectively.

The fluorescence spectrum of TO derivatives containing different substituents in the benzene ring and the TO derivatives labeled with bovine serum albumin (BSA) were measured. The relationship of the substituent categories to the effect on fluorescence strength and its light quantum efficiency were studied. The synergetic effect was investigated on TO–COOH modified with chitosan oligosaccharide at the end of the alkyl.

Fluorescence Spectrum Characteristic of the TO Derivatives Marked with BSA. The fluorescence spectrum of TO derivatives introducing –Cl, –CH_3, and –NO_2 groups, respectively, were measured in the benzene ring at the 5 or 6 position as well as the TO derivatives marked by BSA as above mentioned. The results were shown in Figures 2 and 3 and Table 8.

From Table 8, it is shown that TO derivatives modified by different substitutes can shift the values of maximal fluorescence emission λ_{ex}, fluorescence integral strength F, and fluorescence quantum yield Φ_F of TO derivatives to a certain extent. We can observe that the TO derivatives containing the nitro group has a higher fluorescence integral strength (F) and fluorescence quantum yield Φ_F than others; the nitro group at 5 or 6 does not change the special characteristics of their fluorescence obviously.

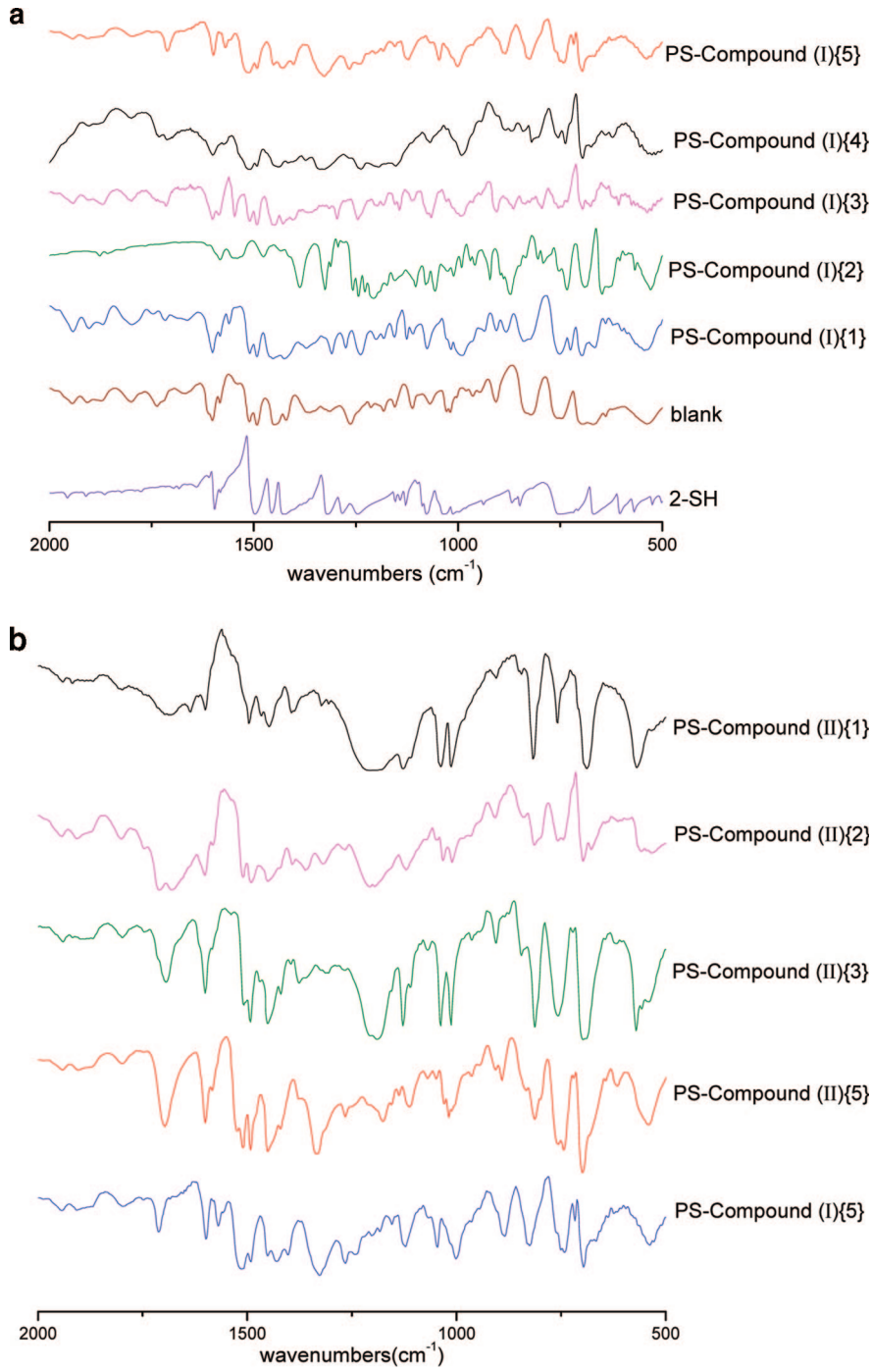


Figure 1. IR spectrum of PS-compound (I) and PS-compound (II).

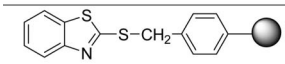
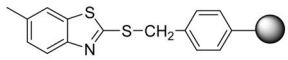
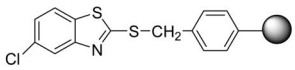
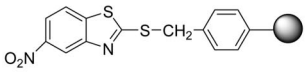
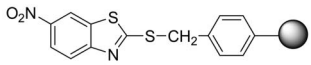
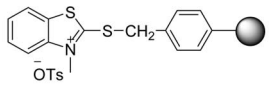
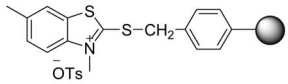
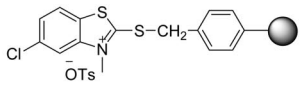
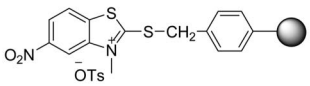
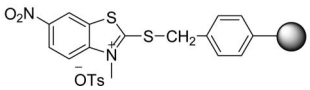
Study of Chemical Structure Character of TO–COOH–CTS. The product of TO–COOH reacting with CTS was characterized by IR and differential scanning calorimetry (DSC) for the character of its chemical structure; the results are shown in Figures 4 and 5.

From the above curves of TO–COOH–CTS and CTS, we can find many differences of the absorbing peaks between the infrared spectroscopy. For instance, the data of 1504, 1464, and 1556 cm^{-1} are new spectrum peaks compared to the spectroscopy curve of CTS, which may be the skeletal vibration of Ar, and the stretching vibration of C–O in 1199 and 754 cm^{-1} is vested in the skeletal vibration of the interior hydrogen bond of benzene. It is shown that this is an ortho substitution of the benzene. As shown above, TO–COOH is modified by CTS to gain TO–COOH–CTS.

The detective results show that there are similar UV curves and ^1H NMR spectra between the PS-compound TO–COOH–CTS and the mixture of TO–COOH and CTS. So, we are trying to find their differences of the characteristic chemical structure by the DSC thermograms.

As shown in Figure 5, we can find that there is a sharp character endothermal peak at 172 °C and a radiative peak at 211 °C in the DSC thermograms of TO–COOH and CTS, respectively. They can also be found in the DSC curve of the mixture of TO–COOH and CTS but cannot be found in the DSC thermograms of TO–COOH–CTS, in which there

Table 7. Element Analysis Data for PS-Compound (I) and PS-Compound (II)

PS-Compound (I) and (II)	Elemential Analysis (N/%)	Conversion rate (%)
	2.62	89
	2.32	78
	2.55	93
	4.08 [a]	70
	3.81[a]	64
	1.75	87
	1.49	80
	1.57	78
	2.3 [b]	67
	1.99[b]	60

[a] The reaction time is 6 h. [b] The reaction time is 6.5 days.

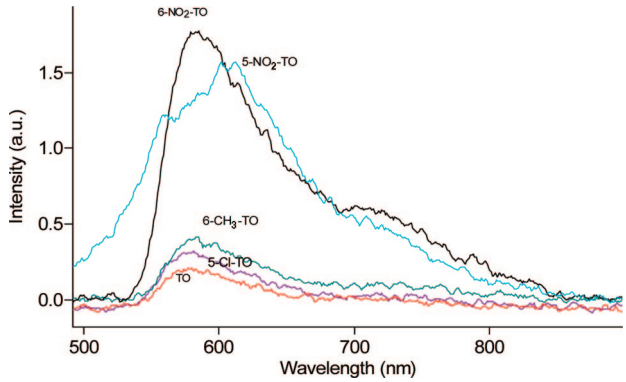


Figure 2. Fluorescence spectra of TO derivatives.

is a new radiative peak. To summarize, TO–COOH–CTS is proved to be a new compound bonded by TO–COOH and CTS and is not the simple mixture of them by infrared spectroscopy and the DSC thermograms.

Fluorescence Characteristic of TO–COOH Modified with CTS. The fluorescence absorption strength of TO–COOH and TO–COOH–CTS was measured for comparing the differences of relevant fluorescence parameters. The results are shown in Figure 6 and Table 9.

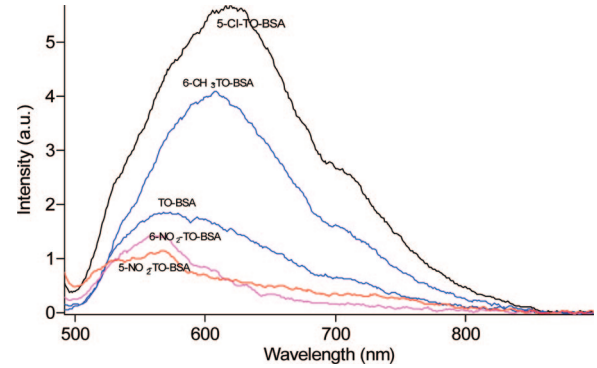


Figure 3. Fluorescence spectra of TO derivatives marked with BSA.

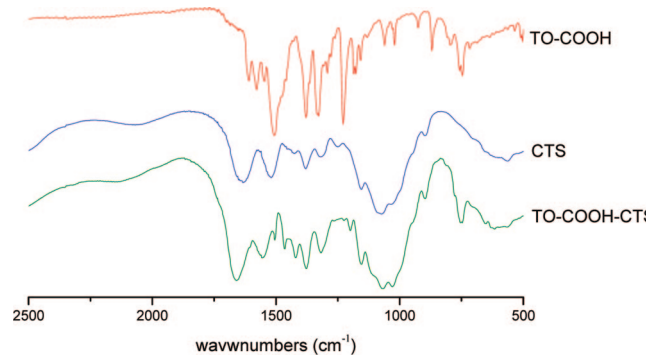


Figure 4. IR spectrum of TO–COOH–CTS and CTS.

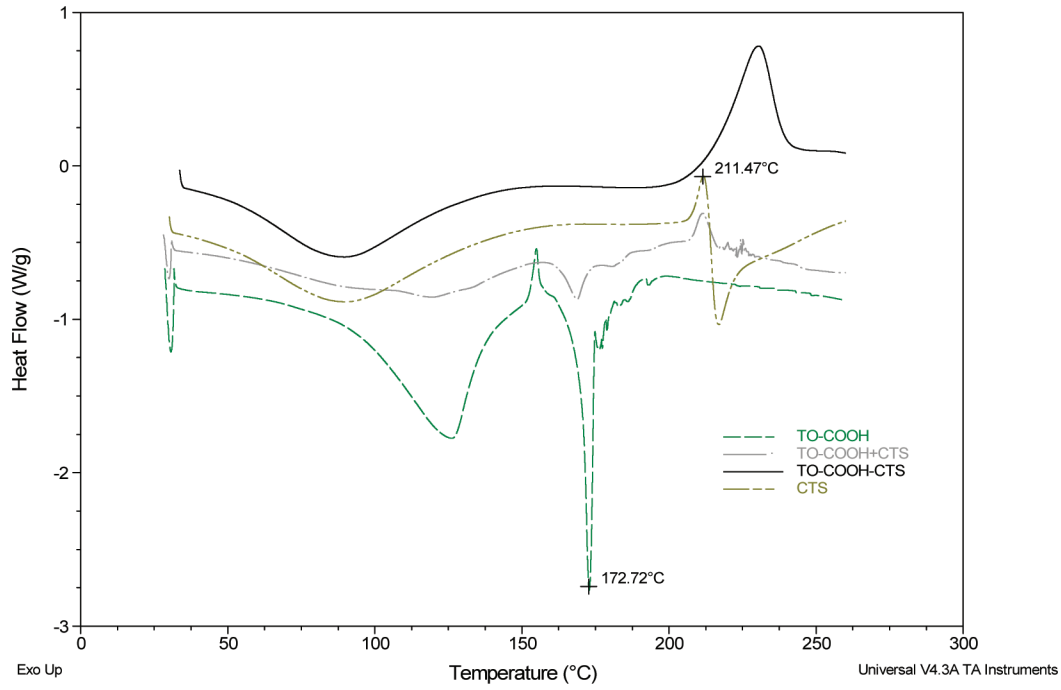
Table 8. Maximal Fluorescence Emission λ_{ex}, Fluorescence Integral Strength, and Fluorescence Quantum Yield of TO Derivatives Labeled with BSA

	TO derivatives			TO marked with BSA		
dye	λ_{ex} (nm)	F (a.u.)	Φ_F	λ_{ex} (nm)	F (a.u.)	Φ_F
TO	568	0.250	8.86×10^{-6}	566	1.906	4.27×10^{-4}
5-Cl–TO	579	0.361	1.48×10^{-5}	618	5.763	2.60×10^{-3}
6-CH_3–TO	591	0.469	2.74×10^{-5}	612	3.401	1.47×10^{-3}
5-NO_2–TO	612	1.645	8.41×10^{-4}	567	1.203	1.89×10^{-3}
6-NO_2–TO	585	1.879	1.39×10^{-4}	563	1.451	5.20×10^{-4}

From Figure 6 and Table 9, we can find the fact that the maximal absorption wavelength of TO–COOH and TO–COOH–CTS are very close. Compared to TO–COOH, the fluorescence integral strength, fluorescence light quantum efficiency, and fluorescence intensity of TO–COOH–CTS are greatly increased, respectively. On the basis of this point, it may be possible to design and make a kind of fluorescence probe of Thiazole Orange having high sensitivity, good biological intermiscibility, and excellent water solubility. The interaction relationship between TO–COOH and CTS are being studied.

Conclusion

Cyanine dye TO and its derivatives created by introducing different substitutes can be designed and synthesized in the solid phase by the traceless cleavage of a Merrifield resin method. In the course of the solid-phase synthesis, solvents, reacting temperature, and reaction time are all of important, influential factors. The electronic effect of the different substitutes influences not only the course of the solid-phase synthesis but obviously also the spectrum of its derivatives



TO-COOH+CTS: the mixture of TO-COOH and CTS

Figure 5. DSC thermograms of TO–COOH–CTS, CTS, TO–COOH + CTS, and TO–COOH.

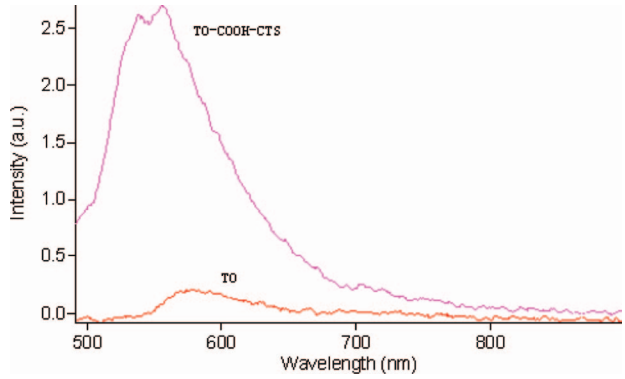


Figure 6. Fluorescence spectra of TO–COOH and TO–COOH–CTS.

Table 9. Maximal Fluorescence Emission Wavelength λ_{ex}, Integral Strength F, and Fluorescence Quantum Yield Φ_F of TO–COOH, TO–COOH–CTS

dye	λ_{ex} (nm)	F (a.u.)	Φ_F
TO–COOH	568	0.250	8.86×10^{-6}
TO–COOH–CTS	556	2.800	1.69×10^{-3}

and fluorescence spectrum of the derivatives marked by BSA. It was shown that the characteristic of increasing of fluorescence intensity can extensively exist in TO derivatives modified with CTS, which can improve the sensitivity of the fluorescent probe in the field of the marked biological tissue.

Experimental Section

Materials and Instruments. Fluorescence spectra were scanned on a fluorescence analysis instrument, Cary Eclipse. DSC thermograms were record on a differential scanning calorimeter produced by TA Co., LTD, American. Element analyses were determined on the Heraeus CHN-O RAPID, Germany. Mass spectral analyses were obtained using an electrospray ionization (ESI) mass spectrometer. Infrared spectra were recorded on a Bruker-EQUINOX55, Bruker Company, Germany, with the following parameters: sample scan 6; frequency range 400–4000 cm^{-1}. Melting points were taken on a Yanaco apparatus and are uncorrected. ^1H NMR spectra were recorded on a Bruker AC-P300 (300 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from TMS (0 ppm). Chemical shifts are reported in parts per million (ppm) downfield from TMS, using D_2O or DMSO-d_6 as a solvent. Rhodamine B was purchased from Tianjin Industrial Technique Graduate School of Medicine; BSA was purchased from Peking Dingguo Biology Technique Co., LTD (China). Merrifield resin (1% DVB cross-linking, 100–200 mesh, loading of 2, 8 mmol/g) was purchased from Tiajin Nankai Hecheng Science & Technology Co., Ltd. Chitosan oligosaccharide (DAC ≥ 85%,) was purchased from Jinan Haidebei Marine Bioenineeing Co., LTD (China). All the reagents are analytically pure.

General Procedure for PS-Compound (I){1–5}. 2-Mercaptobenzothiazole derivatives (11.2 mmol) and K_2CO_3(1.066 g, 7.8 mmol) were added to a vessel containing 1.0 g Merrifield resin swelled for 4 h in EtOAc. After refluxing for 4 h, the reaction mixture was cooled to room temperature. The precipitate was filtrated, washed, and dried to afford PS-compound (I).

General Procedure for PS-Compound (II){1–5}. PS-compound (I) obtained as described above and methyl 4-methylbenzenesulfonate (0.76 g, 4.10 mmol) was added to xylene (10 mL). After being refluxed for 4 days, the reaction mixtures were cooled to room temperature, filtrated, and washed with xylene and acetone sequentially and dried to afford solid PS-compound (II).

General Procedure for PS-Compound (III){1–3}. 1-(3-Bromopropyl)-4-methylquinolinium Bromide PS-Compound (III){1–3}. 4-Methylquinoline (2.50 mL, 18.96 mmol) and 1,3-dibromopropane (13.00 mL, 128 mmol) were added to a 100 mL round-bottomed flask, with stirring at room temperature in the dark for 8 h to afford an off-white solid, which was washed with ether and dried to afford 4.65 g off-white solid. yield 71%; m.p. 147–149 °C; ^{1}H NMR (300 MHz, D_2O): δ 2.52 (t, J = 6.70 Hz, 2H), 2.89 (s, CH_3, 3H), 3.43 (t, J = 6.40 Hz, 2H), 5.02 (t, J = 6.70 Hz, 2H), 7.75 (d, J = 5.10 Hz, 1H), 7.88 (t, J = 7.05 Hz, 1H), 8.09 (t, J = 6.90 Hz, 1H), 8.29 (d, J = 6.60 Hz, 1H), 8.35 (d, J = 6.60 Hz, 1H), 8.98 (d, J = 6.30 Hz, 1H). MS (cESI) m/e: 264.31 (M^+), 265.31 ($M^+ + 1$), 266.31 ($M^+ + 2$).

The experimental results show that 4-methylquinoline reacts with 3-bromopropionic acid in refluxing acetone for 8 h and with 3-bromopropylamine in ethanol at 40 °C for 8 h to produce 1-(2-carboxylethyl)-4-methylquinolinium bromide and 1-(3-aminopropyl)-4-methylquinolinium bromide, respectively.

1-(2-Carboxylethyl)-4-methylquinolinium Bromide Compound (III){2}. yield 39%; m.p. >286 °C; ^{1}H NMR (300 MHz, D_2O): δ 2.84 (s, 3H), 3.04 (t, J = 6.45 Hz, 2H), 5.08 (t, J = 6.00 Hz, 2H), 7.70 (d, J = 6.00 Hz, 1H), 7.82 (t, 1H, J = 7.80 Hz), 8.04 (t, J = 7.20 Hz, 1H), 8.20 (d, J = 8.40 Hz, 1H), 8.31 (d, J = 8.40 Hz, 1H), 8.98 (d, J = 6.00 Hz, 1H). MS (cESI) m/e: 216.24 (M^+), 217.24 ($M^+ + 1$).

1-(3-Aminopropyl)-4-methylquinolinium Bromide Compound (III){3}. yield 46%; m.p. 225–226 °C; ^{1}H NMR (300 MHz, D_2O): δ 2.36 (t, J = 6.75 Hz, 2H), 2.91 (s, 3H), 3.10 (t, J = 7.05 Hz, 2H), 4.98 (t, J = 7.20 Hz, 2H), 7.79 (d, J = 6.00 Hz, 1H), 7.92 (t, J = 6.90 Hz, 1H), 8.12 (t, J = 7.05 Hz, 1H), 8.28 (d, J = 8.70 Hz, 1H), 8.39 (d, J = 7.80 Hz, 1H), 8.97 (d, J = 5.70 Hz, 1H). MS (cESI) m/e: 201.20 (M^+), 202.20 ($M^+ + 1$), 203.98 ($M^+ + 2$).

General Procedure for TO{1–11}. A 250 mL, three-necked, round-bottomed flask was equipped with a mechanical stirrer, a thermometer, and a condenser. Excessive PS-compounds (II) and (III) (0.087 mmol) were added in the distilling flask containing chloroform (20 mL). After stirring for 2 h, KI (0.022 g, 0.13 mmol) was added into the reaction system. The mixture was continually stirred for 30 min in addition to being filtrated and evaporated under reduced pressure to give the title compound.

TO{1}(TO). yield 97%; m.p. 202–204 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.42 (t, J = 6.75 Hz, 2H), 3.66 (t, J = 6.30 Hz, 2H), 4.03 (s, 3H), 4.69 (t, J = 6.75 Hz, 2H), 6.94 (s, 1H), 7.36–7.46 (m, 2H), 7.62 (t, J = 8.10 Hz, 1H), 7.75 (d, J = 7.80 Hz, 1H), 7.80 (d, J = 8.70 Hz, 1H), 8.00 (t, J = 7.35 Hz, 1H), 8.07 (d, J = 7.80 Hz, 1H), 8.14 (d, J = 8.70 Hz, 1H), 8.60 (d, J = 7.20 Hz, 1H), 8.81 (d, J = 8.40 Hz, 1H). MS (cESI) m/e: 411.66 ($M^+ - 1$), 413.66 ($M^+ + 1$), 414.36 ($M^+ + 2$).

TO{2}(TO–NH$_2$). yield 95%; m.p. 191–193 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.35 (t, J = 5.85 Hz, 2H), 2.98 (s, 3H), 3.12 (t, J = 5.40 Hz, 2H), 5.18 (t, J = 6.75 Hz, 2H), 7.01 (t, J = 7.65 Hz, 1H), 7.10 (d, J = 9.00 Hz, 1H), 7.29 (t, J = 7.80 Hz, 1H), 7.52 (d, J = 9.00 Hz, 1H), 8.03 (t, J = 7.65 Hz, 2H), 8.26 (t, J = 7.95 Hz, 1H), 854 (d, J = 9.00 Hz, 1H), 8.67 (d, J = 9.00 Hz, 1H), 9.40 (d, J = 6.00 Hz, 1H). MS (cESI) m/e: 348.33 (M^+), 349.26 ($M^+ + 1$), 350.25 ($M^+ + 2$).

TO{3}(TO–COOH). yield 97%; m.p. 171–173 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.74 (t, J = 6.45 Hz, 2H), 3.99 (s, 1H, CH_3), 4.74 (t, J = 6.10 Hz, 2H,), 6.88 (s, 1H), 7.32 (d, J = 6.9 0Hz, 1H), 7.40 (t, J = 7.65 Hz, 1H), 7.60 (t, J = 7.80 Hz, 1H), 7.75–7.78 (m, 2H), 7.96 (t, J = 7.8 Hz, 1H), 8.00 (d, J = 7.8 Hz, 1H), 8.12 (d, J = 9.00 Hz, 1H), 8.69 (d, J = 9.00 Hz, 1H), 8.77 (d, J = 9.00 Hz, 1H). MS (cESI) m/e: 363.25 (M^+), 364.25 ($M^+ + 1$).

TO{4}(6-CH$_3$–TO). yield 96%; m.p. 150–152 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.39 (t, J = 6.70 Hz, 2H), 2.42 (s, 3H), 3.66 (t, J = 6.45 Hz, 2H), 4.00 (s, 3H), 4.66 (t, J = 7.20 Hz, 2H), 6.87 (s, 1H), 7.28 (d, J = 7.20 Hz, 1H), 7.42 (d, J = 8.70 Hz, 1H), 7.74 (d, J = 7.50 Hz, 1H), 7.68 (d, J = 8.70 Hz, 1H), 7.84 (s, 1H), 7.98 (t, J = 7.65 Hz, 1H), 8.09 (d, J = 8.40 Hz, 1H), 8.54 (d, J = 9.00 Hz, 1H), 8.76 (d, J = 9.00 Hz, 1H). MS (cESI) m/e: 445.86 ($M^+ - 1$), 447.48 ($M^+ + 1$), 449.42 ($M^+ + 3$).

TO{5}(6-CH$_3$–TO–COOH). yield 95%; m.p. 207–210 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.47 (s, 3H), 2.65 (t, J = 6.90 Hz, 2H), 3.99 (s, 3H), 4.80 (t, J = 5.10 Hz, 2H), 6.85 (s, 1H), 7.33 (d, J = 7.80 Hz, 2H), 7.37 (d, J = 8.70 Hz, 1H), 7.57 (d, J = 8.10 Hz, 1H), 7.73 (d, J = 9.00 Hz, 2H), 7.96 (d, J = 7.80 Hz, 1H), 8.08 (d, J = 8.40 Hz, 1H), 8.70 (t, J = 6.00 Hz, 2H). MS (cESI) m/e: 397.41 (M^+), 398.41 ($M^+ + 1$), 399.41 ($M^+ + 2$), 400.51 ($M^+ + 3$).

TO{6}(5-Cl–TO). yield 96%; m.p. 190–192 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.43 (t, J = 6.90 Hz, 2H), 3.66 (t, J = 6.30 Hz, 2Hr), 3.99 (s, 3H), 4.73 (t, J = 6.75 Hz, 2H), 6.92 (s,1H), 7.37–7.47 (m, 2H), 7.78 (t, J = 7.65 Hz, 1H), 7.93 (s, 1H), 8.04 (d, J = 9.00 Hz, 2H), 8.17 (d, J = 9.00 Hz, 1H), 8.68 (d, J = 9.00 Hz, 1H), 8.82 (d, J = 9.00Hz, 1H). MS (cESI) m/e: 427.22 ($M^+ + 1$), 428.24 ($M^+ + 2$), 429.27 ($M^+ + 3$).

TO{7}(5-Cl-TO–COOH). yield 95%; m.p. 209–211 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.30 (t, J = 6.50 Hz, 1H), 3.93 (s, 1H), 4.74 (t, J = 5.85 Hz, 2H), 6.84 (s, 1H), 7.31 (d, J = 6.90 Hz, 1H), 7.39 (d, J = 8.7 Hz, 1H), 7.73 (t, J = 7.35 Hz, 1H), 7.77 (d, J = 9.00 Hz, 1H), 7.87 (s, 1H), 7.98 (d, J = 9.00 Hz, 1H), 8.14 (d, J = 9.30 Hz, 1H), 8.77 (t, J = 7.95 Hz, 2H). MS (cESI) m/e: 377.33 (M^+), 378.35 ($M^+ + 1$), 379.36($M^+ + 2$).

TO{8}(5-NO$_2$–TO). yield 92%; m.p. 206–208 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.44 (t, J = 6.60 Hz, 2H), 3.68 (t, J = 6.45 Hz, 2H), 4.04 (s, 3H), 4.75 (t, J = 6.90 Hz, 2H), 6.94 (s, 1H), 7.11 (d, J = 7.80 Hz, 1H), 7.50 (d, J = 8.10 Hz, 1H), 7.80 (t, J = 7.05 Hz, 1H), 8.05 (t, J = 7.65 Hz, 1H), 8.18–8.22 (m, 2H), 8.50 (s, 1H), 8.78–8.86 (m, 2H). MS (cESI) m/e: 456.42 (M^+), 458.33 ($M^+ + 2$), 459.33 ($M^+ + 3$), 460.31 ($M^+ + 4$).

TO{9}(5-NO$_2$–TO–COOH). yield 92%; m.p. 182–184 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.91 (t, J = 7.05 Hz, 2H), 4.04 (s, 3H), 4.89 (t, J = 6.90 Hz, 2H), 6.97 (s, 1H), 7.12 (d, J = 8.40 Hz, 1H), 7.48 (d, J = 8.10 Hz, 2H), 8.22–8.27 (m, 2H), 8.48 (s, 1H), 8.78–8.86 (m, 3H). MS (cESI) m/e: 408.38 (M^+), 409.40 ($M^+ + 1$), 410.39 ($M^+ + 2$).

TO{10}(6-NO_2–TO). yield 94%; m.p. 228–230 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.45 (t, J = 7.65 Hz, 2H), 3.68 (t, J = 6.6 Hz, 2H), 4.01 (s, 3H), 4.80 (t, J = 6.75 Hz, 2H), 7.02 (s, 1H), 7.12 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 8.10 Hz, 2H), 7.83–7.87 (m, 2H), 8.08 (t, J = 7.35 Hz, 1H), 8.25 (d, J = 8.40 Hz, 1H), 8.86 (d, J = 7.20 Hz, 2H). MS (cESI) m/e: 457.21(M^+), 458.21 ($M^+ + 1$), 459.22 ($M^+ + 2$), 460.21 ($M^+ + 3$).

TO{11}(6-NO_2–TO–COOH). yield 93%; m.p. 238–240 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.97 (t, J = 6.15 Hz, 2H), 4.02 (s, 3H), 4.91 (t, J = 6.15 Hz, 2H), 7.04 (s, 1H), 7.52 (d, J = 6.90 Hz, 1H), 7.85 (t, J = 9.00 Hz, 2H), 8.08 (t, J = 7.40 Hz, 1H), 8.29 (d, J = 9.00 Hz, 1H), 8.42 (d, J = 8.70 Hz, 1H), 8.89 (t, J = 8.10 Hz, 2H), 9.00 (d, J = 8.10 Hz, 1H). MS (cESI) m/e: 408.40 (M^+), 409.42 ($M^+ + 1$), 410.39 ($M^+ + 2$).

Modification of TO–COOH with Chitosan Oligosaccharide. An 8 mL portion of H_2O solution containing 600.00 mg (M_n = 3000) of chitosan oligosaccharide and 4.00 mL DMSO solution containing 56 mg (0.40 mmol) *N*,*N*-diisopropylethylamine (DIEA) were dropped into a 20 mL dimethylformamide (DMF) solution containing TO–COOH (41.66 mg, 0.085 mmol), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTu) (41.20 mg, 0.109 mmol), and *N*-hydroxybenzotriazole (HOBt) (14.72 mg, 0.109 mmol) which was cooled at −5 °C. The reaction mixture was stirred at room temperature for 48 h and extracted with ether and EtOAc in turn after being washed with acetone to give a floccule, which was isolated by filtration followed by washing with acetone and drying to afford a rose solid (624.20 mg).

Fluorescence Spectrum Characteristics of the TO Derivatives Marked with BSA. The DMSO solutions of 0.10 mg/mL TO derivatives and TO–COOH–CTS were prepared, respectively. The 0.10 mg/mL BSA solution was made in pH = 8 buffer solutions of Na_2HPO_4–NaH_2PO_4 and kept in 4 °C refrigerators. It was mixed 3 mL BSA aqua with 0.50 mL of the TO derivatives mentioned above and shaken for 30 min to wait for measurement.

The data of the absorption spectrum and the fluorescence spectrum of the combination above-mentioned TO derivatives and BSA were measured at 480 nm. With the dye of rhodamine B as a standard material, we can calculate the data of fluorescence quantum yield.

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References and Notes

(1) Wang, L. Q.; Peng, X. J. *Dyestuff Ind.* **2002**, *39*, 8.
(2) Southwick, P. L.; Ernst, L. A.; Tauriello, E. W. *Cytometry* **1990**, *11*, 418.
(3) Timcheva, I. I.; Maximova, A. V.; Deligeorgiev, G. T.; Gadjev, I. N.; Sabnis, W. R.; Ivanov, G. I. *FEBS Lett.* **1997**, *405*, 141.
(4) Timtcheva, I. I.; Maximova, A. V.; Deligeorgegiev, G. T. *J. Photochem. Photobiol.* **2000**, *58*, 130.
(5) Cai, Y.; Pailthorpe, M. T.; David, S. K. *Textile Res. J.* **1999**, *69*, 440.
(6) Skeidsvoll, J.; Ueland, P. M. *Anal. Biochem.* **1995**, *231*, 359.
(7) Timtcheva, I.; Maximova, V.; Deligeorgiev, T.; Zaneva, D.; Ivanov, I *J. Photochem. Photobiol. A: Chem.* **2000**, *130*, 7.
(8) Brooker, L. G.; Keyes, G.; Williams, W. *J. Am. Chem. Soc.* **1942**, *64*, 199.
(9) Rye, S. H.; Yue, S.; Wemmer, E. D.; Quesada, A. M.; Haugland, P. R.; Mathies, A. R.; Glazer, N. A. *Nucleic Acids Res.* **1992**, *20*, 2803.
(10) Glazar, N. A. DNA complexes with dyes designed for energy transfer as fluorescent markers. U.S. Patent no. 5,401,847, **1995**.
(11) Song, J.; Feng, R. X.; Chen, L; Wang, J. B.; Xie, Y. *Fine Chem.* **2002**, *19*, 469.
(12) Gao, Z.Y.; Xue, M. Z.; Liu, Y. G.; Huang, D. Y. *Photogr. Sci. Photochem.* **2002**, *20*, 269.
(13) Deligeorgiev, G. T.; Gadjev, I. N. *Dyes Pigments* **1995**, *29*, 315.
(14) Deligeorgiev, G. T.; Gadjev, I. N.; Timtcheva, I. I; Maximova, A. V.; Katerinopoulos, E. H.; Foukaraki, E *Dyes Pigments* **2000**, *44*, 131.
(15) Gadjev, N. I.; Deligeorgiev, G.T.; Kim, H. S. *Dyes Pigments* **1999**, *40*, 181.
(16) Isacsson, J.; Westman, G. *Tetrahedron Lett.* **2001**, *42*, 3207.
(17) Mason, J. S.; Hake, L. J.; Nairne, J.; Cummins, J. W.; Balasubramanian, S. *J. Org. Chem.* **2005**, *70*, 2939.

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