Synthesis of Benzo[a]phenoxazin-5-one Derivatives and Their Interactions with DNA

ZHANG, Peng[a](张鹏) MENG, Ji-Ben*,[a](孟继本) LONG, Jiang[a](龙江)
MATSUURA, Teruo[b](松浦辉男) WANG, Yong-Mei[a](王永梅)
[a]*Department of Chemistry, Nankai University, Tianjin 300071, China*
[b] *21-26 Kawashima-Gondencho, Saikyoku, Kyoto 615-8195, Japan*

The spectroscopic properties of benzo[a]phenoxazin-5-one derivatives (3a—3m) including newly synthesized 3k—3m from 4-nitrosoaniline hydrochlorides and ethyl 1, 3-dihydroxy-2-naphthoate were studied. Compound 3l was converted into a covalent product with DNA, which had a blue shift of the fluorescence maximum. Compounds 3a—3k were found to undergo interaction with DNA and their complexes with DNA had a red shift of the fluorescence maximum and showed increasing melting temperature of DNA. Compound 3m-DNA had a blue shift of the fluorescence maximum to 3m and showed decreasing melting temperature of DNA.

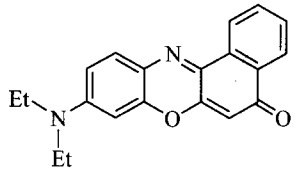
Keywords benzo[a]phenoxazin-5-one, fluorescence, interaction, DNA

Introduction

There has been substantial interest in DNA modified by fluorescent compounds applying for detection and sequencing.[1-5] Currently, the method of four-color DNA sequencing fluorescent dye-labeled primers or terminators is commonly used.[6-10] However, the sensitivity of DNA fluorescence detection with the use of visible fluorophores remains lower than that which has been achieved with radioactive labels because of fluorescence background in studying biological system.[11] To eliminate the high fluorescence background encountered in visible fluorescence analyses, the near-infrared fluorophores labeling biomolecules have been studied broadly.[12-15] The fluorescence region of 600—1000 nm is suitable because this region is inherently low in biological interferences and thus allows a reduced background noise. At present, there are some commercially available dyes having near-infrared fluorescence such as cyanine derivatives.[16]

The Nile red (Scheme 1) derivative[17] are available dyes having near-infrared fluorescence. Although they have strong fluorescence and high stability and the emission wavelengths are above 600 nm,[18,19] their application of modifying nucleic acids was little reported. To develop novel near-infrared fluorogenic labels, we prepared a series of Nile red analogues, benzo[a]phenoxazin-5-one derivatives, and carried out their interactions with DNA.

Scheme 1



Nile red

Experimental

Melting points were determined with a Yanagimoto MP-35 melting point apparatus and uncorrected. ^{1}H NMR spectra were measured with a BRUKER AC-200 spectrometer using tetramethylsilane as the internal standard. Coupling constants are given in Hertz. Mass spectra were recorded on a 7070E-HE spectrometer operating in electron impact mode at 70 eV. IR spectra were recorded on a

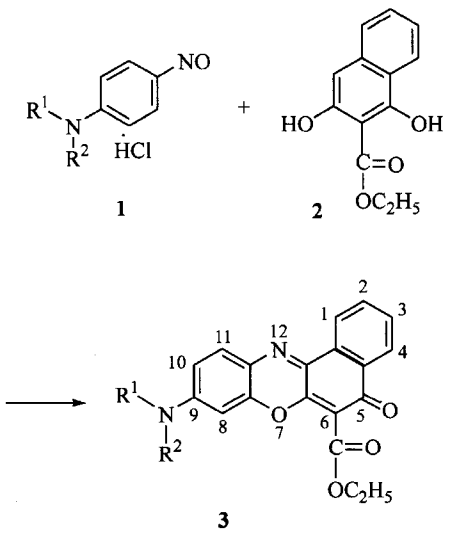
* E-mail: mengjiben@eyou.com
Received July 17, 2001; revised October 23, 2001; accepted January 18, 2002.
Project supported by the National Natural Science Foundation of China (Nos. 29872015 and 29832030).

Bio-Rad FTS135 spectrophotometer. A YANACO CHN CORDER MT-3 apparatus was used for elemental analysis. A Shimadzu UV-240A and a 2101PC spectrometers were used for electronic absorption spectra. Fluorescence spectra were measured on a Shimadzu RF-540 spectrometer.

Synthesis

The starting materials were all commercially available. *N*, *N*-Disubstituted-4-nitrosoaniline hydrochloride[20] and ethyl 1,3-dihydroxy-2-naphthoate[21] were prepared according to known procedures. Compounds **3a—3j** were synthesized as known reported previously (Scheme 2)[22] and compounds **3k—3m** were synthesized as shown in Schemes 2 and 3.

Scheme 2

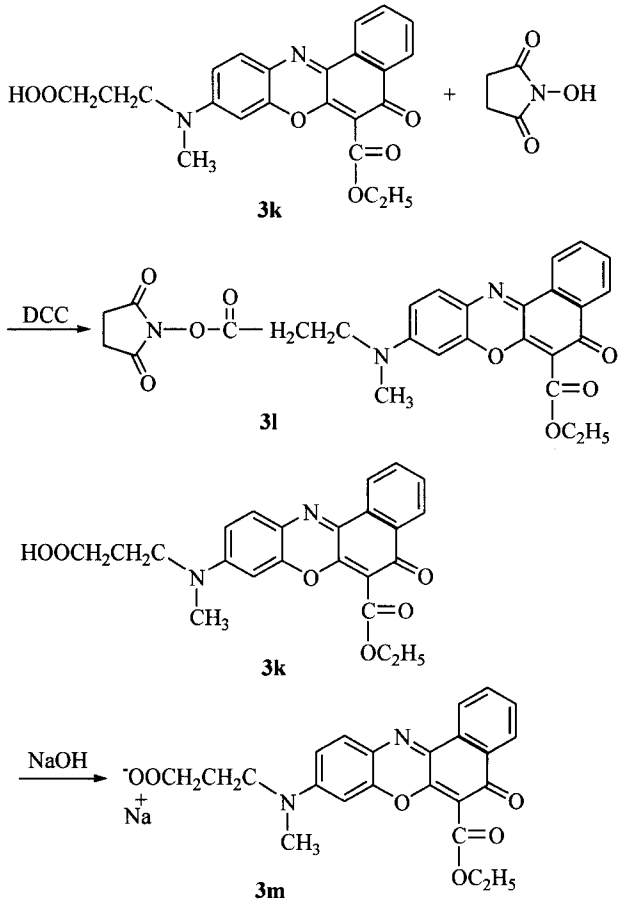


a: R^1=CH_3, R^2=CH_3; **g**: R^1=C_2H_5, R^2=*n*-C_3H_7;
b: R^1=CH_3, R^2=C_2H_5; **h**: R^1=C_2H_5, R^2=*n*-C_4H_9;
c: R^1=CH_3, R^2=*n*-C_3H_7; **i**: R^1=*n*-C_3H_7, R^2=*n*-C_3H_7;
d: R^1=CH_3, R^2=*n*-C_4H_9; **j**: R^1=CH_3, R^2=CH_2CH_2OH;
e: R^1=CH_3, R^2=*n*-C_5H_{11}; **k**: R^1=CH_3, R^2=CH_2CH_2COOH
f: R^1=C_2H_5, R^2=C_2H_5;

6-Carbethoxy-9-(N-methyl-N-carboxyethyl)-5H-benzo[a]phenoxazin-5-one (**3k**)

In 250 mL-three-neck flask, a mixuure of *N*-methylaniline (10.7 g, 0.1 mol) and 3-chloropropionic acid (10.9 g, 0.1 mol) was heated for 3 h at 120 ℃ in an oil bath. After cooling to 0 ℃, concentrated hydrochloric acid (30 mL) and water (100 mL) were added. An aqueous sodium nitrite solution (8.3 g, 0.12 mol, in 40 mL water) was dropwise added during 30 min. The mixture was stirred for 3 h and filtered, and the solid of *N*-methyl-*N*-carboxyethyl-4-nitrosoaniline hydrochloride (16 g) was obtained, which was used for the next reaction without further purification.

Scheme 3



A mixture of *N*-methyl-*N*-carboxyethyl-4-nitrosoaniline hydrochlorides (1.04 g, 4.25 mmol) and ethyl 1,3-dihydroxy-2-naphthoate (0.80 g, 3.45 mmol) in 60 mL of acetonitrile was refluxed for 6 h. After evaporation of the solvent, the residue was purified by column chromatography on silica gel (eluent, acetone:petroleum ether = 1:2, *V*:*V*) to give **3k** (0.64 g, 45%) as a brown solid. m.p. 230—232 ℃; ^{1}H NMR (DMSO-d_6, 200 MHz) δ: 1.35 (t, *J* = 7.3 Hz, 3H, CH_2CH_3), 2.62 (t, *J* = 6.2 Hz, 2H, NCH_2), 3.10 (s, 3H, NCH_3), 3.78 (t, *J* = 6.2 Hz, 2H, $HOOCCH_2$), 4.37 (q, *J* =

7.3 Hz, 2H, OCH_2CH_3), 6.69 (d, J = 2.4 Hz, 1H, 8-H), 7.02 (dd, J = 9.3, 2.3 Hz, 1H, 10-H,), 7.70—7.90 (m, 3H, 11-H, 2-H, 3-H), 8.13 (d, J = 7.3 Hz, 1H, 4-H), 8.61 (d, J = 7.2 Hz, 1H, 1-H); IR (KBr) ν: 3060, 2960 (OH), 1726,1612 (C=O) cm^{-1}; EIS-MS m/z (%): 420 (M^+, 66). Anal. calcd for $C_{23}H_{20}N_2O_6$: C 65.71, H 4.79, N 6.66; found C 65.69, H 4.75, N 6.69.

6-Carbethoxy-9-[N-methyl-(N-succinimidyl propionate)-yl]-5H-benzo[a]phenoxazin-5-one (**3l**)

A mixture of **3k** (0.42 g, 1 mmol), *N*-hydroxysuccinimide (0.15 g, 1.5 mmol) and DCC (dicyclohexylcarbodiimide, 0.26 g, 1.5 mmol) in 10 mL of dimethyl formamide was stirred for 24 h at room temperature. After filtration and evaporation of the filtrate *in vacuo*, the residue was dissolved in ethyl acetate, washed with a saturated $NaHCO_3$ solution and then with water, purified by column chromatograph on silica gel (eluent, acetone: petroleum ether = 1:4, V:V) to give **3l** (0.43 g, 89%) as a violet solid. m.p. 165—166 ℃; ^1H NMR (DMSO-d_6, 200 MHz) δ: 1.36 (t, J = 7.6 Hz, 3H, CH_2CH_3), 2.66 (t, J = 7.2 Hz, 2H, NCH_2), 2.87 (s, 4H, CH_2), 3.12 (s, 3H, NCH_3), 3.85 (t, J = 6.4 Hz, 2H, $OOCCH_2$), 4.37 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 6.71 (d, J = 2.4 Hz, 1H, 8-H), 7.05 (dd, J = 9.3, 2.3 Hz, 1H, 10-H), 7.74—7.90 (m, 3H, 11-H, 2-H, 3H), 8.16 (d, J = 7.4 Hz, 1H, 4-H), 8.62 (d, J = 7.4 Hz, 1H, 1-H); IR (KBr) ν: 1815, 1779, 1732, 1630 (C=O) cm^{-1}; Anal. calcd for $C_{27}H_{23}N_3O_8$: C 62.67, H 4.45, N 8.12; found: C 62.61, H 4.51, N 8.18.

6-Carbethoxy-9-(N-methyl-N-carboxyethyl)-5H-benzo-[a]phenoxazin-5-one sodium salt (**3m**)

A mixture of **3k** (0.064 g, 0.15 mmol) and NaOH (0.006 g, 0.15 mmol) in 40 mL of ethanol was stirred for 2 h at room temperature. After filtration and evaporation of the filtrate, **3m** (0.066 g) was yielded as a green solid. ^1H NMR (D_2O, 200 MHz) δ: 1.31 (t, J = 6.6 Hz, 3H, CH_2CH_3), 2.17 (t, J = 6.8 Hz, 2H, NCH_2), 2.45 (s, 3H, NCH_3), 3.78 (t, J = 6.6 Hz, 2H, $OOCCH_2$), 4.34 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 5.37 (s, 1H, 8-H), 5.85 (d, J = 9.3 Hz, 1H, 10-H), 6.60 (d, J = 9.2 Hz, 1H, 11-H), 7.20—7.85 (m, 4H, 1-H, 2-H, 3-H, 4-H); IR (KBr) δ: 1625, 1405 (COO^-) cm^{-1}.

Covalent linking of **3l** *and* DNA

Calf thymus DNA (**ct-DNA**) was transaminated with 1,6-hexanediamine essentially as described previously.[23] The transaminated solution (1 mol/L $NaHSO_3$, 3 mol/L 1,6-hexanediamine, 5 mmol/L hydroquinone) was adjusted to pH 6 by adding concentrated hydrochloric acid. Prior to the transamination reaction, **ct-DNA** (1 μg/μL, 40 μL) was denatured at 98 ℃ for 10 min. After denaturation 360 μL of transaminated solution was added, the reaction was allowed to proceed at 42 ℃ for 3 h. After overnight dialysis against four exchanges of 5 mmol/L sodium phosphate (pH 7.5), the DNA solution was centrifuged, purified by ethanol precipitation and dissolved in 400 μL water.

A mixture of 370 μL of transaminated DNA solution, 45 μL of 1 mol/L $NaHCO_3$ and 35 μL of **3l** (in DMSO, 10 mg/mL) was reacted for 3 h at room temperature. After precipitation by ethanol, the precipitate was separated by centrifugation, washed with 70% of ethanol and anhydrous ethanol and dried to give **3l-DNA** as a pale violet solid.

Intercalation of **3a—3k** *and* **3m** *with* DNA

A mixture of 50 μL of **ct-DNA** solution (in water, 1 μg/μL), 50 μL of **3a** solution (in DMSO, 10^{-4} mol/L) and 250 μL of DMSO was reacted for 24 h at 37 ℃. After precipitation by ethanol, the precipitate was separated by centrifugation, washed with 70% of ethanol and anhydrous ethanol and dried to give **3a-DNA** as a pale red solid. The reactions of **ct-DNA** and compounds **3b—3k** were done by a similar process to that for **3a**.

A mixture of 50 μL of **ct-DNA** solution (in water, 1 μg/μL), 50 μL of **3m** solution (in H_2O, 10^{-4} mol/L) and 250 μL of H_2O was reacted for 24 h at 37 ℃. After precipitation by ethanol, the product was centrifuged, washed with 70% of ethanol and anhydrous ethanol to give **3m-DNA**.

Absorption and fluorescence spectra

All absorption and fluorescence spectra of com-

pounds **3a**—**3m** and their products after reaction with DNA were measured under the same conditions in DMSO-H_2O (V: V = 5:1) containing 20 mmol/L NaCl. The fluorescent excitation wavelength (λ_{ex}) was 580 nm. Their fluorescence quantum yields (φ_Y) were obtained according to the literatures method which used the quinoline sulfate as reference compound (λ_{ex} = 336 nm, φ_Y = 0.56)[24,25] Absorbencies of **ct-DNA**, **3a-DNA**, **3k-DNA**, **3m-DNA** at different temperatures were measured (Fig. 1) and their melting points (t_m) were estimated from absorbance changes with temperatures. The effect of alternations in the ionic and acidic concentrations on the interaction of DNA with the substrate was measured by increasing the ionic strength of Na^+ (5, 12, 20, 25, 30 and 35 mmol/L) and changing pH (pH = 4, 5, 6, 7, 8 and 9). The result is given in Table 1.

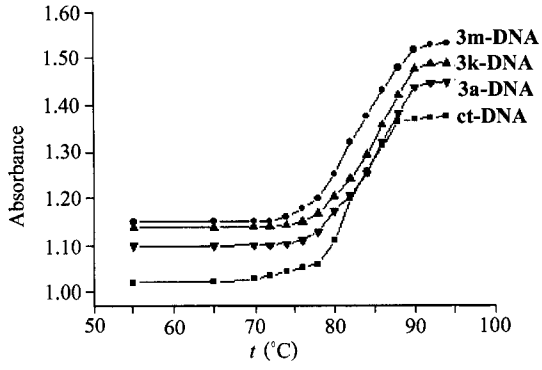


Fig. 1 Changes of absorbance (260 nm) at different temperatures for **ct-DNA**, **3a-DNA**, **3k-DNA** and **3m-DNA**.

Results and discussion

Synthesis of benzo[a]phenoxazin-5-ones (**3a**—**3m**)

Compounds **3a**—**3j** were synthesized as described report previously.[22] Compound **3k** was synthesized from *N*-methyl-*N*-carboxyethyl-4-nitrosoaniline hydrochloride and ethyl 1,3-dihydroxy-2-naphthoate by refluxing in acetonitrile, and **3k** was derived into **3l** and **3m** as Scheme 2. It is a classical reaction that **3l** was synthesized by reacting **3k** and *N*-hydroxysuccinimide with DCC. The succinimidyl ester of **3l** is a reactive group and can be used for modifying DNA.

Absorption spectra of **3a**—**3m**

The absorption and fluorescence maxima of **3a**—**3m** are listed in Table 2. In general, these compounds have 5%—10% decrease in fluorescence intensity in comparison with that of Nile Red. The shift in the wavelength of absorption and emission maxima due to their structural change are summarized as follows. When an *N*-alkyl group on the 9-substituent was methyl or ethyl group (**3a**—**3h**), the increase in the carbon number of another alkyl group caused the absorption maximum to shift to a longer wavelength. When both of the *N*-alkyl groups in the 9-substituent are lengthened symmetrically (**3i**), the absorption maximum red shifted toward the region in agreement with the higher electron-releasing inductive effect of longer chains. If the alkyl groups in the 9-substituent were replaced with 2-hydroxyethyl (**3j**) or 2-carboxyethyl (**3k**) group, the maximum shifted to the blue region because of their electron-withdrawing inductive effect. In comparison of **3l** with **3k**, when a heterocyclic group was introduced to the 9-position, the absorption maximum of **3l** shifted to the red region. The absorption maximum of sodium salt **3m** shifted to the red region markedly.

Fluorescence spectra of **3a**—**3m**

The maxima of emission wavelength of **3a**—**3m** are also listed in Table 2. There is the same substituent effect on their emission wavelengths. The emission wavelengths had a red shift from **3a** to **3j**. When 9-substituent was replaced with 2-hydroxyethyl (**3j**) or 2-carboxyethyl (**3k**) group, the maximum shifted to the blue region. The maximum of **3m** has the red shift to **3k** markedly, but there is no change of emission wavelength between **3k** and **3l**. Their fluorescence quantum yields (φ_Y) are also shown in Table 2. They were obtained by the method of quinoline sulfate as reference compound.[24,25] The fluorescence quantum yields had an increase from **3a** to **3i**. When 9-substituent was replaced with 2-hydroxyethyl (**3j**) or 2-carboxyethyl (**3k**) group, the fluorescence quantum yields had a decrease.

Fluorescence shift of **3a**—**3k** *and* **3m** *in the presence of* **ct-DNA**

A spectroscopic analysis is often used for a study on interaction of a compound with DNA. The electronic absorption and fluorescence spectra are changed after it interacts with DNA.[26] DNA had an absorption peak at 260

nm in its electronic absorption spectrum. Compound **3a-DNA** had a red-shifted absorption at 270 nm. Modified DNA by **3b—3m** had also similar red-shifted absorptions to 270—276 nm in electronic absorption spectra. The spectroscopic change suggested that there were some interactions between compounds **3a—3m** and DNA.

Table 2 shows the maxima of the emission wavelengths of DNA modified by **3a—3k**. Modified DNA with **3a—3k** had longer emission wavelengths and higher fluorescence quantum yields than that of parent compounds. Figs. 2 and 3 show the emission spectra of **3a**, **3k**, **3a-DNA** and **3k-DNA**.

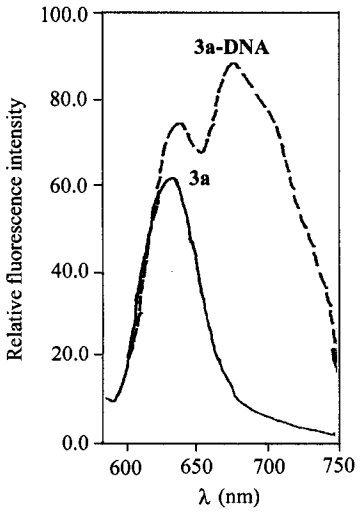


Fig. 2 Fluorescence spectra of **3a** and **3a-DNA**.

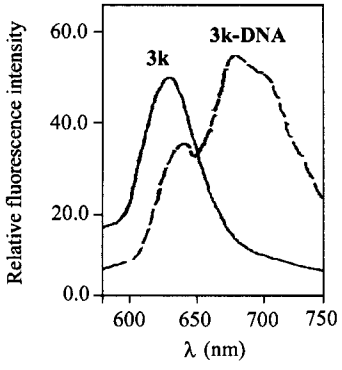


Fig. 3 Fluorescence spectra of **3k** and **3k-DNA**.

The change in fluorescence spectra resulted from interactions of **3a—3k** with DNA. After interaction with DNA, modified DNA might separate from their parents. The excess of parent compounds was removed with ethanol. A specific rangeof acidity and ionic concentrations did not affect the fluorescence intensity of modified DNA. Table 1 shows the relative fluorescence intensity of **3a-DNA** and **3k-DNA** in different pH values and ionic strength. The acidity range of pH 4—9 and ionic strength range of 5—35 mmol/L NaCl are suitable, which do not markedly affect the fluorescent intensity. The suitable acidity is propitious to DNA interaction, but DNA will be denatured under the stronger acidity. Besides, specified concentration of NaCl can eliminate electrostatic influence of phosphate so that the modified DNA is more stable.[26]

Compound **3m** has electrostatic interaction with DNA. The fluorescent maxima of **3m-DNA** had blue shift 15 nm relative to **3m** (Table 2). Fig. 4 shows the fluorescence spectra of **3m** and **3m-DNA**.

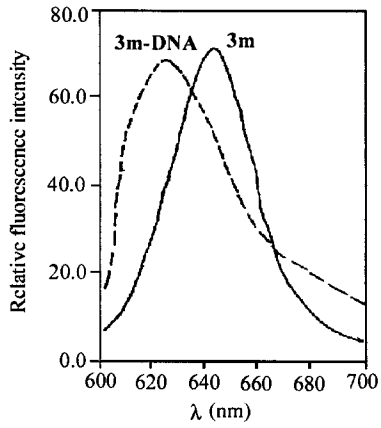


Fig. 4 Fluorescence spectra of **3m** and **3m-DNA**.

Covalent linking of **3l** to **ct-DNA**

In DNA modification studies, a fluorescent molecule is normally bound either covalently or noncovalently.[27] A compound having an active group, such as isothiocyanide, succinimidyl ester and aldehyde groups, can react covalently with DNA modified with an amine.[28,29] The first step for the modification of DNA with **3l** is the introduction of reactive amino groups into DNA by transaminating in the presence of sodium bisulphate and 1,6-hexanediamine. By this reaction (Scheme 4), cytosine bases in single-strand nucleic acids might be converted to N^4-substituted derivatives as reported.[30,31] The transaminated DNA was reacted with succinimidyl ester of **3l** by the method described previously.[29] An excess of **3l** was used to complete the reaction. Prolonging the transamination

Table 1 Relative fluorescence intensity of **3a-DNA** and **3m-DNA** in different pH values[a] and ionic strength[b]

pH	4	5	6	7	8	9
3a-DNA	87.9	89.0	88.4	88.6	88.8	88.9
3k-DNA	53.1	54.3	53.8	54.0	53.8	53.6
NaCl (mmol/L)	5	12	20	25	30	35
3a-DNA	88.6	88.8	89.0P	89.1	88.5	88.7
3k-DNA	54.0	54.2	54.5	54.2	53.8	53.8

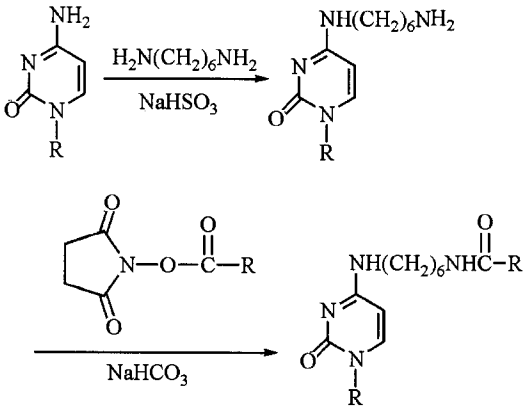
[a] Solvent was DMSO-H_2O (V: V = 5:1) containing 20 mmol/L NaCl. [b] DMSO-H_2O (V: V = 5:1).

Table 2 Maxima of the absorption and emission and their fluorescence quantum yields of **3a—3m** and modified DNA[a]

Compound	λ_{max} (nm)	λ_{em} (nm)	φ_Y	Modified DNA	λ_{em} (nm)	φ_Y
3a	553	619	0.49	**3a-DNA**	672	0.58
3b	561	621	0.51	**3b-DNA**	675	0.59
3c	561	622	0.52	**3c-DNA**	675	0.60
3d	562	622	0.52	**3d-DNA**	675	0.60
3e	564	623	0.52	**3e-DNA**	675	0.60
3f	566	622	0.53	**3f-DNA**	675	0.61
3g	568	623	0.55	**3g-DNA**	675	0.61
3h	569	624	0.55	**3h-DNA**	676	0.60
3i	569	625	0.56	**3i-DNA**	676	0.61
3j	558	620	0.45	**3j-DNA**	678	0.58
3k	556	624	0.41	**3k-DNA**	680	0.52
3l	565	622	0.43	**3l-DNA**	611	0.42
3m	587	640	0.62	**3m-DNA**	625	0.54

[a] λ_{ex} = 580 nm, solvent was DMSO-H_2O (V: V = 5:1) containing 20 mmol/L NaCl.

Scheme 4



did not result in higher labeling degree. Fig. 5 shows the fluorescence spectra of **3l** and **3l-DNA**. The maximum of **3l-DNA** had a blue shift 11 nm relative to **3l** (Table 2). The succinimidyl ester of **3l** is a reactive group, it can form amide bond with the amino group of transaminated DNA. This method is often used for DNA modification.[32] The emission wavelength of modified DNA is above 600 nm. The covalent product **3l-DNA** has been found to be stable at −20 ℃ or +4 ℃ for at least one year.

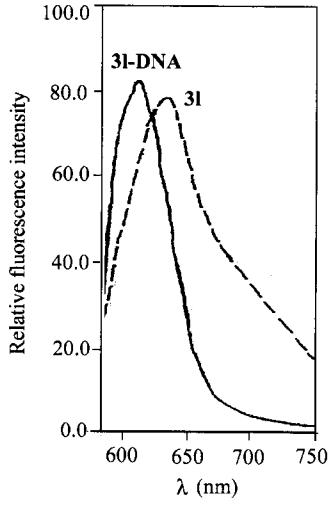


Fig. 5 Fluorescence spectra of **3l** and **3l-DNA**.

Melting point estimation of modified DNA *with fluorescent compounds*

The effect of several compounds (**3a**, **3k** and **3m**) on the thermal stability of DNA duplexes was studied. Their melting temperatures (t_m) were be obtained by the curves of the absorbencies at 260 nm vs. different tem-

peratures as described.[33] The unmodified **ct-DNA** was 82.9 ℃. From Fig. 1, t_m of **3a-DNA** and **3k-DNA** were obtained as 84.0 ℃ and 84.7 ℃, increased melting temperature of DNA by 1.1 ℃ and 1.8 ℃, respectively. It shows that modified DNA with **3a** or **3k** can increase the thermal stability of DNA duplexes and has a higher melting temperature than pure DNA. The melting temperature of **3m-DNA** decreased by 0.8 ℃ compared with DNA. It resulted from electrostatic interaction and decreased the thermal stability of DNA duplexes.

Conclusion

In addition to the previously known **3a—3j**, compounds **3k**, **3l** and **3m** were synthesized. Compound **3l** could form a covalent product with DNA, and **3l-DNA** had a blue shift of fluorescence maximum. Compounds **3a—3k** had interactions with DNA and their products with DNA had a red shift of fluorescence and increased the melting temperature of DNA. Compound **3m-DNA** had a blue shift of fluorescence maximum relative to that of **3m** and decreased the melting temperature of DNA. The result provides a new near-infrared fluorescent probe for DNA modification.

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(E0107174 SONG, J. P.)