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ORIGINAL ARTICLE

Synthesis and Application of Water-Soluble Oxazine Dyes for Detection of PHAs-Producing Bacteria

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Abstract
Derivatives of oxazine dyes were synthesized on mulitigram scales via efficient synthetic strategies. One practical route was selected to prepare compounds **6**, **9** and **10**, especially water-soluble compound **6** was obtained in better yield than reported, and compound **10** was insoluble in aqueous media in absence of phenolic-OH. Compounds **3** and **9** were found to be clear pH-dependent between pH = 4.0 and 10.0, and could be used as acid-base indicators to measure intracellular pH. Compounds **6**, **9**, **10** all have carboxylic acid functionalities, which could be activated and used to conjugate the dyes to biomolecules. In addition, compounds **6** and **9** with good solubility in aqueous media were used to develop a simple, quick, safe, highly sensitive staining method to detect PHAs-producing bacteria on heat-fixed smears, which was confirmed by fluorescence images of PHAs granules of bacteria.

Keywords Oxazine dyes · Carboxyl groups · Good solubility · Staining method · PHAs-producing bacteria

Abbreviations
PHAs Poly(3-hydroxyalkanoic acids)

Introduction

Oxazine dyes have been widely used as labels in biotechnology, especialy as biological stains [1–10]. Nile Red is a solvatochromic fluorescent probe and traditionally used to stain lipid droplets in cell and bacteria [11–14]. Unfortunately, Nile Red has very poor solubility in aqueous media, which limited its application for labeling most biomolecules. Nile Blue has good photochemical stability and strong fluorescence in the red region, which exhibits the potential utility in biological application [15–17]. However, the tendency of Nile Blue to aggregate in aqueous environments has also limited its biological applications [18, 19].

Nile Red and Nile Blue have been widely used to stain Poly(3-hydroxyalkanoic acids) which are a group of polymeric esters functioning as an energy and carbon reserve in bacteria. Environmenal pollution has aroused interests in the development of PHAs for producing novel biodegradable plastic materials [20–27]. PHAs are being investigated in many laboratories, which would greatly benefit from a reliable, fast and sensitive method for the dectection of PHAs in colonies [14, 28–33]. Although many methods have been reported, many of them require time-consuming manupulations and lipophilic oxazine dyes dissolved in ethanol or acetone to kill the bacteria or DMSO toxic to workers [34–40].

In this paper, two different routes were developed for the preparation of oxazine dyes and the spectral properties of these compounds have been investigated. And water-soluble compounds **6** and **9** with dicarboxylic acid were applied in staining PHA(s)-producing bacteria, which was confirmed by images of stained bacteria stranules of the fluorescence microscope.

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Experiments

Materials

3-aminophenol, methyl acrylate, 1-aminonaphthalene, 1-naphthol, 1,6-naphthalenediol and all of organic solvent used were of analytical grade.

The H_3BO_3 buffer (pH = 8.5) was prepared by adding 1 mol/L HCl solution to 0.05 mol/L $Na_2B_4O_7$ solution to the required pH value. Phosphate buffered saline buffer (pH = 7.39) was prepared by dissolving 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 8.00 g NaCl in 800 mL distilled water and adjusting the pH with 0.1 mol/L HCl solution, then diluted to 1000 mL by distilled water. After sterilized at 120 °C, the buffer was stored at the room temperature. Working standard of compound **6** or **9**: compounds **6** and **9** were dissolved in PBS buffer as 100 μg/mL, respectively, and then diluted to 0.5 or 0.1 μg/mL as the working solution.

Luria-Bertani (LB) Agar Broth was prepared by adding 6 g of tryptone, 3 g of yeast extract, 6 g of NaCl and 9 g agar powder to 600 mL distilled water. After sterilized for 30 min at 120 °C, LB Agar Broth was poured into dishes to cultivate bacteria when cooled to 50 °C.

Bacteria used in this study: *E. coli*, *Soil D. Bacillus Polyfermenticus* and *Motile unit cell rod-shaped Bacteria* from Henan Key Laboratory of Crop Pest Control of Hong-Yan Liu researcher.

Characterization

^{1}H NMR was performed on a INOVA 500 spectrometer with tetramethylsilane as internal reference. UV-vis absorption spectra were measured on a computer-controlled Shimadzu UV-2450 spectrometer. Fluorescent microscope images of bacteria stranules stained with compound **6** or **9** were recorded with the invert fluorescent microscope IX71-A12FL/PH. The pH values were determined with a pHS-25 acidimeter calibrated with standard aqueous buffer solutions. The fluorescence spectra were measured in standard quartz cuvettes on a Cary Eclipse or JASCO FP-750 spectrofluorimeter.

Syntheses of Compounds 1–10

Two synthetic routes of diesters **3**, **4** and **5** and dicarboxylic acids **6**, **9** and **10** are outlined in Schemes 1 and 2.

Dimethyl 3,3′-((3-hydroxyphenyl)azanediyl)dipropionate (1) [41]

A mixture of 3-aminophenol (2.18 g, 20.0 mmol), CuCl (0.37 g, 3.7 mmol), acetic acid (2.40 g, 40.0 mmol) and methyl acrylate (5.34 g, 62.1 mmol) was refluxed for 20 h. After cooling to the room temperature, the mixture was diluted with dichloromethane (10 mL) and consecutively washed with H_2O, 10% ammonia and H_2O. The organic phase was dried over Na_2SO_4 and evaporated. The crude product was purified by column chromatography on silica eluting with dichloromethane: petroleum ether: ethyl acetate = 6:5:1 to afford the title compound **1** as yellow oil (3.65 g, 65%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 2.51 (t, J = 7.0 Hz, 4H), 3.49 (t, J = 7.0 Hz, 4H), 3.59 (s, 6H), 6.07–6.11 (m, 3H), 6.93 (s, 1H), 9.05 (s, br, 1H).

Dimethyl 3,3′-((3-hydroxy-4-nitrosophenyl)azanediyl) dipropionate (2) [2]

Sodium nitrite (2.06 g, 29.9 mmol) in water (15.2 mL) was stepwise added to a solution of compound **1** (7.82 g, 27.8 mmol) in HCl (9.38 mL, 12.0 M) and water (4.70 mL) with stirring for 4 h in an ice bath. The mixture was filtered to remove residual impurities. The filtrate was evaporated under reduced pressure. The crude product dissolved in methanol was dried with $MgSO_4$, then filtered and concentrated to obtain compound **2** (6.48 g, 75.1%). This somewhat unstable nitroso compound was directly used without further purification for the next step.

9-(bis(3-methoxy-3-oxopropyl)amino)-5H-benzo[a] phenoxazin-5-iminium chloride (3)

Compound **2** (2.12 g, 6.8 mmol), 1-naphthylamine (0.98 g, 6.8 mmol), and HCl (2.0 mL, 12.0 M) in 50 mL methanol were refluxed for 7 h. The solvent was evaporated and the residue was isolated by column chromatography on silica eluting with the gradient elution of dichloromethane:methanol = 60:1~40:1~30:1 to give the title compound **3** (2.29 g, 80.8%) as metallic green powder.

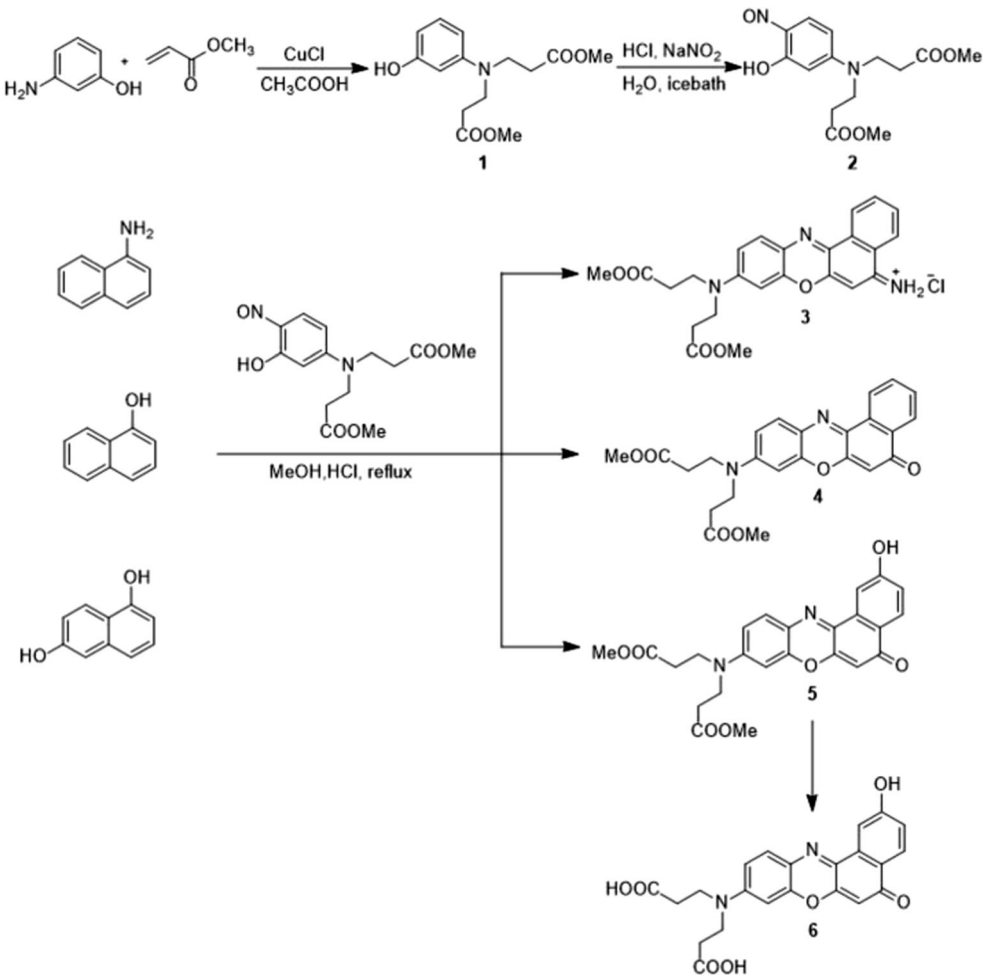
^{1}H NMR (500 MHz, DMSO-d_6): δ 2.70 (t, J = 7.0 Hz, 4H), 3.35 (s, 6H), 3.85 (t, J = 7.0 Hz, 4H), 6.99 (s, 1H), 7.07 (s, 1H), 7.22 (d, J = 9.5 Hz, 1H), 7.85 (d, J = 9.5 Hz, 1H), 7.89 (t, J = 8.0 Hz, 1H), 7.99 (t, J = 8.0 Hz, 1H), 8.55 (d, J = 8.0 Hz, 1H), 8.78 (d, J = 8.0 Hz, 1H).

Dimethyl 3,3′-((5-oxo-5H-benzo[a]phenoxazin-9-yl) azanediyl)dipropionate (4)

Compound **4** was simillarily synthesized as compound **3**. And compound **2** (5.16 g, 16.7 mmol), 1-naphthol (2.34 g, 16.7 mmol), HCl (4.87 mL, 12.0 M) and methanol (50 mL). The residue was isolated by column chromatography on silica eluting with dichloromethane:methanol = 150:1 to give the title compound **4** (1.84 g, 58.5%) as red powder.

^{1}H NMR (500 MHz, DMSO-d_6): δ 2.63 (t, J = 7.0 Hz, 4H), 3.35 (s, 6H), 3.73 (t, J = 7.0 Hz, 4H), 6.29 (s, 1H), 6.73 (s, 1H), 6.84 (d, J = 9.0 Hz, 1H), 7.63 (d, J = 9.0 Hz, 1H), 7.72 (t, J = 7.0 Hz, 1H), 7.81 (t, J = 7.0 Hz, 1H), 8.11 (d, J = 7.5 Hz, 1H), 8.54 (d, J = 7.5 Hz, 1H).

Scheme 1 Synthetic scheme for oxazine dyes **3**–**6**



Dimethyl 3,3'-((2-hydroxy-5-oxo-5H-benzo[a] phenoxazin-9-yl)azanediyl)dipropionate (5)

The process of synthesis of compound **5** was the same as that of compound **3**.And compound **2** (4.56 g, 16.2 mmol), 1,6-naphthalenediol (2.59 g, 16.2 mmol),HCl (2.26 mL, 12.0 M)and methanol (45 mL). The residue was isolated by column chromatography on silica eluting with dichloromethane:methanol = 120:1 to give the title compound **5** (4.48 g, 70.2%) as red powder.

^{1}H NMR (500 MHz, DMSO-d_6): δ 2.63 (t, J = 7.0 Hz, 4H), 3.61 (s, 6H), 3.71 (t, J = 7.0 Hz, 4H), 6.13 (s, 1H), 6.66 (s, 1H), 6.78 (d, J = 9.5 Hz, 1H), 7.07 (t, J = 2.5 Hz, 1H), 7.09 (t, J = 2.5 Hz, 1H), 7.56 (d, J = 9.0 Hz, 1H), 7.81–7.86 (m, 1H), 7.94 (d, J = 9.0 Hz, 1H).

3,3'-((2-hydroxy-5-oxo-5H-benzo[a]phenoxazin-9-yl) azanediyl)dipropionic acid (6) [2]

A solution of K_2CO_3 (0.83 g, 6.0 mmol) dissolved in 10 mL of water was added to a solution of **5** (0.26 g, 0.6 mmol) in 10 mL of methanol/water (1:1). The reaction mixture was heated to 40 °C for 36 h. The solvent was evaporated under reduced pressure; the crude mixture was then dissolved in water (20 mL) and washed with ethyl acetate (3 × 5 mL). The aqueous layer was acidified with HCl (4–5 drops, 12.0 M) to pH 4–5. This aqueous layer was extracted with dichloromethane/2-propanol (3 × 5 mL, 1:1) and the organic layer was evaporated under reduced pressure to give compound 6 (0.15 g, 59.3%) as a red solid.

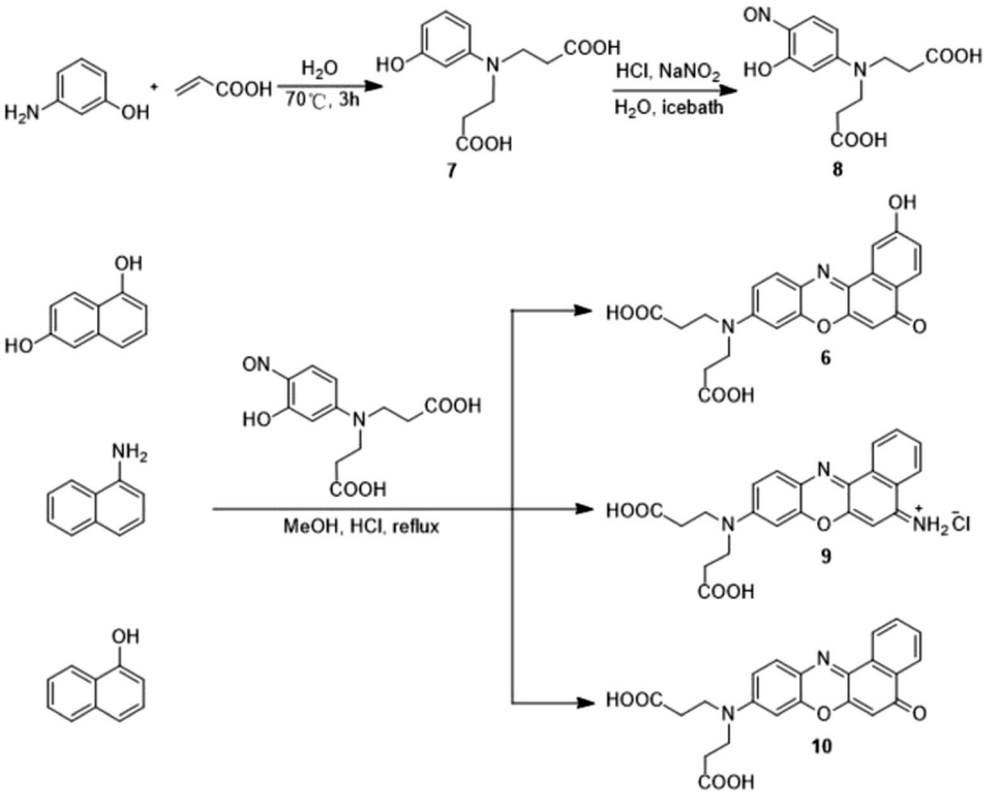
^{1}H NMR (500 MHz, DMSO-d_6): δ 2.59–2.63 (m, 4H), 3.76–3.80 (m, 4H), 6.14 (s, 1H), 6.66 (s, 1H), 6.79 (dd, J = 6.5, 2.5 Hz, 1H), 7.08 (dd, J = 6.0, 2.5 Hz, 1H), 7.57 (d, J = 9.0 Hz, 1H), 7.84 (d, J = 2.5 Hz, 1H), 7.94 (d, J = 9.0 Hz, 1H), 10.61 (s, 1H).

The synthetic routes of diesters **3, 4** and **5** and dicarboxylic acids **6** are outlined in Scheme 1.

3,3'-((3-hydroxyphenyl)azanediyl)dipropionic acid (7) [42]

A solution of 3-amino-phenol (5.45 g, 50 mmol) in acrylic acid (9.72 g, 150 mmol) and water (4.65 mL) was heated to 70 °C for 3 h ($CHCl_3$: CH_3OH: H_2O = 12: 7: 1, R_f = 0.5).The reaction mixture was cooled and ethanol (9 mL) was added and kept at 4 °C for 48 h. The white precipitate that formed was filtered, washed with ethanol (2.5 mL), and dried to obtain dicarboxylic acid (10.88 g, 75.26%). The product was directly used in the next step.

Scheme 2 Synthetic scheme for oxazine dyes **6**, **9**, **10**



3,3′-((3-hydroxy-4-nitrosophenyl)azanediyl)dipropionic acid (8)

Sodium nitrite (0.75 g, 10.94 mmol) in water (12 mL) was added, over a period of 1 h, via a syringe pump at the rate of 0.2 mL per min, to a solution of 1 (2.43 g, 9.60 mmol) in HCl (12 mL, 12.0 M) and water (6 mL) at 0 °C. The mixture was stirred for 2.5 h at 0 °C and filtered to remove residual impurities. The filtrate was evaporated under reduced pressure, the residue dissolved in methanol, dried with magnesium sulfate, and filtered, and methanol was evaporated to yield 2 (2.08 g, 76.8%). Then hygroscopic nitroso compound was used in the next step without further purification.

9-(bis(2-carboxyethyl)amino)-5H-benzo[a] phenoxazin-5-iminium chloride (9)

The solution of compound **8** (1.21 g, 4.3 mmol), 1-naphthylamine (0.61 g, 4.3 mmol) and HCl (1.25 mL, 12.0 M) in methanol (35 mL) was refluxed for 7 h with stirring. The mixture was concentrated, and subjected to column chromatography on silica with dichloromethane: methanol = 40:1 to yield metallic green powder (1.57 g, 91.3%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 2.72 (t, J = 7.0 Hz, 4H), 3.86 (t, J = 7.0 Hz, 4H), 6.94 (s, 1H), 7.10 (s, 1H), 7.26 (d, J = 9.0 Hz, 1H), 7.42 (d, J = 7.5 Hz, 1H), 7.91 (t, J = 7.5 Hz, 1H), 8.02 (t, J = 7.5 Hz, 1H), 8.53 (d, J = 8.5 Hz, 1H), 8.83 (d, J = 8.0 Hz, 1H), 10.15 (s, 1H), 10.28 (s, 1H).

3,3′-((2-hydroxy-5-oxo-5H benzo[a]phenoxazin-9-yl) azanediyl)dipropionic acid (6)

Compound **6** was simillarily synthesized as compound **9**. And compound **8** (1.21 g, 4.29 mmol), 1,6-naphthalenediol (0.69 g, 4.29 mmol), HCl (0.70 mL, 12.0 M) and methanol (33 mL). The residue was isolated by column chromatography on silica eluting with dichloromethane:methanol = 100:1 to give the title compound **6** (0.92 g, 58.8%) as red powder.

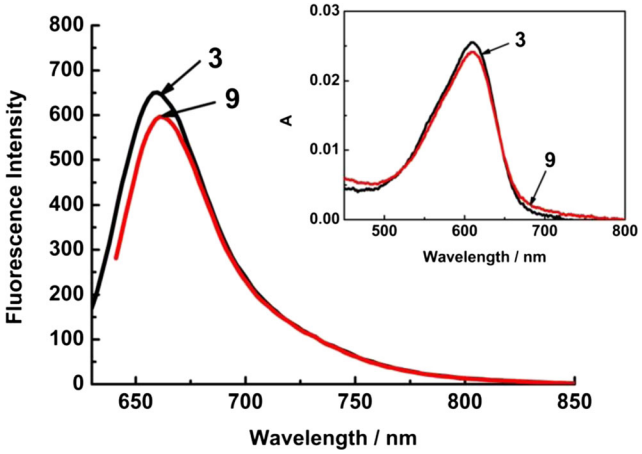


Fig. 1 Fluorescence and UV-Vis spectra of compounds **3** and **9** in ethanol ($\lambda_{ex} = \lambda_{ab\ maxima}$)

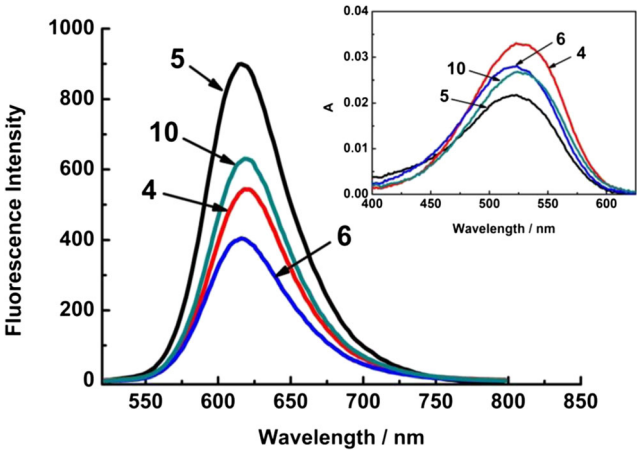


Fig. 2 Fluorescence and UV-Vis spectra of compounds **4**, **5**, **6** and **10** in ethanol ($\lambda_{ex} = \lambda_{ab\ maxima}$)

Compound 6: ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.61 (t, J = 7.5 Hz, 4H), 3.74 (t, J = 7.5 Hz, 4H), 6.09 (s, 1H), 6.47 (s, 1H), 6.69 (dd, J = 6.0, 2.0 Hz, 1H), 7.06 (dd, J = 6.0, 2.0 Hz, 1H), 7.46 (d, J = 9.0 Hz, 1H), 7.81 (d, J = 2.5 Hz, 1H), 7.92 (d, J = 8.5 Hz, 1H), 10.47 (s, 1H).

*3,3'-((5-oxo-5H-benzo[a]phenoxazin-9-yl)azanediyl) dipropionic acid (**10**)*

The process of synthesis of compound **10** was the same as that of compound **9**.And compound **8** (1.21 g, 4.29 mmol), 1-naphthol (0.62 g, 4.29 mmol),HCl (0.70 mL, 12.0 M)and methanol (33 mL). The residue was purified by column chromatography on silica eluting with petroleum ether: ethyl acetate = 4:1 to give the title compound **10** (1.50 g, 77.9%) as red powder.

Compound 10: ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.63 (t, J = 7.0 Hz, 4H), 3.72 (t, J = 7.0 Hz, 4H), 6.30 (s, 1H), 6.71 (s, 1H), 6.84 (dd, J = 8.5, 2.5 Hz, 1H), 7.64 (d, J = 9.0 Hz, 1H), 7.72 (t, J = 7.5 Hz, 1H), 7.81 (t, J = 7.5 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 8.54 (d, J = 7.5 Hz, 1H).

The synthetic routes of dicarboxylic acids **6**, **9** and **10** are outlined in Scheme 2.

Dicarboxylic Acid 6 or 9 Staining PHAs-Producing Bacteria

The cytotoxicity of compounds **6** and **9**: *E. coli* was used as the given bacteria, and 18 mL LB Agar Broth and a certain amount of the PBS buffer of 100 μg/mL compounds **6** or **9** were mixed in order to obtain 0.5, 0.3 and 0.1 μg/mL, respectively. The mixture was poured into the dish, and then 100 μL *E. coli* suspension was extracted, coated and cultured. After three days, the minimum inhibitory concentration (MIC) was determined.

Method 1: Bacteria was collected from the culture and incubated in the PBS buffer of 0.5 μg/mL compound **6** or **9** for 10 min at 55 °C. Bacteria were repeatedly washed with PBS buffer and centrifuged until the last traces of the dye disappeared. And then the bacteria was heat fixed on the smear and covered with a coverslip to observe PHAs granules of bacteria under the fluorescent microscopy.
Method 2: A series of PBS buffers of compound **6** or **9** was prepared at 0.01, 0.03, 0.06, 0.10, 0.15, 0.30, 0.50 and 1.00 μg / mL. Bacteria was took out from the culture and heat fixed on the smear. In order to choose the optimum concentration, a drop of the above solutions was added on the smear, heated for several seconds, and then covered with a coverslip to observe under the fluorescent microscopy.

Results and Discussion

Syntheses of Derivatives of Oxazine Dyes 3, 4, 5, 6, 9, 10

As shown in Scheme 1, double Michael addition of methyl acrylate with 3-aminophenol effectively proceeded to compound **1**, followed by nitrosation to yield compound **2**. By condensation of nitrosocompound **2** with 1-naphthylamine, 1-naphthol and 1,6-naphthalenediol, respectively, compounds

Table 1 Spectroscopic properties of derivatives of oxazine dyes

Substrate	3 [b]	9 [b]	Nile blue	RhB	4[a]	5[a]	6[a]	10[a]
λ_{ab} (nm)	608	608	641	544	523	523	524	523
ε(mol/cm L × 10^4)	2.73	2.93	4.12	7.85	2.64	1.26	3.14	1.85
λ_{ex}(nm)	608	608	641	544	523	523	524	523
λ_{em} (nm)	660	661	678	565	618	616	618	618
Stokes shift (cm^{-1})	1295.6	1318.8	851.4	683.2	2939.2	2886.7	2902.7	2886.7
Φ	0.03	0.045	0.01	0.50	0.38	0.29	0.34	0.30
Emission band halfwidth (nm)	50	50	50	36	64	66	64	66
Solvent	pH = 7.4 phosphate buffer					Ethanol		

[a] standard used for fluorescent quantum yield measurement of rhodamine B (Φ = 0.5 in ethanol) [43]

[b] standard used for fluorescent quantum yield measurement of Nile Blue (Φ = 0.01 in PBS buffer) [44]

3, **4**, **5** were synthesized and isolated via column chromatography in satisfactory yields. Aqueous hydrolysis of compound **5** gave the corresponding diacid compound **6** that was obtained using a simple acid base extraction procedure. Compoumd **6** had the better yield than that was reported by Jiney Jose and Kevin Burgess [2] because of short synthesis steps. However, compound **6** had the better yield in Scheme 1 than that in Scheme 2, which is that the product of Michael addition crystallized out of the reaction mixture and the only column chromatography step used in the two-step syntheses.

Spectroscopic Properties of Derivatives of Oxazine Dyes

From Fig. 1, the emission and the absorption wavelength maxima of compounds **3** and **9** are basically the same, respectively, which caused by similar substituent groups at the N-terminate. However, in comparison with compound 3, the fluorescent spectra of compound 9 produce a slight red shift because of the presence of methyl groups.

From Fig. 2, the emission and the absorption wavelength maxima of compounds **4**, **5**, **6** and **10** are essentially the same, respectively, because of the chemical structure is maily the same in addition to the differences in carboxylic acid or carboxylic ester at the N-terminate.

The spectroscopic properties of compounds **3**, **4**, **5**, **6**, **9**, **10** were summarized in Table 1. The spectral properties of compounds **3** and **9** were investigated by comparison with Nile Blue (Fig. 3), their fluorescent and UV-vis spectra produced a blue shift of 17–18 nm and 33 nm, respectively, and their molar absorptivity was lower than that of Nile blue, which is mainly due to the modifications at the 9-amino of Nile Blue. Nile Blue with the diethylamino group readily forms sandwich-like H-aggregate type of dimers or even higher. But compounds **3** and **9**, which were substituted by dicarboxylic ester and dicarboxylic groups, respectively, increased water solubility and reduced the tendency of aggregate formation in aqueous solution. Therefore, compounds **3** and **9** have a higher fluorescent quantum yield and larger Stokes shift than Nile Blue. The fluorescent quantum yields, the absorption wavelength maxima and molar absorptivity of Nile Red derivatives, compounds **4**, **5**, **6** and **10**, are lower than those of rhodamine B. However, their fluorescence emission

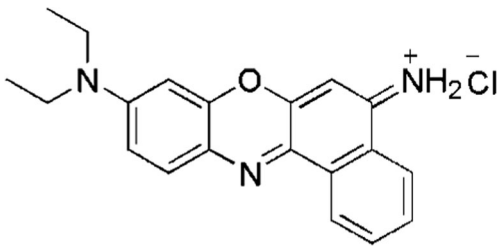


Fig. 3 The chemical structure of Nile blue

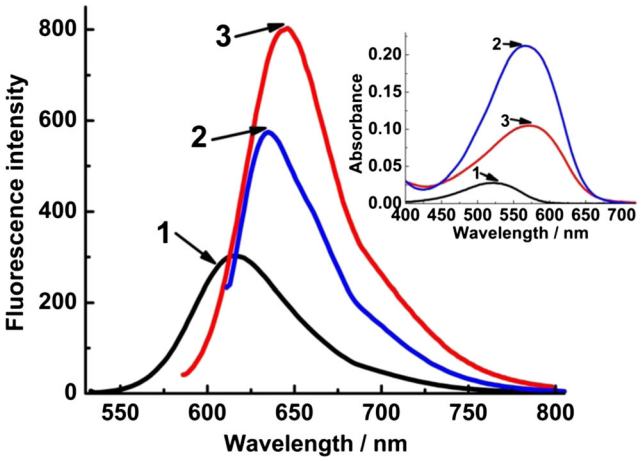


Fig. 4 Fluorescence spectra and UV-Vis spectra of compound **6** in different solvents (1. ethanol, λ_{ex} = 532 nm; 2. phosphate buffer, λ_{ex} = 610 nm; 3. borate buffer, λ_{ex} = 584 nm)

wavelength maxima and Stokes shifts are larger than those of rhodamine B, which can effectively reduce the interference of the background fluorescence and improve the resolution of the detection of biomolecule.

The fluorescence spectra of dicarboxylic acid **6** which has good solubility in ethanol, phosphate buffer at pH = 7.39 and borate buffer at pH = 8.5 are shown in Figs. 4 and 5. In both neutral and basic buffers, the emission maxima were red-shifted, but by only 24 nm relative to the ethanolic solution of the dye. Surprisingly, only lack of a phenolic-OH, compound **10** was insoluble in aqueous medium, and it had a similar absorption and emission wavelength maxima and the similar emission band halfwidth as compound **6**. However, compound **6** had a slightly better quantum yield than compound **10** except that the molar absorptivity was approximately double that of it in ethanol.

The relationship between the fluorescence intensity and pH was studied in this paper. As shown in Fig. 4, compound **9**

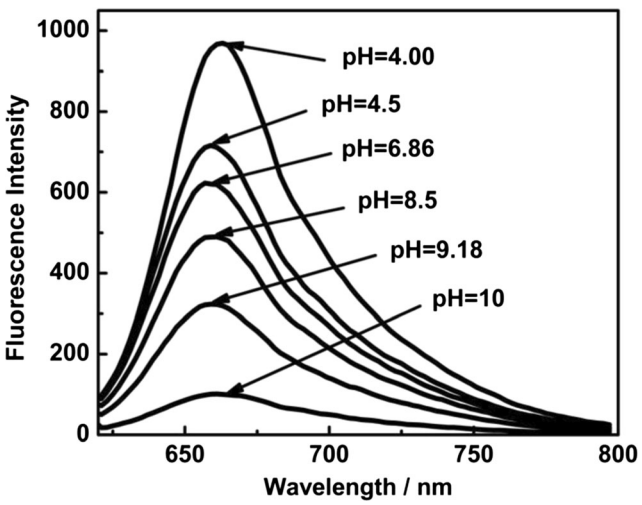


Fig. 5 Emission spectra of compound **9** in buffered solution of various pH (λ_{ex} = 608 nm)

shows clear pH-dependence on the fluorescence intensity. Under the condition of pH = 10.0, compound **9** nearly loses of its fluorescence. With the decrease in pH value, fluorescence intensity of compound **9** increases, but the range of increment was different. When pH is 4.0, the fluorescence intensity reaches the maximum. Compound **3** has the same situation as compound **9**. Hence, compounds **3** and **9** can be used as acid-base indicators. By taking advantage of the pH dependence of their fluorescence, compounds **3** and **9** could be used to measure intracellular pH [45].

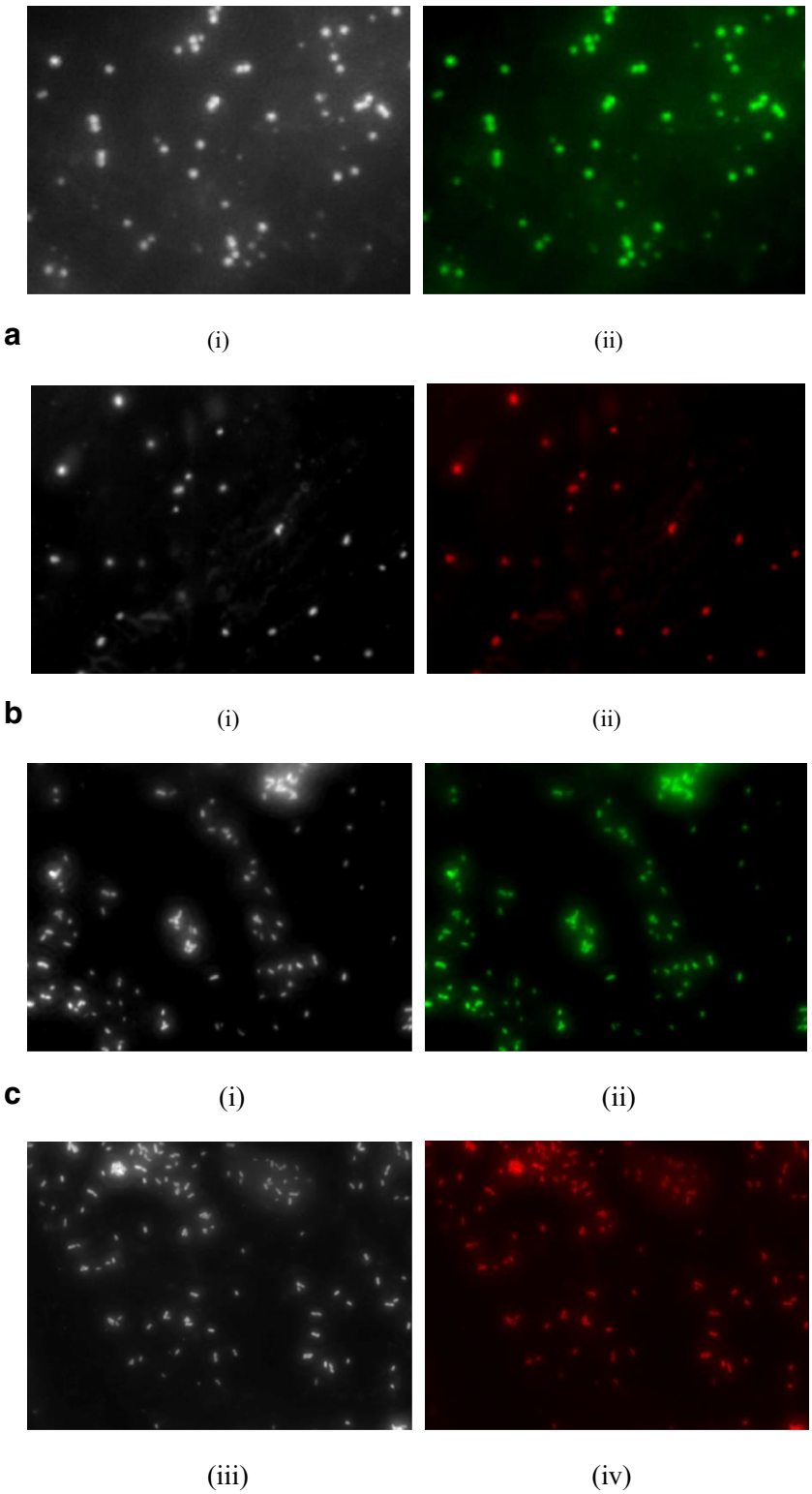


Fig. 6 a Soil D. Bacillus Polyfermenticusafter treated with compound **6** (i) Bright fileld image (ii) Fluorescence image of PHAs granules which were seen as green fluorescence dots b *E. coli* after incubation with compound **9** (i) Bright fileld image (ii) Fluorescence image of PHAs granules which were seen as red fluorescence dots. c Motile unit cell rod-shaped bacteria after staining with compound **6** and **9** (i) Bright fileld image staining with compound **6** (ii) Fluorescence image of PHAs granules which were seen as green fluorescence dots staining with compound **6** (iii) Bright fileld image staining with compound **9** (iv) Fluorescence image of PHAs granules which were seen as red fluorescence dots staining with compound **9**. (The bacteria with PHAs, stained with compound **6**, was depicted in green; stained with compound **9**, was depicted in red)

Dicarboxylic Acid Compound 6 or 9 Staining for the Detection of PHAs-Producing Bacteria

The PBS buffer of 0.50 μg/ml compound **6** or **9** showed antimicrobial ability against *E. coli* to some extent, while 0.3 and 0.1 μg/mL compounds **6** or **9** had no bacteriostatic effect.

Staining bacteria was rinsed to remove residual compound **6** or **9**, which could reduce background color during the observation of the fluorescent microscope in Method 1. 0.10 μg/ml compound **6** or **9** was chosen as the optimum concentration in Method 2. A higher dye concentration did not enhance fluorescence intensity of PHAs granules of bacteria only caused background color. It is obvious that the application of two staining methods for screening large numbers of individual colonies is time-saving. In addition, the PBS buffer of compound **6** or **9** allowed original colonies to be tested without the risk of cells being killed by ethanol, which should be a satisfactory stain for colonies on the agar plate. Thus, compound **6** or **9** can directly monitor the growth of PHAs in the colony and reflect the activity of PHAs in the bacteria.

Images of PHAs granules of bacteria of the fluorescent microscope are shown in Fig. 6.

Conclusion

Simple and efficient synthetic strategies were developed for six derivatives of oxazine dyes on multigram scales. The route outlined in Scheme 2 was the better method than reported for producing water-soluble compound **6**. Compounds **6**, **9** and **10** have carboxylic functionalities which could be activated and used to conjugate biomolecules. Compounds **3** and **9** could be used as acid-base indicators with higher fluorescent quantum yields in contrast to Nile Blue. In addition, compounds **6** and **9** with good solubility in aqueous media were used to develop a simple, quick, safe, highly sensitive staining method to detect PHAs-producing bacteria on heat-fixed swears. The feasibility of the staining method was confirmed by fluorescent images of PHAs granules of bacteria. The results demonstrated that compounds **6** and **9** could be directly dissolved in agar medium to develop a viable-colony staining method, which could directly monitor the occurrence of PHAs in colonies and reflect the in vivo activity of PHAs syntheses. From the above analysis, we believe compounds **6** and **9** will have latent applications as biological markers in biotechnology.

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