Synthesis and Properties of a Maleimide Fluorescent Thiol Reagent Derived from a Naphthopyranone

Jun-Rui Yang and Margaret E. Langmuir*

Covalent Associates, Inc., 52 Dragon Court,
Woburn, MA 01801

Received February 2, 1991

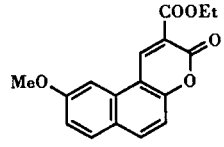
N-(2-Carbomethoxy-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-10-yl)maleimide **2** was designed and synthesized as a new maleimide fluorescent thiol reagent. The optical properties of **2** were investigated with and without the addition of glutathione, GSH. We have found **2** is twice as sensitive as DACM and is of comparable sensitivity to CPM for detection of thiols. The emission maximum for the GSH adduct of **2** is 513 nm which is at longer wavelength than the GSH adducts of both DACM and CPM.

J. Heterocyclic Chem., **28**, 1177 (1991).

As the fundamental importance of the thiol functional group in biological systems continues to unfold, detection and quantitation of biological thiol compounds have become critical issues. Among the analytical methods available now, fluorescent thiol reagents have found wide applications in the study of protein structural and microenvironmental properties, micro assays for choline esterases or glutathione *S*-transferase, histochemical staining, hplc analysis of thiol compounds, and flow cytometry [1]. Fluorescent maleimides, which undergo facile addition to thiols, were first designed and synthesized by Kanaoka to study protein structures [2]. After careful studies of the reactivity, hydrolytic behavior, and fluorescence properties of a series of *N*-substituted maleimides, Kanaoka concluded that it is possible to design thiol reagents which carry a variety of fluorescent reporters but have similar reactivities toward thiol compounds [3].

A successful candidate for a fluorescent thiol reagent should have a fluorogenic group with fluorescence emission maximum higher than 480 nm, in order to avoid the background fluorescence of many biological materials [4]. In addition, a large Stokes' shift, a high extinction coefficient, and a high quantum yield are preferred properties. *N*-(7-Dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM) reacts with thiols to yield fluorescent derivatives with emisson maxima at 477 nm [5]. However, routine use of DACM is limited by its high cost [6]. *N*-(4'-(7-Diethylamino-4-methylcoumarin-3-yl)phenyl)maleimide (CPM) is less expensive than DACM, but it still has short wavelength absorption and emission maxima [6]. The thiol derivative of fluorescein maleimide emits at 515 nm [7], but it suffers from concentration quenching due to its small Stokes' shift. Other available long emission wavelength thiol reagents include eosin maleimide [8], rhodamine maleimide [9], and *N*-(6-Butterflyaminocoumarin-3-yl)-maleimide (BACM), a coumarin based maleimide recently reported in this journal [10]. An application study of microfluorometric agent found DACM to be superior among other long emission wavelength thiol reagents including eosin-5-maleimide [1]. Therefore, further research is needed to develop new fluorescent thiol reagents with improved spectroscopic properties as well as to meet a variety of requirements in various fields of biological research.

While we were involved in a systematic study of fluorescence properties of naphthopyranones in our laboratory, we prepared compound **1**. The superior optical properties of **1** prompted us to prepare the maleimide derivative **2**, and to examine its potential as a thiol fluorophore.



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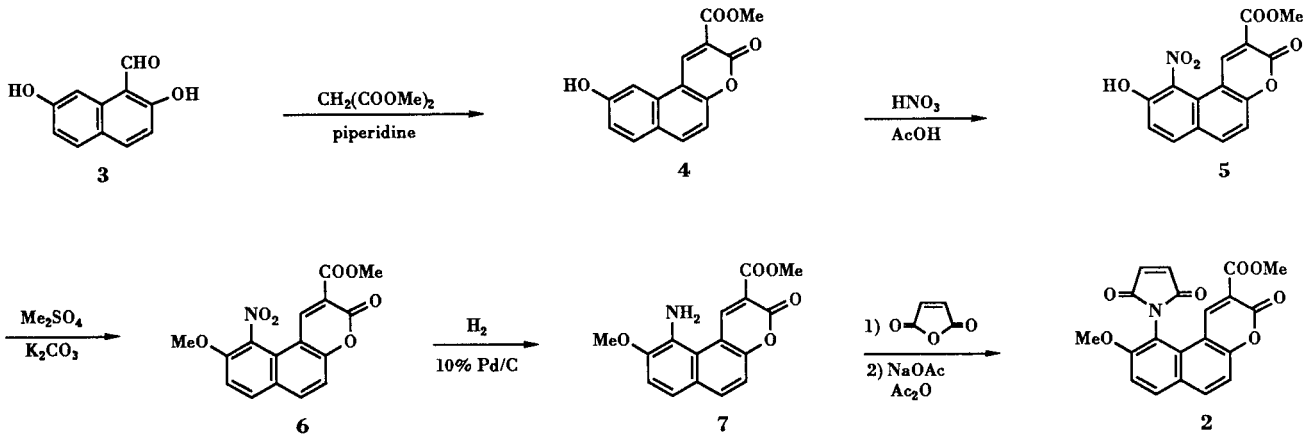
Synthesis.

The introduction of the nitrogen substituent is always a key step in the syntheses of coumarinyl maleimides. In the case of DACM, both positions (6- and 8-positions) *ortho* to the 7-dimethylamino group are similarly activated toward electrophilic substitution. In the original synthesis of DACM [5], nitration of 7-dimethylamino-4-methylcoumarin yielded three isomers which had to be separated chromatographically, causing the low yield and high cost of DACM. An alternative synthesis of DACM reported recently [11] incorporated an acetamido group during the construction of the coumarin ring to avoid the isomeric mixture. However, in that case the yield of the key step was only 12%. We have carefully designed our target molecule so that by using the proper reagent, we can selectively nitrate the desired position of the naphthopyranone. This strategy avoids the time consuming and costly separation of isomers. The synthetic procedures leading to the target molecule **2** are summarized in Scheme 1.

The first stage of our synthesis involves the construction of the naphthopyranone ring. Among the preparation methods available for various substituted benzopyranones (coumarins), the Perkin synthesis [12] and the Pechmann

Scheme 1



condensation [13] are most widely used. Generally, the Pechmann condensation requires a phenol derivative as one of the starting materials and it often leads to 4-methyl substituted coumarins which have been shown to undergo photo-oxidation [14]. The Perkin synthesis, on the other hand, calls for an *o*-hydroxyarylaldehyde as one of the substrates. We chose the Perkin synthesis for preparation of our starting naphthopyranone. The condensation of the aldehyde **3** [15] with dimethyl malonate in the presence of piperidine yielded the naphthopyranone **4**. The product **4** bears a carbomethoxy group at the 2-position, which serves to block this position toward further attack by nitrating reagents, therefore reducing the possible number of isomers in the subsequent nitration reaction. In addition, **4** does not contain a vinylic methyl group which is known to accelerate the photodegradation of 4-methyl coumarins in the presence of oxygen [14].

When 9-hydroxynaphthopyranone **4** was treated with concentrated nitric acid in glacial acetic acid, the 10-nitro derivative **5** was obtained in high yield as the only product. This exclusive regio selectivity can be attributed to both the proper reaction condition and the difference in reactivity between the α- and β-positions of naphthalene derivatives toward electrophilic substitution. When nitration was carried out in glacial acetic acid, the solvent served to limit the steady state concentration of nitronium ion, NO_2^+, the active nitrating agent. For naphthalene derivatives, the α-position (10-position in our case) is known to be more susceptible to aromatic electrophilic substitution than the β-position (8-position in our case).

Conversion of 9-hydroxy-10-nitro compound **5** to its 9-methoxy analogue **6** was accomplished with dimethyl sulfate and potassium carbonate. The 10-nitro group of **6** was reduced to the 10-amino group of **7** by hydrogenation with 10% palladium/carbon as catalyst. The final reaction of amino derivative **7** with maleic anhydride followed by maleimide ring closure with acetic anhydride and sodium acetate was carried out as described by Machida, *et al.* [20].

Fluorescence Study.

The optical properties of the maleimide resulting from this synthesis, **2**, were investigated with and without the addition of glutathione, GSH. The absorption spectrum of the maleimide has a broad long wavelength band with a maximum at 384 nm and an extinction coefficient of 8.86 x 10^3 L·mole^{-1}·cm^{-1}. Addition of GSH does not change the absorption spectrum but has a remarkable effect on the emission which is shown in Figure 1. Figure 1 illustrates that the maleimide alone (curve 4) has a very little fluorescence (ϕ_f = 0.016), but when both *p*H 7.2 phosphate buffer and GSH are added there is an instantaneous increase in fluorescence to curve 1 (ϕ_f = 0.65, corrected λ max =

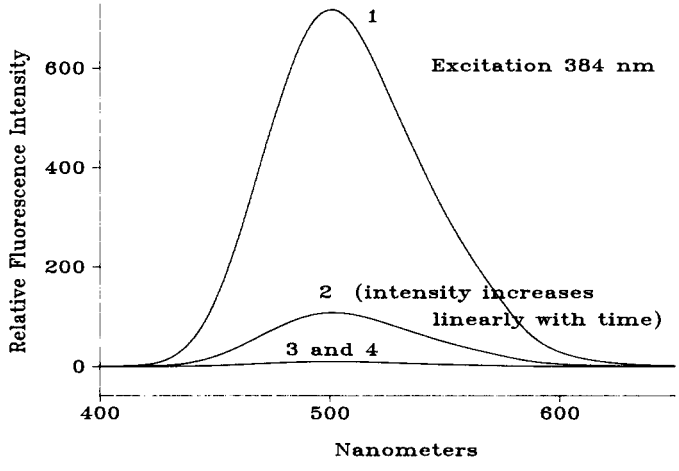


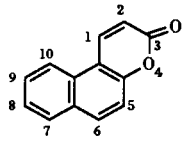
Figure 1. Fluorescence spectra of compound 2 in aqueous solution. (wavelength uncorrected)
Curve 1, compound 2 with GSH and pH 7.2 phosphate buffer;
Curve 2, compound 2 with GSH, no buffer;
Curve 3, compound 2 with buffer, no GSH;
Curve 4, compound 2 only, no buffer, no GSH.

513 nm). In the absence of buffer, the reaction is much slower and the curve 2 fluorescence linearly increases with time. The source shutter was closed and reopened during the experiment to determine that the linear intensity change was not due to a photochemical reaction but solely to the reaction between GSH and the maleimide. The rate determining step in this reaction is the formation of the thiolate anion of GSH which is *p*H dependent. In the presence of buffer but without GSH (curve 3) there is no fluorescence increase and the response is the same as for curve 4.

The extinction coefficient is also an important factor in the sensitivity of a fluorophore. In fact, the product of the extinction coefficient and the quantum yield ($\epsilon * \phi$) is used as a rule of thumb for comparing relative sensitivities. On this basis, we can show that **2** is twice as sensitives as DACM and is of comparable sensitivity to CPM for detection of thiols. But the thiol adduct of **2** has the advantage of longer wavelength emission. In addition, because of its large Stokes' shift, **2** would be a better substitute for fluorescein maleimide. In summary, our new thiol reagent should find use in applications where a large Stokes' shift and long wavelength fluorescence are important.

EXPERIMENTAL

The ir spectra were recorded on an IBM FT/32 Fourier transform infrared instrument. The ^{1}H nmr spectra were obtained either on a Varian XL-300 spectrometer or on a Varian EM360A spectrometer. The uv spectra were run on a Hitachi U-2000 spectrophotometer. Fluorescence spectra were obtained on a Hitachi F-3010 Spectrofluorometer fitted with a red sensitive Hamamatzu R928F photomultiplier tube and controlled by SpectraCalc software (Galactic Industries Corp., Salem, NH). Fluorescence emission spectra were corrected for instrument response using Oxazine-725 (Exciton, Inc., Dayton, OH) and the method of Kopf and Heinze [16]. Fluorescence quantum yields were determined by the method of Parker and Rees [17] using quinine sulfate as a standard. The IUPAC numbering system used in naming the compounds and making the ^{1}H nmr assignments is shown below.



3*H*-Naphtho[2,1-*b*]pyran-3-one

2-Carbomethoxy-9-hydroxy-3*H*-naphtho[2,1-*b*]pyran-3-one (**4**).

To a suspension of **3** (42.21 g, 0.224 mole) in 100 ml of ethanol were added dimethyl malonate (31.07 g, 0.235 mole) and piperidine (2.5 ml). The mixture was refluxed overnight and cooled. The yellow precipitate formed was filtered and washed with ethanol and water. Recrystallization from dimethylformamide and methanol afforded **4** in 57% yield, mp 270.5-271.5°; ir (potassium bromide): 3376, 1755, 1717, 1566, 1516, 1256, 1206 cm^{-1}; ^{1}H nmr (DMSO-d_6): δ 9.08 (s, 1H, H_1), 8.13 (d, 1H, H_6 or H_7), 7.89 (d, 1H, H_6 or H_7), 7.62 (d, 1H, H_{10}), 7.26 (d, 1H, H_5), 7.17 (d of d, 1H, H_8), 3.90 (s, 3H, OCH_3).

Anal. Calcd. for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73. Found: C, 66.62; H, 3.77.

2-Carbomethoxy-9-hydroxy-10-nitro-3*H*-naphtho[2,1-*b*]pyran-3-one (**5**).

To a suspension of **4** (26.76 g, 0.099 mole) in 200 ml of glacial acetic acid was added concentrated nitric acid (7.5 ml, 0.119 mole). After stirring at room temperature for six hours, the reaction was quenched by addition of 800 ml of water, The orange precipitate was filtered and washed with water until the wash was neutral. Drying the precipitate in a vacuum oven overnight gave 28.52 g (91% yield) of **5**. Recrystallization from dimethylformamide gave an analytically pure sample, mp 247-249°; ir (potassium bromide): 3252, 1763, 1725, 1698, 1562, 1512, 1265 cm^{-1}; ^{1}H nmr (DMSO-d_6): δ 8.59 (s, 1H, H_1), 8.34 (d, 1H, H_6 or H_7), 8.16 (d, 1H, H_6 or H_7), 7.50 (d, 1H, H_5 or H_8), 7.43 (d, 1H, H_5 or H_8), 3.85 (s, 3H, OCH_3).

Anal. Calcd. for $C_{15}H_9NO_7$: C, 57.15; H, 2.88; N, 4.44. Found: C, 57.25; H, 2.92; N, 4.51.

2-Carbomethoxy-9-methoxy-10-nitro-3*H*-naphtho[2,1-*b*]pyran-3-one (**6**).

To a suspension of **5** (9.46 g, 0.030 mole) in 300 ml of anhydrous acetone were added dimethyl sulfate (3.78 g, 0.030 mole) and potassium carbonate (8.29 g, 0.060 mole). The reaction mixture was refluxed under nitrogen overnight, cooled and filtered. The light brown precipitate was washed with 0.1 *N* sodium hydroxide and water, and dried in a vacuum oven. Recrystallization from dimethylformamide yielded 6.35 g (64%) of **6**, mp 314-314.5°; ir (potassium bromide): 1771, 1557, 1524, 1262, 1237 cm^{-1}; ^{1}H nmr (DMSO-d_6): δ 8.63 (s, 1H, H_1), 8.47 (d, 1H, H_6 or H_7), 8.41 (d, 1H, H_6 or H_7), 7.84 (d, 1H, H_5 or H_8), 7.63 (d, 1H, H_5 or H_8), 4.10 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3).

Anal. Calcd. for $C_{16}H_{11}NO_7$: C, 58.37; H, 3.37; N, 4.25. Found: C, 58.26; H, 3.39; N, 4.23.

10-Amino-2-carbomethoxy-9-methoxy-3*H*-naphtho[2,1-*b*]pyran-3-one (**7**).

Compound **6** (5.78 g, 0.0176 mole) and 10% palladium/carbon (1.916 g, 0.0018 mole) were suspended in 200 ml of dimethylformamide and stirred under hydrogen overnight. After removing dimethylformamide on a rotary evaporator, the residue was stirred well in chloroform and filtered. The filter cake was washed with chloroform repeatedly until the filtrate was pale yellow. The solvent was removed from the combined filtrates to afford crude **7**. This sample was used directly in the next reaction.

N-(2-carbomethoxy-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-10-yl)maleimide (**2**).

Chloroform (350 ml) and maleic anhydride (3.45 g, 0.0352 mole) were added to the flask containing **7**. After refluxing for twenty hours, the solvent was removed. The brown syrup was then dissolved in methylene chloride. The yellow precipitate which formed after stirring for a few minutes was filtered, washed with methylene chloride and dried. This material was then mixed with acetic anhydride (90 ml) and sodium acetate (0.38 g, 0.0046 mole). The reaction mixture was refluxed for one hour, cooled and added dropwise to 300 ml of cold water with vigorous stirring. The precipitate was filtered, washed with water and dissolved in methylene chloride. The organic solution was washed

with aqueous potassium hydroxide and water, and dried over magnesium sulfate. Removal of solvent afforded 3.03 g (45% yield from **6**) of **2**. An analytical sample was prepared by twice recrystallizing from ethanol, mp 243-244°; ir (potassium bromide): 1748, 1717, 1700, 1555, 1265 cm^{-1}; ^1H nmr (deuteriochloroform): δ 9.16 (s, 1H, H_1), 7.92 (d, 1H, H_6 or H_7), 7.78 (d, 1H, H_6 or H_7), 7.26 (d, 1H, H_5 or H_8), 7.12 (d, 1H, H_5 or H_8), 6.98 (s, 2H, maleimide H), 3.88 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3).

Anal. Calcd. for $C_{20}H_{13}NO_7$: C, 63.33; H, 3.45; N, 3.69. Found: C, 63.20; H, 3.47; N, 3.65.

Acknowledgement.

This work was partially supported by NIH Grant No. 1 R43 GM38982-01.

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