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The 3-maleimidocoumarin BACM (**2d**) is a new tag designed to react with thiols in biological materials to form fluorescent derivatives whose emission is in the 500 nm (green) region with good resistance to fading. The fluorescence properties of its *N*-acetyl-L-cysteine derivative are described. BACM itself is non-fluorescent, as is its predecessor, DACM (**1a**). A synthesis of BACM is reported which avoids the formation of nitrocoumarin isomers obtained in the reported synthesis of DACM. The synthesis of the related 3-(4-maleimido-phenyl)coumarin is also described.

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Fluorescent stains containing functional groups reactive towards amino or thiol functions in biological substances are being used increasingly in both research and clinical assays. In research a wide range of disease-causing microorganisms has been identified, localized and quantified by fluorescent-antibody staining. In clinical assays, the technique is used in screening tests for the detection of syphilis, lupus, rheumatoid arthritis, myasthenia gravis, Herpes Simplex viruses and streptococcal infections [1].

A common requirement for an ideal fluorophore is that the fluorogenic group should have a fluorescence emission maximum (Fl max) at longer wavelengths than 480 nm, in order to bypass the background fluorescence of many biological materials, which is "blue" (430-470 nm) in appearance [2]. Although the presently used fluorophore, fluorescein, as its isothiocyanate (FITC), does meet this criterion, since the Fl max of its conjugate with gamma globulin is 525 nm [3], it suffers from some serious drawbacks such as (1) concentration quenching because it has a small Stokes shift [4,5], (2) its fluorescence is sensitive to pH, to the point where the quantum yield of fluorescence falls below 1% at pH values found in some regions of biological cells [6] and (3) it is photochemically reactive, decomposes rapidly, and its fluorescence fades and/or changes from green to blue, forcing rapid and error-prone judgements on the appearance of slide preparations. Slides cannot be stored and reexamined later.

Machida *et al.* [8] have reported that *N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide ("DACM", **1a**), itself non-fluorescent, is a useful reagent for tagging thiols in biological materials to form fluorescent derivatives whose emission is in the 500 nm (green) region, and have good resistance to fading, a large Stokes' shift, and a high extinction coefficient [9]. However, Jones *et al.* [10] have reported that the fluorescence quantum efficiency (FQE) and photochemical stability of 4-substituted coumarin dyes related to DACM, **1b** and **1c**, are sharply reduced in polar solvents when the 7-amino substituents are free to rotate, while the coumarins **2b** and **2c** with rigidized 7-amino groups maintained their high quantum yield and

photochemical stability in such solvents, as is desirable for biological tags. The 4-methyl group reduces the photochemical stability of coumarins, while 3-aryl groups enhance it [32].

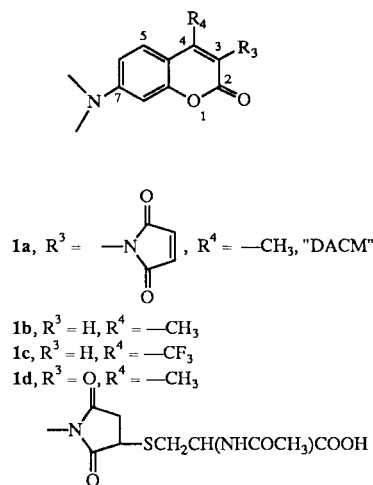
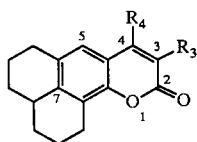


Figure 1. Structures of Coumarins with Freely-Rotating 7-Amino Substituent

Developments in the field of laser dyes demonstrated that a certain series of coumarins with rigidized 7-amino groups including **2a**, **2e** and **2g**, when exposed to xenon lamp flashes in polar solvents, exhibited at least 2,500 times the stability of fluorescein, 1,000 times the stability of 7-diethylamino-4-methylcoumarin (the fluorophore of DACM), and 50 times that of rhodamine 6G [7]. In an effort to prepare a fluorescent probe devoid of the disadvantages of either fluorescein or DACM, we synthesized a pair of 3-arylcoumarins containing the fluorophores in **2a** and **2e**, respectively: "*N*-(6-Butterflyaminocoumarin-3-yl)maleimide" (BACM, **2d**) and its 3-phenyl analog **2f**, whose thiol derivatives would be expected to show a bathochromic shift of 14 nm and equally high quantum yield (95%), based on the values for the parent compounds [7].



2a, $R^3 = R^4 = H$

2b, $R^3 = H, R^4 = -CH_3$

2c, $R^3 = H, R^4 = -CF_3$

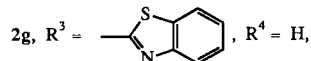
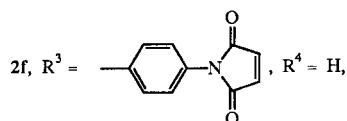
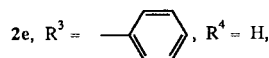
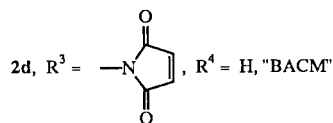
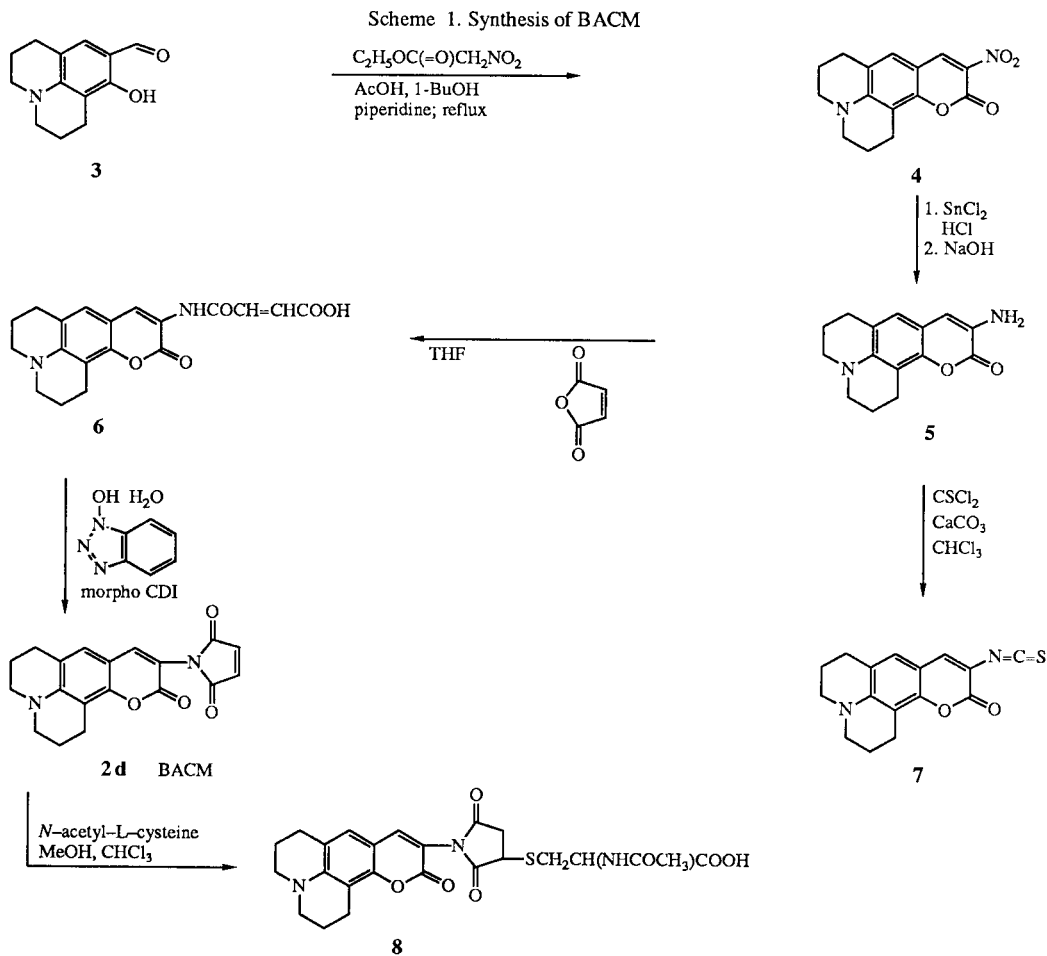


Figure 2. Structures of Coumarins with Rigidized 7-Amino Substituent

Syntheses.

The key intermediate in our synthesis (Scheme 1) is the 3-nitrocoumarin derivative **4**. With few exceptions, 3-nitrocoumarins have always been prepared by nitration. However, only 4-hydroxy- and 4-aminocoumarins are sufficiently activated in the 3-position of the pyran ring to give high yields of the 3-nitro isomers [12,13]. The fluorescent coumarins usually have 7-amino or 7-hydroxy substituents [14] which activate the carbocyclic ring towards nitration, so that clean 3-nitro derivatives are not obtainable. For example, 7-hydroxycoumarin gave a 73% yield of 3,6,8-trinitrocoumarin [15]. The ethyl carbamate of 7-amino-4-methylcoumarin gave the 6-nitro isomer as the major product [16]. In the synthesis of DACM (**1a**) from 7-dimethylamino-4-methyl-3-aminocoumarin, nitration of the 7-dimethylamino-4-methylcoumarin gave a mixture of the 3-, 6-, and 8-isomers, which had to be separated chromatographically. Furthermore, the desired 3-nitro isomer was obtained in a rather low yield (30%) [17]. Dauzonne and Royer found that condensation of methyl nitroacetate with salicylaldehyde in toluene, catalyzed by an equimolar amount of diethylammonium chloride, gave good yields of 3-nitrocoumarins [18]. These researchers obtained 3-nitrocouma-



rin with an electron releasing substituent (methoxy) in the 7-position, in 65% yield in 96 hours, with collection of water by means of a Dean and Stark trap. They later reported that 3-nitrobenzo[*f*] and 3-nitrobenzo[*h*]coumarins could be prepared in the same manner [19]. None of their compounds contained 7-amino or 7-hydroxy substituents. We found independently at about the same time that condensation of ethyl nitroacetate with the 7-aminosalicylaldehyde **3** in 1-butanol, catalyzed by about 1 mol% piperidinium acetate, gave an 84% yield of the 3-nitrocoumarin compound **4** in 43 hours, when the by-product water was stripped by allowing the refluxing solvent to pass through 4 Å Molecular Sieve (hence driving the reaction forward) in a Soxhlet extractor. Earlier work had demonstrated the effectiveness of Molecular Sieve in shortening reaction times for the formation of the coumarin **2g**. Without Molecular Sieve a 61% yield of crude material was obtained after 96 hours of reflux; with it 62% was obtained after 4 hours reflux at the same temperature.

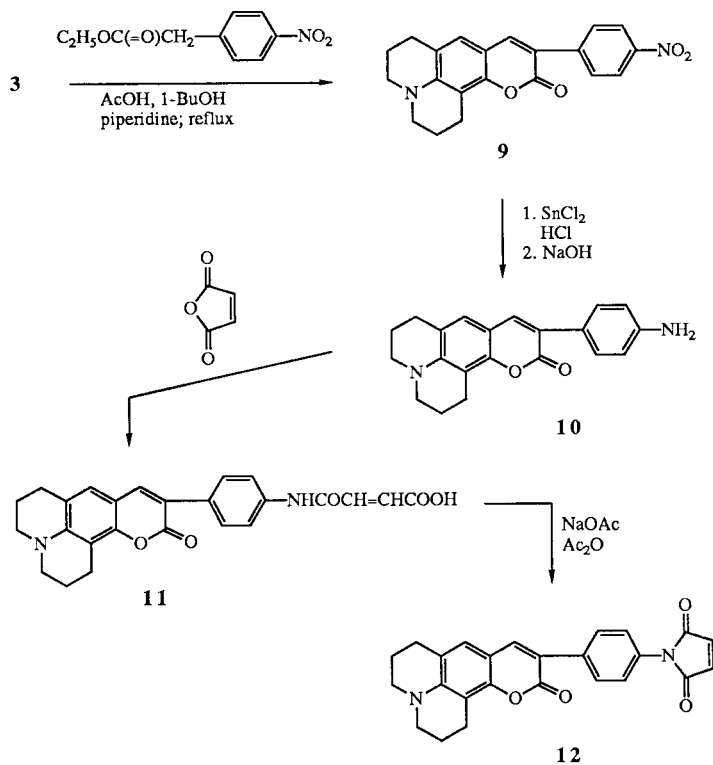
Reduction of the 3-nitro group to the 3-aminocoumarin **5** was accomplished with tin(II) chloride in 12 *M* hydrochloric acid in 93% yield. We note that 3-aminocoumarin has been reported recently by hydrolysis of 3-acetamidocoumarin, itself prepared by condensation of *N*-acetylglycine with salicylaldehyde [20]. Reaction of compound **5** with maleic anhydride, in a manner analogous to that of Machida *et al.* [17], gave the corresponding maleamic acid

derivative **6**.

Cyclization of **6** to give the target compound **2d** was first attempted by the analogous method of Machida *et al.* [17]. However, this method did not give a good result, owing to side reactions (probably due to the higher reactivity of the maleimide under the reaction conditions) to give polymers, and/or due to the formation of isomaleimide [21]. Therefore, another method of cyclization, analogous to that of Trammer and Hendrick [21] was tried with the following modifications: (a) a water soluble form of *N,N'*-dicyclohexylcarbodiimide (DCC), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate (morpho CDI) was used instead of DCC itself. The advantage of using morpho CDI instead of DCC is that both the quaternary salt and the urea co-product were freely soluble in water and in dilute acid [22]. This greatly facilitated the separation of the desired maleimide **2d** from the urea derivative as well as from excess of any unreacted coupling reagent; and (b) a 4-mol-equivalent amount of morpho CDI was used instead of 1.1 in the analogous reaction by Trammer and Hendrick [21]. Reaction using 1.1, 2.5 and 3.5 mol-equivalent of this coupling reagent gave a relatively poor yield (34% in case of 3.5 mol-equivalent) of the maleimide.

A small amount of *N*-acetyl-L-cysteinyl derivative **8** was also synthesized for the purpose of studying fluorescent characteristics of BACM. The synthetic route (Scheme 1)

Scheme 2. Synthesis of 3-Phenylcoumarins



involving Michael type condensation was developed in our laboratory.

Our attempt to synthesize the 3-phenylmaleimide derivative **12**, (Scheme 2) by cyclization of the 3-phenylmaleamic acid **11** following the method of Machida *et al.* [17] or by the modified method of Trammer and Hendrick [21] as used for **2d** above, was unsuccessful, as we were unable to get a clean product. Therefore, another method of cyclization analogous to that of Cava *et al.* [23] was tried using acetic anhydride and sodium acetate. A reasonably high reaction temperature (80°) and presence of acetate is reported to prevent the formation of any isomaleimide [21,24] in this method. The infrared spectrum of the maleimide **12** was similar to that of the corresponding maleimide derivative **2d**, however, no satisfactory elemental analysis for C and N were obtained for this compound. Also, a clean proton magnetic resonance spectrum of this product could not be obtained. However, experimental details have been provided for guidance of others.

Fluorescent Studies.

Like DACM, BACM does not fluoresce by itself. Their *N*-acetyl-L-cysteinyl derivatives **1d** and **8** are strongly fluorescent (Table 1), in agreement with an empirical observation about maleimides and their thiol derivatives [25]. The BACM derivative had a somewhat higher FQE in ethanol and much higher FQE in phosphate buffer, following Jones' [10] observations for coumarins with rotating and rigidized 7-amino groups. The emission peak of the BACM derivative was bathochromically shifted 37 nm from that of DACM to 510 nm, a bright green which resisted fading for at least a one month exposure to daylight (in a 1:1::water:2-propanol solution).

Table 1

Fluorescence Characteristics of the *N*-Acetyl-L-cysteine derivatives of DACM (**1d**) and BACM (**8**)

Compound No.	Solvent	Ex [a]	Em [b]	FQE [c]
1d [d]	ethanol	390 nm	457 nm	67%
1d [d]	0.1 M phosphate buffer, pH 7.0	390	477	11
8	95% ethanol	412	489	82 [e]
8	0.005 M phosphate buffer, pH 7.5	412	510	65 [e]

[a] Excitation maximum. [b] Emission maximum. [c] Fluorescence quantum efficiency. [d] Values from Machida *et al.* [8]. [e] Determined by the dilute solution method [31] where the reference compound was Coumarin 510 (dye **8** in [7]) in 95% ethanol and its FQE was taken as 85%.

BACM was used successfully to label the protein actin. The analogous isothiocyanate **7** ("BACITC") was prepared by a conventional method from the amine **5** with thiophosgene and calcium carbonate in chloroform, but was not well-characterized. Both compounds were judged

too insoluble in water for convenient use - their only major fault [26,34]. Since the 3-phenyl analog **12** containing 6 additional carbons would be even less soluble in aqueous media, work with it was not pursued. In our view, preparation of an ionic or zwitterionic analog of one of the rigidized coumarins or 4-arylpyridines [33] would be a logical step to overcome this problem.

EXPERIMENTAL

Melting points were determined in capillary tubes in a Thomas Hoover apparatus and needed no correction. The tlc were obtained on Whatman MK6F silica gel plates. The uv spectra were obtained on a Beckman DB-GT grating spectrophotometer or on a Cary 15 spectrophotometer. The ir spectra were obtained on a Beckman IR-4230 spectrophotometer. The ¹H nmr spectra were obtained on a JEOL FX 90Q, 90 MHz or on an IBM NR-80, 80 MHz spectrometer, using tetramethylsilane as internal standard, and the chemical shifts are reported in ppm. The Chemical Abstracts numbering used in naming the coumarins and in making ¹H nmr assignments is shown in Figure 3. Fluorescence spectra were obtained with a Perkin-Elmer MPF-44A spectrometer (operated in ratio mode with Rhodamine B in the reference compartment) in conjunction with a Hitachi corrected spectra unit. Elemental microanalyses were done by Microanalysis Inc., Wilmington, Delaware.

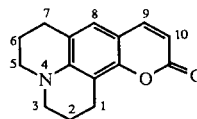


Figure 3. Chemical Abstracts Numbering of Rigidized Coumarins

10-Nitro-2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i*,*j*]quinolizine (**4**).

To a 100 ml, one-necked, round-bottomed flask, equipped with a Soxhlet extractor with a 27 x 80 mm thimble containing 7 g of Molecular Sieve, 4 Å and a reflux condenser, was added 4.76 g (35.8 mmoles) of ethyl nitroacetate, 50 ml of 1-butanol, 7.77 g (35.8 mmoles) of 9-formyl-8-julolidinol (**3**) [27], 0.25 ml of acetic acid and 0.50 ml of piperidine. The reaction mixture was heated at reflux so as to extract Molecular Sieve for 43 hours. The extract on cooling to room temperature gave a royal purple solid that was collected on a filter, washed with 50 ml methanol, and dried in a vacuum oven (80°/20 torr/2 hours) to yield 8.55 g (84%) of **4**, mp 250-251°. A small amount of this compound was recrystallized from dimethylformamide:toluene to give an analytical sample, mp 249.5-250.5°; R_f = 0.48 (ethyl acetate); ir (potassium bromide): ν max 1702 (C=O), 1600, 1560 (NO₂), 1345 (NO₂) and 1240 cm⁻¹.

Anal. Calcd. for C₁₅H₁₄N₂O₄: C, 62.93; H, 4.93; N, 9.79. Found: C, 62.81; H, 4.59; N, 9.94.

10-Amino-2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i*,*j*]quinolizine (**5**).

Into a 250 ml round-bottomed flask equipped with a powerful magnetic stirrer, were placed, in order, 19.6 g (87.0 mmoles) of

stannous chloride dihydrate and 39.2 ml (492 mmoles) of hydrochloric acid. To the stirred mixture was then added 3.50 g (12.0 mmoles) of the nitro compound **4** in small amounts, over a period of 30 minutes. Stirring was continued for 3.5 days. The solution so obtained was then diluted with 200 ml of water and cooled to about 15° on an ice-water bath. To the cooled solution was then added 120 ml of sodium hydroxide (6 M) in small portions with vigorous stirring below 15°, resulting in an off-white precipitate. The precipitate was filtered, washed with 2 x 25 ml of water, and dried under vacuum at 80°/30 torr for 5 hours to give 4.55 g of a dry yellow powder. The crude product was extracted with 140 ml of toluene using a Soxhlet extractor. The extract was allowed to cool to room temperature and the solvent was evaporated on a rotary evaporator to give a brown-red crystalline product. The crystals were dried in vacuum oven at 80°/30 torr for 1.5 hours to give 2.82 g (90%) of the desired amino derivative **5**, mp 123-125°; Rf = 0.79 (ethyl acetate); ir (potassium bromide): ν max 3500-3230 (NH₂), 1685 (C=O), 1608, 1308 and 765 cm⁻¹; ¹H nmr (2%, deuteriochloroform): δ 1.80-2.27 (4H, m, 3-CH₂ and 6-CH₂), 2.62-2.99 (4H, m, 4-CH₂ and 7-CH₂), 3.22 (4H, t, J = 6.1 Hz, 2-CH₂ and 5-CH₂), 3.72-4.25 (2H, br s, deuterium oxide exchangeable, NH₂), 6.65 (1H, s, 9-H) and 6.73 (1H, s, 8-H).

Anal. Calcd. for C₁₅H₁₆N₂O₂: C, 70.29; H, 6.29; N, 10.92. Found: C, 70.34; H, 6.02; N, 10.67.

N-(2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]quinolzin-10-yl)maleamic Acid (**6**).

Into a 100 ml round-bottomed flask were placed 2.5 g (9.8 mmoles) of the 3-aminocoumarin derivative **5** and 35 ml of tetrahydrofuran. Magnetic stirring was begun and the solution was cooled to 10-12° on an ice-water bath. To the cooled, stirred solution was added a solution of 1.24 g (5.0 mmoles) of maleic anhydride [28] in 8 ml of tetrahydrofuran. Color of the reaction mixture changed to deep orange-red almost immediately, and a precipitate started to appear within 8 minutes. Stirring was continued for 4 hours, and the orange-red crystalline product so obtained was collected on a filter, washed with 2 x 10 ml of tetrahydrofuran, and dried in a vacuum oven (80°/30 torr/3 hours) to give 3.15 g (91%) of **6**, mp 226-229° [29]; Rf = 0.61 (ethyl acetate:acetic acid::4:1); ir (potassium bromide): ν max 3600-3100 (OH), 1715 (C=O), 1675 (C=O), 1610, 1585 and 850 cm⁻¹; neutralization equivalent, Required: 354.4. Found: 352.1; ¹H nmr (2%, DMSO-*d*₆): δ 1.60-2.05 (4H, m, 3-CH₂ and 6-CH₂), 2.30-2.85 (4H, m, 4-CH₂ and 7-CH₂), 3.25 (4H, t, J = 6.1 Hz, 2-CH₂ and 5-CH₂), 3.45-3.85 (1H, br s, NH), 6.40 (1H, d, J = 9.3 Hz, =CH-), 6.60 (1H, d, J = 9.3 Hz, =CH-), 7.08 (1H, s, 8-H), 8.45 (1H, s, 9-H) and 10.21 (1H, br s, COOH).

Anal. Calcd. for C₁₉H₁₈N₂O₅: C, 64.34; H, 5.12, N, 7.90. Found: C, 64.17; H, 5.02; N, 7.63.

N-(2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]quinolizin-10-yl)maleimide (**2d**, BACM).

Into a 100 ml round-bottomed flask were added 0.35 g (1.0 mmoles) of the maleamic acid derivative **6** and 25 ml of dichloromethane. Stirring was started and the reaction mixture was cooled to 2° on an ice-water bath. To this stirring, cooled suspension, was added in order, 0.13 g (1.0 mmoles) of 1-hydroxybenzotriazole hydrate and 1.67 g (4.0 mmoles) of morpho CDI. After 40 hours of stirring, the reaction mixture was diluted with 30 ml of dichloromethane and extracted successively with 2 x 70 ml of water, 1 x 40 ml of 1 N hydrochloric acid and 1 x 40 ml of potassi-

um hydrogen carbonate (1 N). The organic phase was dried (sodium sulfate) and the solvent was evaporated on a rotary evaporator to give a brown viscous residue. Further drying of this residue in a vacuum oven (70°/30 torr/2.5 hours) resulted in 0.24 g of crude maleimide as a fluffy, yellow-brown solid. Purification by column chromatography over silica-gel (60-200 mesh, 30 g, eluent ethyl acetate and chloroform, 4:1) gave 0.14 g (44%) of **2d** in an analytically pure form, mp 204-207° [29]; Rf = 0.58 (ethyl acetate: chloroform, 4:1); uv (dichloromethane): λ max nm (ϵ) 414 (2.6 x 10⁴) and 250 (1.2 x 10⁴); ir (potassium bromide): ν max 3050, 1777 (C=O), 1710 (C=O), 1615 and 765 cm⁻¹; ¹H nmr (2%, DMSO-*d*₆): δ 1.70-2.05 (4H, m, 3-CH₂ and 6-CH₂), 2.57-2.82 (4H, m, 4-CH₂ and 7-CH₂), 3.11 (4H, t, J = 6.1 Hz, 2-CH₂ and 5-CH₂), 4.95 (2H, s, CH=CH), 6.60 (1H, s, 8-H) and 6.78 (1H, s, 9-H).

Anal. Calcd. for C₁₉H₁₆N₂O₄: C, 67.84; H, 4.80; N, 8.32. Found: C, 67.92; H, 5.06; N, 8.04.

S-(*N*-(2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]10-quinolizinyl)-3-succinimido)-*N*-acetyl-L-cysteine (**8**).

To a stirred solution of 0.1 g (0.3 mmole) of the maleimide derivative **2d** in 10 ml of chloroform under nitrogen was added 0.09 g (0.6 mmole) of *N*-acetyl-L-cysteine dissolved in 12 ml of methanol. The reaction mixture was heated at reflux for 6 hours and allowed to cool to room temperature. The solvents were evaporated on a rotary evaporator to give an yellow-orange product, which was dissolved in 25 ml of chloroform and extracted with 5 x 30 ml of sodium hydrogen carbonate (2%). The aqueous extract was cooled to 2-3° on an ice-water bath, and the free acid was precipitated by slow addition of 5% hydrochloric acid until pH = 2. The precipitate was cooled overnight at -15°, collected on a filter, and dried under vacuum (70°/30 torr/3 hours) to give 0.07 g (50%) of compound **8** as a bright, yellow crystalline solid, mp 195-205° dec; Rf 0.35 (ethyl acetate:acetic acid, 4:1); uv (methanol): λ max nm (ϵ) 412 (2.8 x 10⁴) and 264 (1.1 x 10⁴); ir (potassium bromide): ν max 3620-3120 (OH), 1788 (C=O), 1720 (C=O), 1685 (C=O), 1625 and 765 cm⁻¹; see also note [30].

10-(4-Nitrophenyl)-2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]quinolizine (**9**).

This compound was prepared in 86% yield by condensation of 9-formyl-8-julolidinol (**3**) with ethyl *p*-nitrophenylacetate essentially as described for compound **4** above; mp 277-280°; Rf = 0.72 (ethyl acetate); uv (dichloromethane): λ max nm (ϵ) 456 (4.96 x 10⁴) and 228 (2.28 x 10⁴); ir (potassium bromide): ν max 2910, 2815, 1675 (C=O), 1560 (NO₂) 1320 (NO₂), 1295 and 835 cm⁻¹; ¹H nmr (5%, deuteriochloroform): δ 1.82-2.11 (4H, m, 3-CH₂ and 6-CH₂), 2.68-3.05 (4H, m, 4-CH₂ and 7-CH₂), 3.22 (4H, t, J = 5.8 Hz, 2-CH₂ and 5-CH₂), 6.93 (1H, s, 8-H), 7.75 (1H, s, 9-H), 7.92 (2H, d, J = 8.6 Hz, 2',6' ArH) and 8.22 (2H, d, J = 8.6 Hz, 3',5' ArH).

Anal. Calcd. for C₂₁H₁₈N₂O₄: C, 69.60; H, 5.00; N, 7.73. Found: C, 69.85; H, 5.22; N, 7.93.

10-(4-Aminophenyl)-2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]quinolizine (**10**).

This compound was prepared in 93% yield by reduction of the nitro compound **9** as described for compound **5** above; mp 214.5-216.5°; Rf = 0.68 (ethyl acetate); uv (ethanol): λ max nm (ϵ) 418 (4.88 x 10⁴) and 278 (2.52 x 10⁴); ir (potassium bromide): ν max 3440-3280 (NH₂), 2925, 1682 (C=O), 1600 and 825 cm⁻¹; ¹H nmr (2%, deuteriochloroform): δ 1.80-2.20 (4H, m, 3-CH₂ and

6-CH₂), 2.62-3.00 (4H, m, 4-CH₂ and 7-CH₂), 3.25 (4H, t, J = 5.8 Hz, 2-CH₂ and 5-CH₂), 3.50-3.83 (2H, br s, deuterium oxide exchangeable, NH₂), 6.68 (2H, d, J = 8.2 Hz, 3',5' ArH), 6.84 (1H, s, 8-H), 7.49 (2H, d, J = 8.2 Hz, 2',6' ArH) and 7.50 (1H, s, 9-H).

Anal. Calcd. for C₂₁H₂₀N₂O₂: C, 75.88; H, 6.06; N, 8.42. Found: C, 75.91; H, 6.18; N, 8.15.

N-(*p*-{2,3,6,7-Tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]quinolizin-10-yl}phenyl)maleamic Acid (**II**).

This compound was obtained in 87% yield by condensing the amino compound **10** with maleic anhydride in a manner as described for compound **6** above, mp 228-230° [29]; Rf = 0.45 (ethyl acetate:acetic acid, 5:0.5); uv (dichloromethane): λ max nm (ε) 432 (5.12 × 10⁴) and 232 (3.44 × 10⁴); ir (potassium bromide): ν max 3600-3200 (OH), 2935, 1708 (C=O), 1668 (C=O), 1608, 1585 and 835 cm⁻¹; neutralization equivalent, Required: 430.5. Found: 438.5; ¹H nmr (2%, DMSO-*d*₆): δ 1.63-2.05 (4H, m, 3-CH₂ and 6-CH₂), 2.40-2.83 (4H, m, 4-CH₂ and 7-CH₂), 3.25 (4H, t, J = 5.4 Hz, 2-CH₂ and 5-CH₂), 3.42-4.25 (1H, br s, deuterium oxide exchangeable, NH), 6.32 (1H, d, J = 8.2 Hz, -CH=C), 6.42 (1H, s, J = 8.2 Hz, -CH=C), 7.05 (1H, s, 8-H), 7.55 (2H, d, J = 11.5 Hz, 3',5' ArH), 7.65 (1H, s, 9-H), 7.80 (2H, d, J = 11.5 Hz, 2',6' ArH) and 10.90-11.10 (1H, br s, deuterium oxide exchangeable, COOH).

Anal. Calcd. for C₂₅H₂₂N₂O₅: C, 69.76; H, 5.15; N, 6.51. Found: C, 70.04; H, 5.14. N, 6.28.

Attempted synthesis of *N*-(*p*-{2,3,6,7-tetrahydro-11-oxo-1*H*,5*H*,11*H*-benzo[*b*]pyrano[6,7,8-*i,j*]quinolizin-10-yl}phenyl)maleimide (**12**).

Into a 25 ml Erlenmeyer flask were placed 3.74 ml (3.6 mmoles) of acetic anhydride and 0.09 g (1.0 mmole) of anhydrous sodium sulfate. The reaction mixture was heated on a steam-bath for 10 minutes. Into the flask was then introduced 1.0 g (2.3 mmoles) of compound **11**. The resulting suspension was dissolved by swirling and heating on the steam-bath for about 5 minutes. The reaction mixture was allowed to cool to room temperature, diluted with 20 ml of ice-water, and stirred with a glass-rod until the suspension solidified. The brown precipitate thus obtained was collected on a filter, washed with 2 × 10 ml of petroleum ether (30-60°) and dissolved in 20 ml chloroform. The unreacted acid was neutralized by adding 20 ml of 0.5 *M* sodium carbonate monohydrate, in small portions with continuous stirring. The chloroform layer was collected, dried (sodium sulfate) and filtered. To the filtrate was then added 500 ml of petroleum ether (30-60°) resulting in the reprecipitation of a yellow solid residue. The crude residue was collected on a filter, washed with 2 × 20 ml of petroleum ether (30-60°), dried in a vacuum oven (70°/30 torr/4 hours) to give 0.79 g of a solid compound, mp 205-207°. Recrystallization from chloroform/methanol gave 0.36 g (40%) of product **12**, mp 206-208°; uv (dichloromethane): λ max nm (ε) 422 (4.04 × 10⁴) 278 (1.6 × 10⁴) and 232 (3.2 × 10⁴); ir (potassium bromide): ν max 1765 and 1690 (C=O), 1672 (C=O), 1602, 1500 and 820 cm⁻¹.

Anal. Calcd. for C₂₅H₂₀N₂O₄: C, 72.80; H, 4.88; N, 6.79. Found: C, 72.07; H, 5.06; N, 6.28.

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REFERENCES AND NOTES

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- [28] The commercial grade maleic anhydride obtained from Eastman was found to be impure. It was recrystallized by extracting 20 g with 350 ml of 1,1,2-trichloro-1,1,2-trifluoroethane in a Soxhlet extractor for 24 hours. The extract on cooling to room temperature gave 17.4 g of pure maleic anhydride, mp 54.5-55.5°.

[29] The mp of the compound was taken by dipping the capillary into a pre-heated bath at 217°, since a slow heating of the capillary resulted in polymerization of the compound at about 200°.

[30] This product failed to give a satisfactory elemental analysis, probably due to its unstable nature, as judged by change in color from an initial bright-yellow to a dull brown, at room temperature, within 4-5 days.

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[34] BACM is now available from Polysciences, Inc., 400 Valley Rd, Warrington, PA 18976.