Synthesis and Characterisation of Pure Isomers of Iodoacetamidotetramethylrhodamine†

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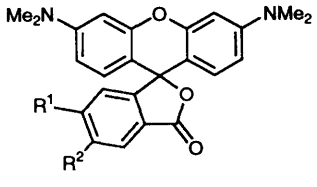
The isomeric benzoylnitrobenzoate esters **5** and **6**, prepared by condensation of 4-nitrophthalic anhydride and 3-(dimethylamino)phenol followed by esterification, were separated by fractional crystallisation and their structures assigned by NOE difference spectroscopy. Reduction of the nitro group in each compound followed by acetylation and ester hydrolysis gave the isomeric acetamido acids **7** and **8**, which were efficiently condensed with 3-(dimethylamino)phenol in the presence of trimethylsilyl polyphosphate to give the acetamidorhodamines **9** and **10**, respectively. These compounds were converted by standard means into the pure 6- and 5-(iodoacetamido)tetramethylrhodamines **1** and **2**. The visible spectroscopic properties of the compounds were examined and accurate extinction coefficients determined.

Rhodamine dyes are used extensively in various aspects of fluorescence microscopy because of the brightness and resistance to photobleaching of the rhodamine fluorophore. These properties are also valuable in more detailed biophysical studies aimed at probing changes in orientation and mobility of proteins using fluorescence anisotropy measurements. For many such studies it is necessary covalently to attach the fluorescent dye to a protein molecule and, when site-selective labelling is required, a common strategy is to use an iodoacetamidorhodamine which reacts selectively with cysteine side-chains.

Iodoacetamidotetramethylrhodamine (IATR) as normally prepared occurs as a mixture of the 6- and 5-substituted isomers **1** and **2** and has been available only from commercial sources without published details of isomeric composition, chemical purity or synthetic route. This lack of information has led to errors and irreproducibility in published work. For example, in papers from the early 1980s the molar extinction coefficients of IATR at its visible absorption maximum, either as the free dye [1] or conjugated to a protein,[2] were estimated to be 23 000 and 24 000 $dm^3\ mol^{-1}\ cm^{-1}$, respectively. We show below that the true value is over 4-fold higher, which implies that the purity of these early samples was < 25%. In recent work [3] using materials claimed (without published evidence) to be the individual isomers **1** and **2**, which were also obtained from a commercial source, the extinction coefficients at 555 nm were reported to be 69 000 and 79 000 $dm^3\ mol^{-1}\ cm^{-1}$ for the isomers **1** and **2**,



1 $R^1 = ICH_2CONH$, $R^2 = H$

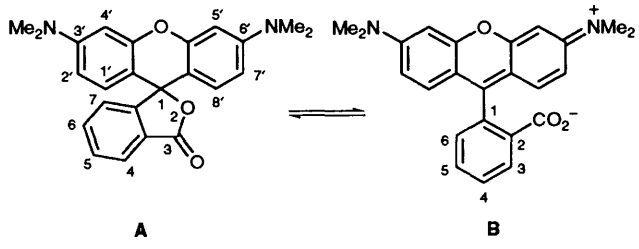
2 $R^1 = H$, $R^2 = ICH_2CONH$

respectively. This paper regrettably contains internal contradictions since the extinction coefficients of the two isomers given in spectra shown graphically are substantially lower than the values quoted in the text. No evidence of homogeneity is given and the purity of these samples is also open to question.

The lack of structural and synthetic information for these rhodamine derivatives is particularly surprising in view of the long known structures of corresponding substituted fluoresceins, which derive from the separation [4] and characterisation [5] of the isomeric 5- and 6-nitrofluorescein diacetates. We became interested in the isomers of IATR through their use to label thiol groups in muscle proteins for fluorescence anisotropy studies of actomyosin crossbridges [6,7] and the present paper details the unambiguous synthesis of each of the isomers of IATR in high purity, and their characterisation.

Results and Discussion

For consistency, rhodamines are shown and named throughout in the spirolactone form **A**, although we recognise that under most conditions they exist in the open-chain form **B** as shown below, together with the numbering system used. The existence of the two valence-bond tautomeric forms of rhodamines is a source of ambiguity and they can be found indexed as derivatives of either the **A** or **B** form. This is



A **B**

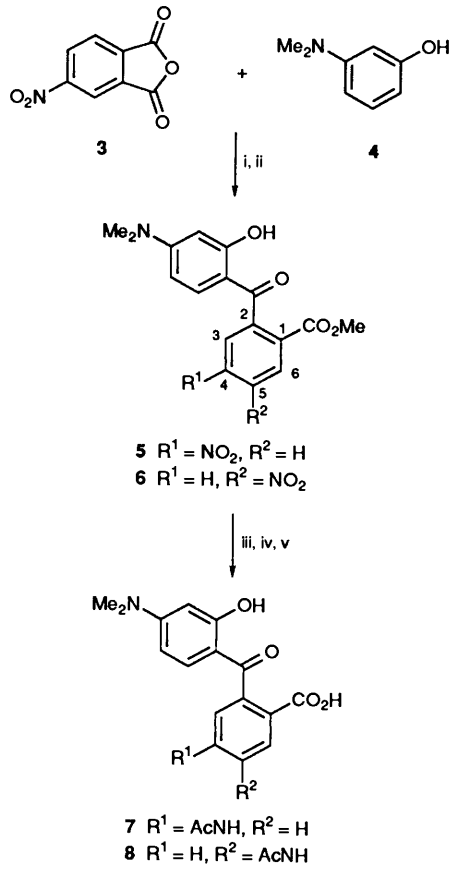
Tautomerism of rhodamines

particularly confusing when substituted derivatives such as the isomers **1** and **2**, numbered as 6- and 5- according to the system for form **A**, are related to open-chain form **B**, in which they would properly be numbered 5- and 4- respectively.

Synthesis of rhodamines involves overall condensation of 2 molecules of a *meta*-aminophenol with a phthalic anhydride. The first acylation of the aminophenol by the anhydride requires no catalyst and leads easily to a substituted benzoylbenzoic acid. Thus 4-nitrophthalic anhydride **3** (prepared from commercial 4-nitrophthalic acid, which was purified from contaminating 3-substituted isomer by published procedures) [8,9] and an equimolar quantity of 3-(dimethylamino)phenol **4** were heated together in toluene and the

† Part of this work is the subject of UK Patent Appl. 9320019.4, filed 28th September 1993.

product was esterified with MeOH–H_2SO_4 to give a mixture of nitro esters **5** and **6** (Scheme 1), in approximately equal



Scheme 1 *Reagents and conditions:* i, heat in toluene; ii, MeOH–H_2SO_4; iii, H_2–Pd on C; iv, Ac_2O–py; v, NaOH–aq. MeOH

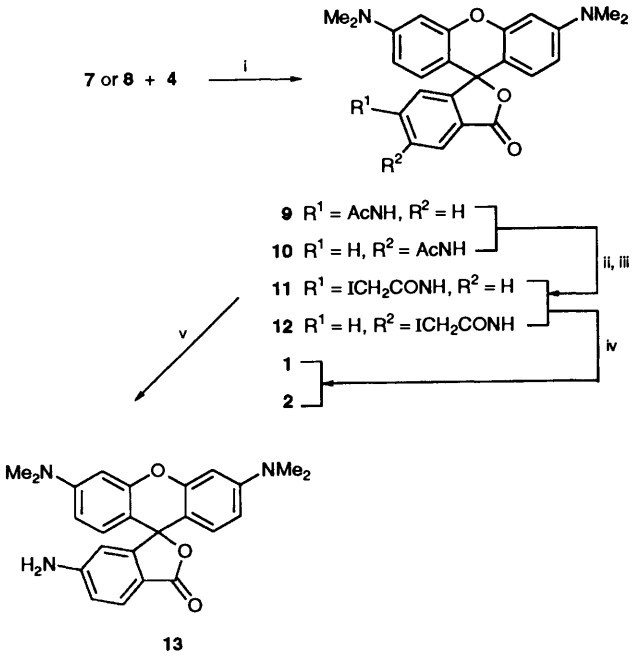
proportions. The two isomers were separated by fractional crystallisation which, although laborious and rather inefficient (see Experimental section), was able to produce sufficient quantities of pure materials to carry forward to give useful quantities of pure IATR isomers. With the individual isomers **5** and **6** in hand it was necessary to assign the structures unambiguously. Simple calculation using incremental chemical shifts[10] suggests that the two compounds should have almost identical ^{1}H NMR spectra, but in fact dramatic differences were evident. Each compound contains two 1,2,4-trisubstituted benzene rings, with one ring bearing 2 electron-donating substituents and the other ring bearing 2 electron-withdrawing substituents. The expected spin systems were therefore readily assigned to the appropriate rings, and in both isomers the signals for the amino-substituted ring appeared at similar chemical-shift values. However, for the nitro-substituted rings, in one isomer the signals were compressed into a narrow range of 0.16 ppm, while for the other isomer they were dispersed over a 1.34 ppm range.

The structures of the isomers **5** and **6** were readily assigned from NOE difference spectra obtained on irradiation of the ester methyl group. Among the low-field signals attributable to protons on the nitro-substituted ring, for compound **5** there was an enhancement of an *ortho*-coupled doublet at δ 8.20 while for compound **6** a *meta*-coupled doublet at δ 8.90 was specifically enhanced. These data are consistent only with placement of the nitro groups of compounds **5** and **6** at the 4- and 5-position respectively. For each compound, the difference spectra also showed enhancements for the phenolic hydroxy group and the *ortho*-coupled 6-proton of the phenolic ring. These additional enhancements indicate that the two compounds each exist in twisted conformations, in which the electron-rich ring and hydrogen-bonded inter-ring carbonyl group lie in one plane, with the nitro-substituted ring orthogonal to this plane. Such a conformation places the 3-proton of the nitro-ring in the shielding cone of the inter-ring carbonyl, which explains the very different ^{1}H NMR spectra of compounds **5** and **6**. Thus, for compound **5** the most deshielded proton would be expected to be 3-H which lies between the nitro and carbonyl substituents. In the orthogonal conformation, this signal is moved upfield into the region of the other two signals arising from this ring. For compound **6**, 3-H would be expected to be the least deshielded, and the anisotropic shielding moves its resonance even further upfield, which gives rise to the wide dispersion of the signals from protons on the nitro-substituted ring. Identical considerations apply to the interpretation of the ^{1}H NMR spectra of subsequent pairs of compounds throughout this work, since in the rhodamine derivatives the xanthylium ring also exerts a shielding effect on the 7-proton (numbered according to the convention outlined above).

With the structures of the isomeric nitro compounds **5** and **6** definitively assigned, their conversion into the required rhodamine derivatives was expected to be straightforward. However, initial experiments in which the nitro compound **5** or its corresponding carboxylic acid, obtained by mild alkaline hydrolysis, were treated with 1 mole equivalent of 3-(dimethylamino)phenol **4** in the presence of Lewis acid catalysts gave complex reaction mixtures which contained poor amounts of rhodamine chromophore, together with other highly coloured species. Although reactions of this type have previously been described to give nitro-substituted rhodamines,[11] no details of yield or purity were reported. We suspected that the complexity of the product mix arose in part from readily occurring oxidation of 3-(dimethylamino)phenol **4** by the nitro group present in the reaction mixture. Accordingly, the nitro groups of compounds **5** and **6** were hydrogenated over Pd–C and the amine products were immediately acetylated with acetic anhydride–pyridine. Alkaline hydrolysis of the crude products gave the acetamido acids **7** and **8** (Scheme 1).

Trial condensations of the acids **7** and **8** with 3-(dimethylamino)phenol **4** in the presence of a variety of protic or Lewis acids gave cleaner reaction products than the previous attempts using the nitro compound **5** or its corresponding free acid (see above) but the yields of rhodamine product were still disappointingly low. However, when trimethylsilyl polyphosphate[12] in dimethylformamide (DMF) was used, the reaction mixtures were much cleaner and from the reaction using the acid **8** the 5-acetamidorhodamine **10** was obtained in 81% crude yield (Scheme 2). Trimethylsilyl polyphosphate has been used previously as a combined Lewis acid and dehydrating agent (see ref. 13 and references therein) and has been particularly recommended to promote intramolecular Friedel–Crafts reactions.[14] The crude 5-acetamidorhodamine **10** was hydrolysed with hot HCl–HOAc and the crude amine was treated with chloroacetyl chloride–DMF. After chromatography, the 5-(chloroacetamido)rhodamine **12** was obtained in 53% overall purified yield from the acid **8**. The 6-chloroacetamido product **11** was obtained by the same procedure in 19% overall yield from the acid **7**. Losses during chromatography were consistently higher in preparations of the 6-chloroacetamido compound. However, only this single chromatographic purification was required in the complete synthesis of each pure IATR isomer **1** and **2**.

Completion of the synthesis required only exchange of iodide for chloride in compounds **11** and **12**. Conventional conditions with a solution of sodium iodide in acetone could not be used here because of the very limited solubility of the two chloro-



Scheme 2 *Reagents:* i, trimethylsilyl polyphosphate–DMF; ii, aq. HCl–HOAc; iii, $ClCH_2COCl$–DMF; iv, NaI–MeOH; v, aq. HCl–EtOH

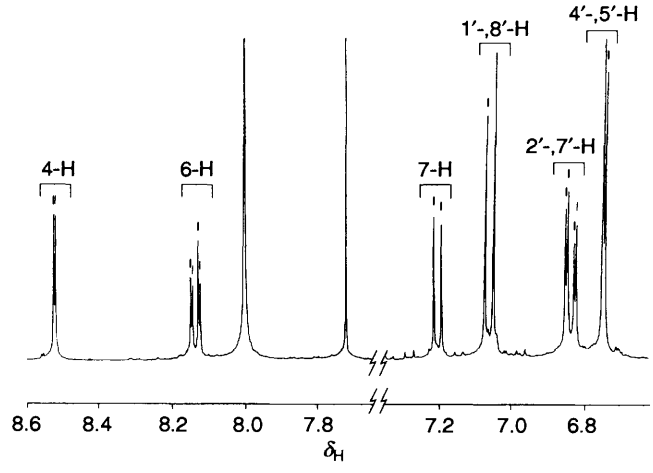
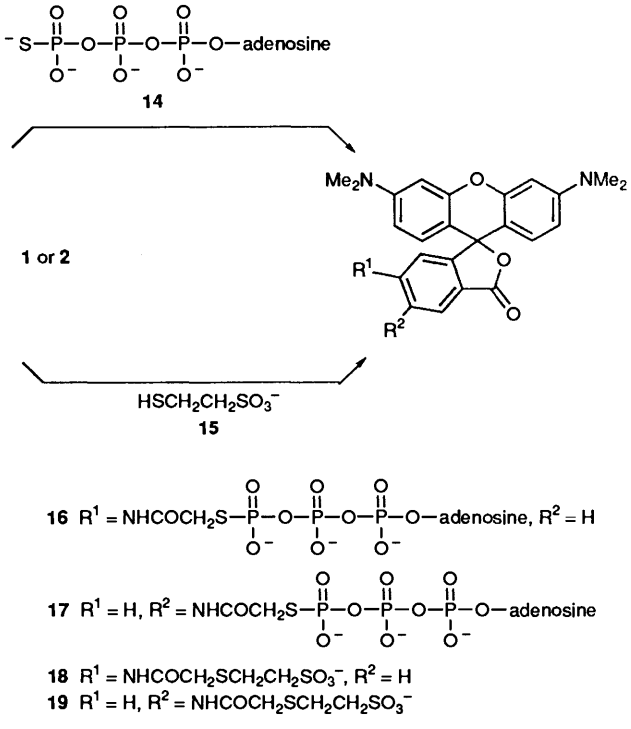


Fig. 1 Partial ^1H NMR spectrum of 5-(iodoacetamido)tetramethylrhodamine **2**. The signals at δ 8.0 and 7.7 are from residual DMF and $CHCl_3$ in the NMR solvents.

acetamides in this solvent. However, the exchange could be effected in methanol, although the rate was much slower than for similar reactions in acetone, and a minimum time of 70 h at room temperature was required to ensure complete reaction. The IATR isomers **1** and **2** were recovered from this reaction in 65–70% yield, with an estimated purity >95% as judged from the ^1H NMR spectrum. As an example, Fig. 1 shows the aromatic region of the ^1H NMR spectrum of the 5-IATR isomer **2**; complete details are reported in the Experimental section. We note that the NMR spectra of the rhodamine derivatives were run in $[^2H_7]$DMF–$CDCl_3$ (3:7) which requires brief comment. Preliminary observations with rhodamine B, which is readily soluble in chloroform, had shown that the signals from the xanthene ring protons gave a well resolved, first-order spectrum in this solvent but collapsed to a single broad line in $(CD_3)_2SO$ ($[^2H_6]$DMSO) or trifluoroacetic acid (TFA). Since the best possible spectral characterisation of the IATR reagents and their precursors was required, we needed to retain the full information content of the spectra, but the IATR compounds were insufficiently soluble in $CDCl_3$ to achieve this. Trials with rhodamine B showed that $[^2H_6]$DMSO–$CDCl_3$ mixtures containing up to 40% $[^2H_6]$DMSO enabled observation of the individual signals from the xanthene protons to be made. However, DMSO readily caused degradation of iodoacetamido compounds at room temperature. This is unsurprising since DMSO at 25 °C has been reported[15] to oxidise other α-halogenocarbonyl compounds, *e.g.* phenacyl bromide is converted into phenylglyoxal, although the present reaction was clearly more complex. In a model experiment, when *N*-(4-nitrophenyl)iodoacetamide was kept in $[^2H_6]$DMSO solution for 24 h at room temperature, the initial singlet signal in the ^1H NMR spectrum at δ 3.88 from the iodoacetyl group slowly diminished, while new singlet resonances appeared at δ 4.06, 4.34, 4.66, 5.12, 10.30 and 10.88. The signals from the aromatic-ring protons became correspondingly complex. No attempt was made to investigate the products of this reaction, but by using deuteriated DMF as the co-solvent instead of DMSO, the solutions for NMR analysis were found to be stable indefinitely.

With the synthesis of the individual IATR isomers completed, we wished additionally to obtain definitive visible absorption spectra of the compounds, since the difficulty of obtaining pure materials has meant that previous determinations of extinction coefficients have been inaccurate, as discussed above. Solutions of rhodamine dyes are known to exhibit a non-covalent monomer–dimer equilibrium,[16] which results in a concentration dependence of their absorption spectra, hence extinction coefficient data presented without corresponding specification of concentrations are insufficient. Resolved monomer and dimer spectra have been published for rhodamine B and related dyes.[16] The monomer–dimer equilibrium is also relevant to the use of rhodamine dyes for fluorescence measurements since, in aqueous solution, fluorescence is almost totally quenched in the dimer[16,17] and the low residual dimer fluorescence, at least in the case of rhodamine B where it has been studied, occurs at longer wavelength than for the monomer.[17]

Our approach to the problem was to prepare a radioactive derivative of known specific activity, for which concentrations could be determined by radioactive counting. As shown in Scheme 3, the 6- and 5-IATR isomers **1** and **2** were treated with



Scheme 3

[^{35}S]adenosine 5′-*O*-(γ-thiotriphosphate) **14** (ATPγS) to give the rhodamine-thionucleotide conjugates **16** and **17** respectively, which were isolated by anion-exchange HPLC. The [^{35}S]ATPγS itself was purified with the same column and its specific activity was determined from its measured radioactivity and concentration, the latter being found spectroscopically from the known[18] extinction coefficient of adenine nucleotides. The direct experimental determination of the specific activity of the [^{35}S]ATPγS at the same time as the isolation of the rhodamine-thionucleotide conjugates avoids inaccuracies which could arise from the short half-life of ^{35}S and the instability of ATPγS. The latter property did however cause a problem, because in addition to the radioactivity which co-eluted with authentic ATPγS from the HPLC column, there was a substantial peak of activity which eluted very early and which tended to tail into the peaks from the rhodamine-thionucleotide conjugates. From an injection of the [^{35}S]ATPγS alone, fractions were collected with elution volumes identical with those for the conjugates **16** and **17**. In view of their identical charges and very similar structures, these two compounds had surprisingly different elution volumes, with the 6-isomer **16** being the first eluted. The result was that in collections across the peak for each compound, the background radioactivity arising from impurities in the [^{35}S]ATPγS was a high proportion of the total collected for compound **16**, while for compound **17** this background activity was only 3.5–4.5% of the total. Large and variable background corrections meant that concentration measurements based on radioactivity determinations could not reliably be made for compound **16**. However, this error for compound **17** was relatively small and measurements gave an estimate of 96 900 ± 5300 dm^3 mol^{-1} cm^{-1} (SD; $n = 3$) for the molar extinction coefficient at 549 nm. The concentration range at which the measurements were made was 8.7 ± 1.1 μmol dm^{-3} and for the 5-isomer it was shown (see below) that the spectra (corrected for dilution) were concentration independent at and below this range.

To examine the concentration dependence of rhodamine spectra, we used adducts of the IATR isomers with 2-sulfanylethanesulfonate **15** (*i.e.*, compounds **18** and **19**). Thus, concentrated solutions of IATR isomers **1** and **2** were allowed to react with an excess of thiol **15** and the reaction mixtures were then diluted by known factors to give a range of concentrations (~4–400 μmol dm^{-3}). Absorbance data were normalised according to the dilution factors and overlaid. For each isomer this procedure gave a set of spectra with an isosbestic point at 528 nm. For the 5-isomer **19** the normalised spectra were invariant at concentrations below 10 μmol dm^{-3} and we conclude that spectra under these conditions are those of the pure monomer. As discussed above, the molar extinction coefficient at 549 nm for the monomer was determined as 96 900 dm^3 mol^{-1} cm^{-1} and from this value the derived extinction coefficient at the 528 nm isosbestic point is 52 000 dm^3 mol^{-1} cm^{-1}. The spectra of the 6-isomer **18** showed concentration-dependent behaviour down to the lowest concentration at which we could make reliable measurements (~1 μmol dm^{-3}) and it was therefore not possible to make an experimental estimate of the extinction coefficient at the isosbestic point. However, since the qualitative behaviour is similar to that for the 5-isomer **19** and the wavelength of the isosbestic point is identical, it seems reasonable that the same value should be used for the molar extinction coefficient of each isomer at this wavelength. The value obtained here for the extinction coefficient at the isosbestic point is similar to the value of ~57 000 dm^3 mol^{-1} cm^{-1} reported for rhodamine B in aqueous solution.[16] It is interesting that our data show a greater tendency to dimer formation for the 6-substituted rhodamine.

Finally, in our study of these spectral properties we examined briefly the effects of salt concentration and pH. Identical dilutions of compounds **18** and **19** were made into ammonium phosphate buffers at pH 7.2 supplemented with NaCl over the range 0–200 mmol dm^{-3}. No effect on the spectra was observed. Similarly, these spectra were identical with those of compounds **18** and **19** at the same concentrations in the pH 6.0 buffer used for HPLC, in which the spectra of the radioactive conjugates **16** and **17** were measured (see above), thereby confirming that these latter measurements could validly be used for solutions at neutral pH.

Although these observations concluded our initial aims, we also examined briefly the preparation of the pure 6-amino compound **13**, since this could be used to prepare other protein-reactive derivatives such as the isothiocyanate or maleimide in isomerically pure form. Insufficient material was available to prepare the corresponding 5-amino compound, but the details are recorded here as a guide to possible future work. Initial attempts directed simply to chromatographic purification of the amine after acid-catalysed hydrolysis of the acetamide **9** were unsatisfactory and we chose instead to purify the acetamide **9**. It was then found that the acetamide could be hydrolysed to the amine under much milder conditions (1 mol dm^{-3} aq. HCl–EtOH at reflux) than had been used earlier, and the product amine **13** was obtained in good yield and purity. In the context of the earlier discussion of the ^{1}H NMR spectra of the compounds synthesised here, it is of interest to note the effect on the 7-proton of hydrolysis of the acetamide group. This step replaces a moderately deshielding group by a strongly shielding one. In consequence, the 7-proton signal is moved greatly upfield and appears at higher field than the protons of the xanthene ring, giving an overall dispersion of 1.4 ppm for the signals from the non-xanthene ring. Although the 5-amino compound was not available for experimental measurement, the spectrum of the related 5-aminofluorescein has been published[19] and shows as expected that the signals of the non-xanthene ring protons are spread over a range of only 0.1 ppm. Thus it seems that the situation which occurs in the spectra of the acylaminorhodamines **1**, **2** and **9**–**12** and in their precursor nitro and acetamido compounds **5**–**8** is reversed in the amino-substituted rhodamines.

Preliminary results of fluorescence anisotropy studies obtained when the pure IATR isomers were used to label muscle proteins have been published as abstracts[20] and show significant differences between the two isomers. Full accounts will be published in due course.

Experimental

Analyses were carried out by the Chemical Analysis Centre, University of Kent, Canterbury. Low-resolution FAB mass spectra were determined on a VG 70-250SE instrument. NMR spectra were, unless otherwise stated, determined in $CDCl_3$ on JEOL FX90Q or Bruker WM400 spectrometers with tetramethylsilane as the internal standard. *J* Values are given in Hz. Visible spectra of rhodamine derivatives to determine isosbestic points were recorded on a Cary 3E spectrophotometer. Other UV–VIS measurements were made on a Beckman DU70 instrument. Merck 9385 silica gel was used for flash chromatography. Anion-exchange HPLC was performed using equipment from Waters Associates on a Whatman Partisphere SAX column (Cat. No. 4621-0505), with 0.17 mol dm^{-3} $NH_4H_2PO_4$ (adjusted to pH 6.0 with NaOH) plus 2.5% acetonitrile (v/v) as mobile phase at 1.5 cm^3 min^{-1} and UV detection at 254 nm. Trimethylsilyl polyphosphate was from Fluka, Gillingham, Dorset. Light petroleum was the fraction boiling at 40–60 °C. 3-(Dimethylamino)phenol **4** was purified by vacuum distillation, b.p. 112 °C (2 mmHg). Triethylammonium hydrogen carbonate buffer (TEAB) was prepared by bubbling CO_2 into 1 mol dm^{-3} aq. triethylamine at 4 °C until

the pH stabilised at ~7.4. Adenosine 5'-*O*-(γ-thiotriphosphate) (ATPγS) was purchased from Boehringer Mannheim UK, Lewes, Sussex, and was purified by chromatography on a DEAE-cellulose column (2 × 45 cm) eluted with a linear gradient of TEAB (400–1000 mmol dm^{-3}, total volume 2 dm^3). Pooled fractions containing ATPγS were evaporated under reduced pressure and freed from residual buffer salts by repeated evaporation of methanol under reduced pressure. [^{35}S]ATPγS was purchased from Amersham International, Amersham, Bucks (initial specific activity 22 TBq $mmol^{-1}$). Readysafe™ scintillation cocktail (Beckman, Fullerton, California), was used for counting radioactive samples on a Beckman LS6000SC scintillation counter. Procedures involving rhodamines were performed under subdued light. Organic extracts were dried over anhydrous sodium sulfate.

4-*Nitrophthalic Anhydride* **3**.—This compound was prepared by modifications of published procedures.[1,9] Thus, a solution of commercial 80% 4-nitrophthalic acid (50 g, 237 mmol) and conc. sulfuric acid (28 cm^3) in methanol (1000 cm^3) was heated under reflux for 8 h. The methanol was removed under reduced pressure and the residue was diluted in diethyl ether (500 cm^3), washed successively with water (3 × 250 cm^3) and saturated aq. $NaHCO_3$ (4 × 200 cm^3), dried, and evaporated under reduced pressure to give dimethyl 4-nitrophthalate as a yellow solid (36 g).

A suspension of this diester (26.9 g, 112 mmol) in aq. NaOH (2.75 mol dm^{-3}; 45 cm^3) was heated under reflux for 1 h, cooled, and acidified below pH 2 with conc. nitric acid. The resulting suspension was extracted with diethyl ether (2 × 100 cm^3; previously washed with 1 mol dm^{-3} aq. NaOH to remove any ethanol) and the combined extracts were dried. The extract was allowed to evaporate to dryness overnight at atmospheric pressure to give 4-nitrophthalic acid as a solid (22. 4 g, 100%). A sample was crystallised from ethyl acetate–light petroleum as pink needles, m.p. 164–165 °C (lit.,[21] 165 °C).

A suspension of 4-nitrophthalic acid (20.2 g, 81 mmol) in acetic anhydride (17 cm^3) was heated for 1 h at 60 °C, then under reflux for 10 min. The mixture was allowed to cool, then was ground in a mortar with dry light petroleum (2 × 80 cm^3). The precipitated solid was filtered off, and dried *in vacuo* to give the anhydride **3** (16.6 g, 90%). A sample sublimed *in vacuo* (50 °C, 2 mmHg) gave clear needles, m.p. 122–123 °C (lit.,[21] 119 °C); δ_H(90 MHz) 8.24 (1 H, d, $J_{5,6}$ 8, 6-H), 8.79 (1 H, dd, $J_{3,5}$ 2, 5-H) and 8.83 (1 H, d, 3-H).

Methyl 2-(4'-*Dimethylamino*-2'-*hydroxybenzoyl*)-4- *and* -5-*nitrobenzoate* **5** *and* **6**.—A solution of 4-nitrophthalic anhydride **3** (28.95 g, 150 mmol) and redistilled 3-(dimethylamino)phenol **4** (21.25 g, 155 mmol) in dry toluene (500 cm^3) was heated under reflux for 6 h and cooled. The toluene was evaporated off under reduced pressure and the residue was dissolved in $CHCl_3$ (500 cm^3), washed successively with dil. aq. HCl (6 × 200 cm^3) and water (200 cm^3), then was extracted with saturated aq. $NaHCO_3$ (6 × 200 cm^3). The combined aqueous extracts were acidified below pH 2 with dil. sulfuric acid and extracted with diethyl ether (3 × 200 cm^3). The combined extracts were washed with water (3 × 200 cm^3) and extracted with saturated aq. $NaHCO_3$ (3 × 200 cm^3), and the aqueous extracts were acidified below pH 2 and extracted with diethyl ether (3 × 200 cm^3). These combined extracts were washed with water (200 cm^3), dried, and evaporated under reduced pressure to leave an orange solid (26.1 g, 53%), which was dissolved in methanol (500 cm^3) containing conc. sulfuric acid (14 cm^3), and the solution was heated under reflux for 8 h and cooled. The methanol was removed under reduced pressure and the residue was dissolved in chloroform (500 cm^3), washed successively with water (300 cm^3) and saturated aq. $NaHCO_3$ (3 × 300 cm^3) and dried. The solvent was evaporated off under reduced pressure to afford the mixed esters **5** and **6** as an orange solid (26.2 g). The solid was crystallised from methanol (1.5 dm^3) to give a yellow-orange solid (15 g). Three further crystallisations from methanol gave the 4-*nitro ester* **5** as yellow needles (3.9 g, 14%), m.p. 177–178 °C (Found: C, 59.3; H, 4.5; N, 8.3. $C_{17}H_{16}N_2O_6$ requires C, 59.3; H, 4.7; N, 8.1%); λ_{max}(EtOH)/nm 259 and 350 (ε/dm^3 mol^{-1} cm^{-1} 11 300 and 23 400); λ_{max}(EtOH–OH^-)/nm 345 (ε/dm^3 mol^{-1} cm^{-1} 15 000); δ_H(400 MHz) 3.07 (6 H, s, NMe_2), 3.78 (3 H, s, OMe), 6.12 (1 H, dd, $J_{5',6'}$ 9 and $J_{3',5'}$ 2.5, 5'-H), 6.19 (1 H, d, 3'-H), 6.85 (1 H, d, 6'-H), 8.20 (1 H, d, $J_{5,6}$ 8.4, 6-H), 8.25 (1 H, d, $J_{3,5}$ 2.2, 3-H), 8.36 (1 H, dd, 5-H) and 12.26 (1 H, s, ArOH).

The mother liquor from the first crystallisation was concentrated by distillation to two-thirds its volume and was cooled on ice. The resulting precipitate, enriched in isomer **6**, was collected by filtration and the process was repeated until the proportion of isomer **5** in the mother liquor was less than 25% (quantified from the signals of the methoxy protons of each isomer in the ^{1}H NMR spectrum). The mother liquor was then evaporated to dryness and the residual orange solid was crystallised four times from ethanol to give the 5-*nitro ester* **6** as orange prisms (0.75 g, 3%), m.p. 164–165 °C (Found: C, 59.2; H, 4.5; N, 8.15%); λ_{max}(EtOH)/nm 258 and 347 (ε/dm^3 mol^{-1} cm^{-1} 10 700 and 23 100); λ_{max}(EtOH–OH^-)/nm 341 (ε/dm^3 mol^{-1} cm^{-1} 18 800); δ_H(400 MHz) 3.07 (6 H, s, NMe_2), 3.81 (3 H, s, OMe), 6.09 (1 H, dd, $J_{5',6'}$ 9.1 and $J_{3',5'}$ 2.4, 5'-H), 6.18 (1 H, d, 3'-H), 6.78 (1 H, d, 6'-H), 7.56 (1 H, d, $J_{3,4}$ 8.3, 3-H), 8.46 (1 H, dd, $J_{4,6}$ 2.2, 4-H), 8.90 (1 H, d, 6-H) and 12.24 (1 H, s, ArOH).

4-*Acetamido*-2-(4'-*dimethylamino*-2'-*hydroxybenzoyl*)*benzoic Acid* **7**.—The 4-nitro ester **5** (2.14 g, 6.26 mmol) was dissolved in acetic acid (600 cm^3) and 5% Pd–C (0.40 g) was added. The mixture was stirred under hydrogen at room temp. and pressure for 5 h, and after disconnection from the hydrogen atmosphere was warmed to 50–60 °C and filtered. The acetic acid was removed under reduced pressure and the residue was suspended in a mixture of pyridine (35 cm^3) and acetic anhydride (35 cm^3) and stirred for 16 h at room temp. The resulting solution was evaporated under reduced pressure and the residue was dissolved in chloroform (130 cm^3), washed successively with 0.2 mol dm^{-3} aq. HCl and water, and concentrated to afford a brown oil, which was dissolved in methanol (40 cm^3). 10% Aqueous NaOH (10 cm^3) was added and the mixture was heated under reflux for 1 h. The methanol was removed under reduced pressure, water (25 cm^3) was added, and the solution was acidified to below pH 2 with 5% aq. sulfuric acid. The precipitate was filtered, washed with water, and dried (50 °C, 2 mmHg) for 24 h to give the *acetamido acid* **7** (1.74 g, 82%), which was crystallised from methanol as brown plates, m.p. 228–230 °C (decomp.) (Found: C, 62.8; H, 5.1; N, 8.0. $C_{18}H_{18}N_2O_5$ requires C, 63.1; H, 5.3; N, 8.2%); λ_{max}(EtOH)/nm 257 and 344 (ε/dm^3 mol^{-1} cm^{-1} 18 000 and 28 900); λ_{max}(EtOH–OH^-)/nm 344 (ε/dm^3 mol^{-1} cm^{-1} 19 700); δ_H{400 MHz; [$^2H_6$]DMSO–$CDCl_3$ (3:7)} 2.13 (3 H, s, MeCO), 3.04 (6 H, s, NMe_2), 6.09 (2 H, m, 5'- and 3'-H), 6.92 (1 H, d, $J_{5',6'}$ 9.5, 6'-H), 7.69 (1 H, d, $J_{3,5}$ 2.5, 3-H), 7.78 (1 H, d, $J_{5,6}$ 8.5, 6-H) and 7.96 (1 H, dd, 5-H).

5-*Acetamido*-2-(4'-*dimethylamino*-2'-*hydroxybenzoyl*)*benzoic Acid* **8**.—The 5-nitro ester **6** (1.61 g, 4.68 mmol) was reduced, acetylated, and saponified as for the ester **5** to give the *acetamido acid* **8** (1.10 g, 69%), which was crystallised from methanol as brown prisms, m.p. 218–220 °C (decomp.) (Found: C, 61.4; H, 5.8; N, 7.6. $C_{18}H_{18}N_2O_5 \cdot 0.5H_2O$ requires C, 61.5; H, 5.45; N, 8.0%); λ_{max}(EtOH)/nm 346.5 (ε/dm^3 mol^{-1} cm^{-1} 28 400); λ_{max}(EtOH–OH^-)/nm 347.5 (ε/dm^3 mol^{-1} cm^{-1} 18 900);

δ_H[90 MHz; [$^2H_6$]DMSO–$CDCl_3$ (3:7)] 2.13 (3 H, s, MeCO), 3.04 (6 H, s, NMe_2), 6.06 (1 H, dd, $J_{5',6'}$ 9.5 and $J_{3',5'}$ 2.5, 5'-H), 6.11 (1 H, d, 3'-H), 6.95 (1 H, d, 6'-H), 7.17 (1 H, d, $J_{3,4}$ 8.5, 3-H), 7.94 (1 H, dd, $J_{4,6}$ 2, 4-H) and 8.15 (1 H, d, 6-H).

5-Chloroacetamido-3',6'-bis(dimethylamino)spiro[1,3-*dihydroisobenzofuran*-1,9'-*xanthen*]-3-*one* **12**.—A solution of the acid **8** (0.96 g, 2.8 mmol), redistilled 3-(dimethylamino)phenol **4** (800 mg, 5.8 mmol) and trimethylsilyl polyphosphate (5 g) in dry DMF (100 cm^3) was heated at 130 °C for 4 h under nitrogen. The reaction mixture was concentrated under reduced pressure to a small volume (~ 10 cm^3), diluted in 1 mol dm^{-3} aq. NaOH (200 cm^3), stirred vigorously for 5 min, and extracted with chloroform (3 × 150 cm^3). The combined extracts were washed with 1 mol dm^{-3} aq. NaOH (2 × 100 cm^3) and evaporated under reduced pressure. The residue was dissolved in conc. HCl–glacial acetic acid (1:1; 200 cm^3) and heated under reflux for 1 h under nitrogen. The reaction mixture was evaporated to dryness under reduced pressure, diluted with water (100 cm^3), and again evaporated to dryness. The residual solid was dissolved in 2 mol dm^{-3} aq. HCl (200 cm^3), and the solution was washed with chloroform (80 cm^3), basified by addition of solid NaOH and extracted with chloroform (4 × 100 cm^3). The combined extracts were washed with 1 mol dm^{-3} aq. NaOH (2 × 100 cm^3), dried, and evaporated under reduced pressure to afford a purple gum (0.90 g, 81%). A portion (720 mg, 1.82 mmol) was dissolved in dry DMF (50 cm^3), chloroacetyl chloride (0.145 cm^3, 1.83 mmol) was added, and the mixture was heated under nitrogen at 75 °C for 3 h. The reaction mixture was concentrated, diluted in methanol–chloroform (1:1; 100 cm^3), and mixed with silica gel (2 g). The solvent was removed under reduced pressure and the silica gel containing the adsorbed compound was added to the top of a flash chromatography column (250 cm^3 silica gel), which was successively eluted with chloroform (500 cm^3), methanol–chloroform (1:19, 250 cm^3), methanol–chloroform (1:9, 250 cm^3) and methanol–chloroform (1:4; 750 cm^3). The major fraction was further purified by flash chromatography (180 cm^3 silica gel) with successive elution by chloroform (500 cm^3), methanol–chloroform (1:9; 250 cm^3) and methanol–chloroform (1:4; 750 cm^3) to afford the 5-chloroacetamido compound **12** as a purple solid (700 mg, 53%) [Found: (M^+ + H), 478. $C_{26}H_{24}ClN_3O_4$ + H requires *m/z*, 478]; δ_H[400 MHz; [$^2H_7$]DMF–$CDCl_3$ (3:7)] 3.34 (12 H, s, NMe), 4.11 (2 H, s, $ClCH_2CO$), 6.84 (2 H, d, $J_{meta'}$ 2.7, 4'- and 5'-H), 6.97 (2 H, dd, $J_{ortho'}$ 9.4, 2'- and 7'-H), 7.20 (2 H, d, 1'- and 8'-H), 7.22 (1 H, d, $J_{6,7}$ 8.2, 7-H), 8.26 (1 H, dd, $J_{4,6}$ 2, 6-H), 8.77 (1 H, d, 4-H) and 11.40 (1 H, s, NHCO).

6-Chloroacetamido-3',6'-bis(dimethylamino)spiro[1',3'-*dihydroisobenzofuran*-1,9'-*xanthen*]-3-*one* **11**.—The acid **7** (1.167 g, 3.4 mmol), when treated in an identical manner to the acid **8**, afforded the 6-chloroacetamido compound **11** as a purple solid (310 mg, 19%) [Found: (M^+ + H), 478. $C_{26}H_{24}ClN_3O_4$ + H requires *m/z*, 478]; δ_H[400 MHz; [$^2H_7$]DMF–$CDCl_3$ (3:7)] 3.15 (12 H, s, NMe), 4.25 (2 H, s, $ClCH_2CO$), 6.63 (2 H, d, $J_{meta'}$ 2.3, 4'- and 5'-H), 6.67 (2 H, dd, $J_{ortho'}$ 9, 2'- and 7'-H), 6.89 (2 H, d, 1'- and 8'-H), 7.80 (1 H, d, $J_{5,7}$ 1.9, 7-H), 7.92 (1 H, dd, $J_{4,5}$ 8.5, 5-H), 8.05 (1 H, d, 4-H) and 10.96 (1 H, s, NHCO).

5-Iodoacetamido-3',6'-bis(dimethylamino)spiro[1',3'-*dihydroisobenzofuran*-1,9'-*xanthen*]-3-*one* **2**.—A solution of sodium iodide (0.56 g, 3.75 mmol) in methanol (5 cm^3) was deoxygenated by bubbling briefly with nitrogen, then was added to the 5-chloroacetamido compound **12** (50 mg, 0.10 mmol). The solution was kept under nitrogen at room temp. for 72 h, then was diluted with chloroform (250 cm^3) and washed successively with 5% aq. sodium thiosulfate (250 cm^3) and water (2 × 250 cm^3). The chloroform solution was diluted with methanol (250 cm^3), dried, and evaporated under reduced pressure to give the 5-iodoacetamido compound **2** as a purple solid (36 mg, 64%); R_f 0.30 [Whatman MK6F silica gel, $CHCl_3$–MeOH (4:1); developed twice] [Found: (M^+ + H), 570. $C_{26}H_{24}IN_3O_4$ + H requires *m/z*, 570]; δ_H[400 MHz; [$^2H_7$]DMF–$CDCl_3$ (3:7)] 3.20 (12 H, s, NMe), 4.00 (2 H, s, ICH_2CO), 6.69 (2 H, d, $J_{meta'}$ 2.4, 4'- and 5'-H), 6.75 (2 H, dd, $J_{ortho'}$ 9.2, 2'- and 7'-H), 6.97 (2 H, d, 1'- and 8'-H), 7.18 (1 H, d, $J_{6,7}$ 8.3, 7-H), 8.09 (1 H, dd, $J_{4,6}$ 2, 6-H), 8.47 (1 H, d, 4-H) and 10.80 (1 H, s, NHCO).

6-Iodoacetamido-3',6'-bis(dimethylamino)spiro[1',3'-*dihydroisobenzofuran*-1,9'-*xanthen*]-3-*one* **1**.—The 6-chloroacetamido compound **11** (50 mg, 0.10 mmol) was treated as for its isomer **12** to give the 6-iodoacetamido compound **1** as a purple solid (40 mg, 71%); R_f 0.22 [$CHCl_3$–MeOH (4:1); developed twice] [Found: (M^+ + H), 570. $C_{26}H_{24}IN_3O_4$ + H requires *m/z*, 570]; δ_H[400 MHz; [$^2H_7$]DMF–$CDCl_3$ (3:7)] 3.30 (12 H, s, NMe), 4.03 (2 H, s, ICH_2CO), 6.81 (2 H, d, $J_{meta'}$ 2.4, 4'- and 5'-H), 6.93 (2 H, dd, $J_{ortho'}$ 9.4, 2'- and 7'-H), 7.13 (2 H, d, 1'- and 8'-H), 7.89 (1 H, d, $J_{5,7}$ 2, 7-H), 7.96 (1 H, dd, $J_{4,5}$ 8.7, 5-H), 8.21 (1 H, d, 4-H) and 10.87 (1 H, s, NHCO).

Molar Extinction Coefficient of 5-IATR–ATPγS Conjugate **17**.—An aliquot of stock [^{35}S]ATPγS (0.02 cm^3) was added to a solution of ATPγS (20 mmol dm^{-3}; 0.30 cm^3) to give an approximate specific activity of 1.2 GBq mol^{-1}. An aliquot of this solution (0.10 cm^3) was added to a mixture prepared from a ~ 20 mmol dm^{-3} methanolic solution of 5-IATR (0.05 cm^3), water (0.03 cm^3) and DMF (0.02 cm^3) and the mixture was kept at room temp. for 24 h. Aliquots (0.02 cm^3) were subjected to anion-exchange HPLC and 40 s fractions (*i.e.*, 1 cm^3) were collected up to 8 min. Fractions spanning the peak corresponding to conjugate **17** were those eluted between 6 and 8 min, and were analysed by visible spectroscopy and by radioactive counting. For the latter, aliquots (0.10 cm^3) were added to a mixture of Readysafe scintillant (14 cm^3) and water (3 cm^3). Reagent radioactive backgrounds were determined by analogous HPLC fractionation of a blank reaction mixture (*i.e.*, containing [^{35}S]ATPγS but no IATR) and were subtracted from the appropriate counts. From the same injection, fractions containing [^{35}S]ATPγS were collected, and quantitated by UV absorbance at 260 nm (lit.,[18] ε/dm^3 mol^{-1} cm^{-1} 15 300) and by radioactive counting as above. An identical reaction and analysis sequence was performed for 6-IATR **1**. Results are given in the Discussion section.

In analytical HPLC runs, the retention times of the various compounds were: 6-IATR **1**, 2.65 min; 5-IATR **2**, 2.60 min; 6-IATR–ATPγS **16**, 4.20 min; 5-IATR–ATPγS **17**, 6.40 min; and ATPγS **14**, 40.0 min.

Concentration Dependence of Rhodamine Spectra.—Aliquots (0.02 cm^3) of ~ 20 mmol dm^{-3} methanolic solutions of each IATR isomer **1** and **2** were mixed with 200 mmol dm^{-3} aq. sodium 2-sulfanylethanesulfonate (0.08 cm^3), 200 mmol dm^{-3} ammonium phosphate, pH 7.2 (0.35 cm^3) and DMF (0.05 cm^3) and kept at room temp. for 24 h. Aliquots of these solutions were diluted 2-, 10-, 20-, 100- and 200-fold into 25 mmol dm^{-3} ammonium phosphate, pH 7.2, which contained 150 mmol dm^{-3} NaCl. The visible spectra were recorded using cells with path lengths of 10, 1 or 0.105 mm as appropriate to the concentrations, and were processed further as described in the Discussion section to obtain the isosbestic-point data.

6-Acetamido-3',6'-bis(dimethylamino)spiro[1',3'-*dihydroisobenzofuran*-1,9'-*xanthen*]-3-*one* **9**.—The acid **7** (1.2 g) was treated with redistilled 3-(dimethylamino)phenol **4** and

trimethylsilyl polyphosphate, and the crude product was obtained as described above for preparation of the chloroacetamidorhodamine **12**. The crude product was dissolved in methanol–chloroform (1:1; 100 cm^3), mixed with silica gel (4 cm^3), and the solvent was removed under reduced pressure. The silica gel containing the adsorbed compound was added to the top of a flash chromatography column (250 cm^3 silica gel) and was eluted successively with chloroform (500 cm^3), methanol–chloroform (250 cm^3; 1:19), methanol–chloroform (250 cm^3; 1:9) and methanol–chloroform (750 cm^3; 1:4). The major fraction was further purified by a second flash chromatography (180 cm^3 silica gel) with successive elution by chloroform (500 cm^3), methanol–chloroform (250 cm^3; 1:9) and methanol–chloroform (750 cm^3; 1:4) to afford the acetamide **9** as a purple solid (400 mg, 26%) [Found: $(M^+ + H)$, 444. $C_{26}H_{25}N_3O_4$ + H requires *m/z*, 444]; δ_H[400 MHz; [$^2H_7$]DMF–$CDCl_3$ (3:7)] 2.10 (3 H, s, COMe), 3.04 (12 H, s, NMe), 6.50 (2 H, dd, $J_{ortho'}$ 8.5, $J_{meta'}$ 2.7, 2′- and 7′-H), 6.52 (2 H, d, 4′-and 5′-H), 6.72 (2 H, d, 1′- and 8′-H), 7.67 (1 H, d, $J_{5,7}$ 1.5, 7-H), 7.77 (1 H, dd, $J_{4,5}$ 8.9, 5-H), 7.90 (1 H, d, 4-H) and 10.40 (1 H, s, NHCO).

6-Amino-3′,6′-bis(dimethylamino)spiro[1′,3′-dihydroisobenzofuran-1,9′-xanthen]-3-one **13**.—A solution of the acetamide **9** (200 mg, 0.45 mmol) in ethanol (100 cm^3) and 1 mol dm^{-3} aq. HCl (100 cm^3) was heated under reflux for 2 h under nitrogen. The reaction mixture was cooled, diluted with 2 mol dm^{-3} aq. NaOH (200 cm^3), and extracted with chloroform (2 × 100 cm^3). The combined extracts were washed 1 mol dm^{-3} aq. NaOH (2 × 100 cm^3), dried, and concentrated to afford the amine **13** as a purple solid (140 mg, 77%) [Found: $(M^+ + H)$, 402. $C_{24}H_{23}N_3O_3$ + H requires *m/z*, 402); δ_H[400 MHz; [$^2H_7$]DMF–$CDCl_3$ (3:7)] 3.04 (12 H, s, NMe), 6.28 (1 H, d, $J_{5,7}$ 1.7, 7-H), 6.51 (2 H, d, $J_{meta'}$ 2, 4′- and 5′-H), 6.52 (2 H, dd, $J_{ortho'}$ 9.7, 2′- and 7′-H), 6.79 (2 H, d, 1′- and 8′-H), 6.83 (1 H, dd, $J_{4,5}$ 8.2, 5-H) and 7.68 (1 H, d, 4-H).

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