Investigation of enzyme activity by SERRS using poly-functionalised benzotriazole derivatives as enzyme substrates

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New methods of measuring biologically relevant concentrations of enzymes are necessary to allow greater understanding of biological systems. We have previously shown that aryl azo benzotriazolyl alkyl esters can act as enzyme substrates, with the progress of the reaction being monitored using SERRS (see *Nat. Biotechnol.*, 2004, **22**, 1133, ref. 1). This is a wholly novel analytical application of SERRS, and the low detection levels of the technique allow for an ultra-sensitive enzyme assay. Masked enzyme substrates are used that are invisible to SERRS until enzymatic hydrolysis. Turnover of the substrate by the enzyme leads to the release of the surface-seeking dye necessary for SERRS, and intense signals are produced. Here we report an improved synthesis of 2*H*-benzotriazolyl alkyl esters *via* nucleophilic substitution of a chloromethyl ester by benzotriazolyl azo dyes, giving up to a ten-fold increase on previously reported yields. Introduction of electron-withdrawing groups to the benzotriazole ring allows control over the SERRS properties of the compounds. This is of great significance in expanding the synthetic flexibility and subsequently the fundamental use of these compounds as ultra-sensitive and selective reporters of enzyme activity.

Introduction

The uses of benzotriazole bestride a wide range of chemical disciplines. Described as a 'tame halogen', it has been extensively employed as a synthetic auxiliary, and used in a number of elegant syntheses.[2,3] Benzotriazole derivatives are also of use as photostabilisers[4] and anti-corrosion inhibitors.[5] The latter is made possible due to the strong affinity benzotriazole has for metal surfaces, and it is this property that makes benzotriazole and its derivatives excellent analytes for SERS and SERRS.[6–9] The surface-enhanced Raman sensitivity of a benzotriazole moiety is reliant on the capacity of the benzotriazole to bind to a silver or gold surface. Through *N*-substitution of the benzotriazole species we can prevent, wholly or partially, this binding to the metal surface and thus mask the SERRS activity of the compound. Through judicious choice of this *N*-substituent, it is possible to prepare a SERRS-inactive species that can be chemically, or in our case enzymatically cleaved to generate a SERRS-active species. Given the high sensitivity of SERRS,[10] this technique has shown the capacity to monitor enzyme turnover at very low substrate concentrations. We have recently demonstrated that a 2-benzotriazol-2-yl alkyl ester azo dye can act as an enzyme substrate, with the rate of enzymatic reaction being monitored by SERRS.[1]

The synthetic organic utility of benzotriazole has been well reported and there is considerable scope available for *N*-functionalisation of the benzotriazole moiety. As a synthetic auxiliary, it has been used for a number of elegant purposes; in syntheses of ketones,[11] esters,[12] ethers[13] and amides[14] to name only a few. *N*-Alkylated benzotriazole derivatives can be formed from halogen displacement in alkyl halides,[15] hydroxyl displacement in alcohols or alkoxy displacement in acetals.[16,17] Benzotriazole can also add across carbonyls in aldehydes,[18] to imines[19] and also to enamines.[20] For the *N*-alkylation of benzotriazole with alkyl halides, sodium hydroxide in DMF,[15] sodium alkoxide bases,[21,22] sodium hydride bases,[23] micellar reactions[24] and phase transfer conditions[25,26] have all been employed. A mixture of 1*H*, 2*H*, and 3*H* isomers can potentially be formed, although in a symmetrically substituted benzotriazole, the 1*H* and 3*H* isomers will be degenerate. This isomerisation can be affected, and to a certain extent controlled, by a number of factors.[25,27,10]

Isomerisation has proven essential in dictating the extent of observed masking of the SERRS. 1*H* alkyl esters have previously shown considerably higher SERRS than the 2*H* isomers, as illustrated in Fig. 1. This could be governed by a number of factors. It may be a steric hindrance factor or it may be an electronic factor. It is known[28] that 2*H* isomers of benzotriazole are weaker

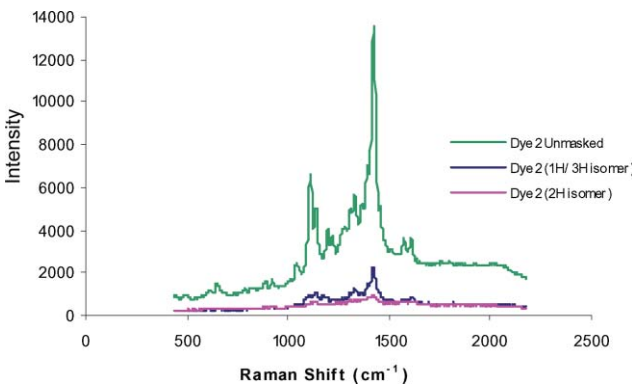

Fig. 1 Superimposed SERRS spectra of dye **2**, the 1*H*/3*H* isomers and the unmasked dye.

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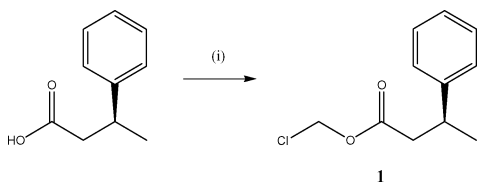
Brønsted bases than their 1*H* counterparts, and it would not be unreasonable to suggest they are also weaker Lewis bases.

From our previous study, the synthesis of benzotriazolyl alkyl esters, as *per* Katritzky *et al.*,[29] yielded a very small amount of the desired 2*H* isomer, with the 1*H* predominant. This method involved acylation of the benzotriazole, then reaction of the *N*-acyl benzotriazole with formaldehyde in the presence of base. We can now report an improved synthetic route to these compounds, with an up to ten-fold increase in yield of the 2*H* isomers. Furthermore, we have now demonstrated that 1*H*-benzotriazolyl alkyl esters can completely mask SERRS by introduction of electron-withdrawing groups to the benzotriazole ring. This opens up the range of substrates that can be used in this approach and provides further understanding on the requirements for the masking of these dyes in terms of SERRS activity.

Results and discussion

2*H*-Benzotriazolyl alkyl ester synthesis

For this study, benzotriazolyl alkyl esters were prepared by nucleophilic substitution of chloromethyl ester **1**, using a sodium benzotriazolate as the nucleophile. A number of factors were found to increase the relative yield of 2*H* isomer; the use of acetone as a solvent, preformation of the benzotriazole anion and anhydrous conditions. Chloromethyl ester **1** was prepared by alkylation of the relevant carboxylic acid using chloromethyl chlorosulfate[30,31] (CMCS), under phase transfer conditions, in yields consistently higher than 90% (Scheme 1).



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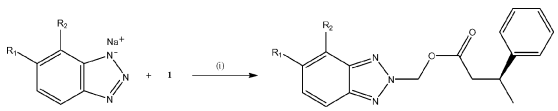
Scheme 1 *Reagents and conditions*: (i) CMCS, $NaHCO_3$, Bu_4NHSO_4, DCM–H_2O.

The starting benzotriazolyl dyes for **2**, **3** and **4** were prepared according to literature procedures.[1,32,33]

The *N*-alkylation of the benzotriazolyl dyes was carried out by reaction of the appropriate sodium benzotriazolate with chloromethyl ester **1**, as outlined in Scheme 2. Acetone was found to be the most effective solvent in terms of overall yield, but acetonitrile, DMF and DMSO were also used with success. NMR analysis of the reactions indicated the presence of all 3 isomers, with the ratio 1 : 2.5 for the 2*H* and combined 1*H* + 3*H* isomers respectively. The 1*H* and 3*H* isomers were not isolated and their structural isomerism not determined, but crude NMR data has been previously obtained.[1] Trace amounts of *N*-acyl benzotriazole were also formed. The yield of benzotriazolyl alkyl ester formed by this route is comparable to the yields reported in literature,[29] but the yield of 2*H* isomer is higher. Outside of our own previous work,[1] 2*H*-benzotriazolyl alkyl esters had only been reported as a trace amount from 1*H* syntheses. Phase transfer conditions, micellar reactions, or organic bases in acetone all led to a reduced proportion of 2*H* isomer. A summary of the compounds prepared and their yields is contained in Table 1.



Scheme 2 *Reagents and conditions*: (i) Acetone.

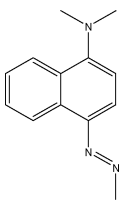
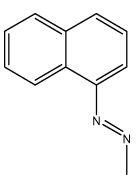
SERRS studies. In the first instance, the masked compounds were interrogated to ascertain that the compounds were indeed SERRS-inactive.

From Fig. 1 it can be seen that the 2*H* isomer of dye **2** gives better masking of SERRS. All spectra recorded were performed using single-scan measurements, 1 second spectral acquisition times with 514.5 nm excitation.

Dye **2** was tested against a range of lipases, which were all succesful in hydrolysing the substrate, but with varying intensity of SERRS response. The results are illustrated in Fig. 2.

It can be seen from Fig. 2 that the *Candida*-derived enzymes seemingly hydrolyse the substrate more rapidly than those from *Pseudomonas*. When dye **3** was tested against the lipase from

Table 1 Summary and yield of 2*H*-benzotriazolyl alkyl esters. Reported yield of 1*H* + 3*H* taken from crude NMR only

Compound	R_1	R_2	Yield of 2*H* (%)	Yield of 1*H* + 3*H* (%)
2		H	21	65
3	NH_2		23	65

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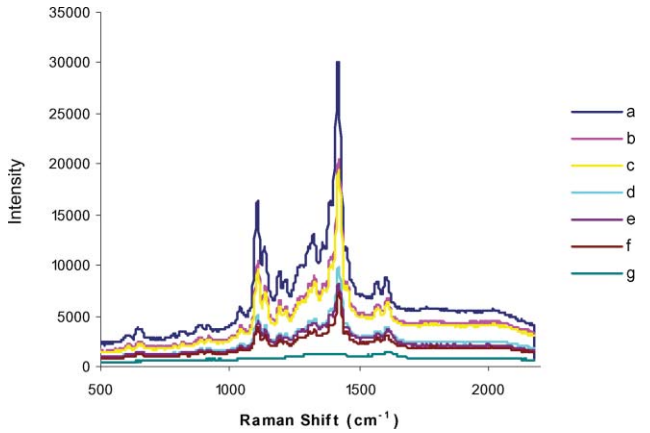


Fig. 2 SERRS spectra of **2** before and after treatment with a selected range of lipases. All experiments were carried out *in situ* in silver colloidal solution, with the substrate present at 1×10^{-7} M and the enzyme at 0.002 mg ml^{-1}. The spectrum was recorded at 1200 seconds after lipase addition. For ease of interpretation the enzymes are listed in decreasing order of size of SERRS response. a) *Candida aspergillus*, b) *Candida cylindracea*, c) *Candida rugosa*, d) *Pseudomonas stutzeri*, e) *Pseudomonas cepacia*, f) *Pseudomonas fluorescens*, g) no enzyme added.

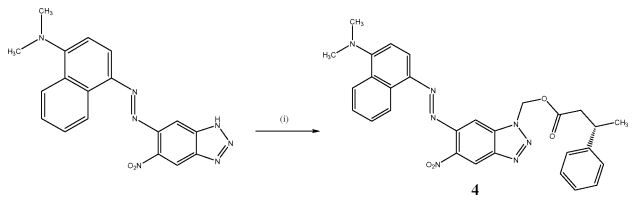
Pseudomonas cepacia, no appreciable turnover could be observed by SERRS. It is suggested that by moving the bulky naphthylazo group from the 5 to the 4 position of the benzotriazole, the ester functionality is hindered from accessing the active site of the enzyme.

Electron-deficient benzotriazolyl alkyl esters

The occurrence of SERRS is dependent on binding to an appropriate metal surface, in this case colloidal silver. As part of the same study, an electron-deficient 3*H*-benzotriazolyl alkyl ester **4** was prepared, to investigate the effect the withdrawing group would have on the binding to silver.[34]

This approach was taken in an attempt to produce masked 1*H*/3*H* substrates that were as well masked to SERRS as the 2*H* compounds. This would allow for simpler chemistry towards our targets, as more synthetic chemistry is known with regards to 1*H* compounds. Accordingly, a nitro group was introduced to the aromatic ring. However, this caused a decrease in nucleophilicity of the benzotriazole anion, and subsequently reaction with the chloromethyl ester as described in Scheme 1 did not occur. Furthermore, the anion was no longer soluble in acetone. A modified alkylation procedure was therefore used, with the chloromethyl ester being converted to the iodomethyl analogue, and DMF used as the solvent. No 2*H* isomer was observed using these conditions, with the 3*H* isomer being isolated in 20% yield, the structure of which was confirmed by X-ray crystallography. The 1*H* isomer was observed by NMR analysis of the crude reaction mixture, in ~20% conversion, but was not isolated to a suitably high degree of purity for characterisation. The synthetic route and yield of the compound are illustrated in Scheme 3.

SERRS studies. Upon interrogation of dye **4**, the SERRS effect is completely masked. The contrast can be seen when the same experiment is carried out with a mixture of 1*H* and 3*H* isomers of **2**, the de-nitro analogue (Fig. 3). Dye **4** gives a similar response to **2** upon treatment with the lipase from *Pseudomonas*



Scheme 3 *Reagents and conditions*: (i) (*S*)-iodomethyl 3-phenylbutanoate, K_2CO_3, DMF, rt, 16% (65% conversion to 1*H*/3*H*).

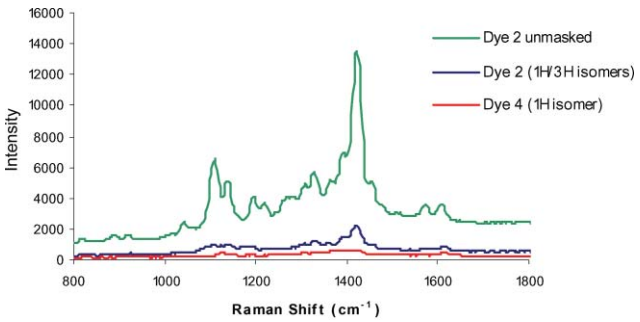


Fig. 3 Superimposed SERRS spectra of 1*H* isomers of **2** and **4**. The electron-deficient benzotriazole **4** gives greater blocking of SERRS.

cepacia, *i.e.* addition of enzyme causes the substrate to become SERRS-active. The parent or "unmasked" dyes of **2** and **4** give similar SERRS spectra, as can be seen in Fig. 4, with the main difference being that a single peak is observed at 1125 cm^{-1} for dye **4**, whereas dye **2** has two bands at 1110 and 1137 cm^{-1}.

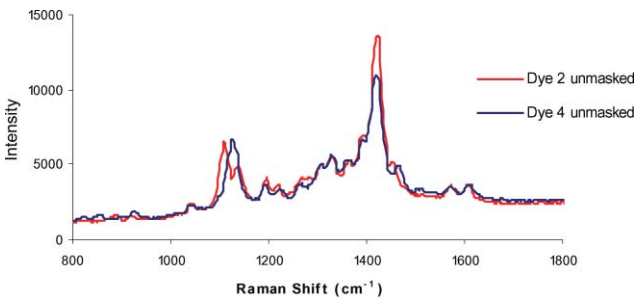


Fig. 4 Superimposed spectra of the 'unmasked' dyes from **2** and **4**.

As has been previously stated, the occurrence of SERRS is dependent on binding to an appropriate metal surface. In the 2*H* compounds discussed above, this metal binding is prevented to a greater extent than with the 1*H* counterparts, thus lower SERRS is obtained. It is proposed that the reduced SERRS from electron-poor benzotriazol-1-yl alkyl esters is because of the inductive effect of the nitro group. The basicity of the triazole ring is reduced, which prevents the binding to the silver surface, and subsequently prevents SERRS to a greater extent than the de-nitro analogue.

Conclusions

A new synthesis of 2*H*-benzotriazolyl alkyl esters has been reported, simplifying the preparation of such compounds and expanding the scope of compounds that can be prepared. The introduction of an electron-withdrawing group to the phenyl ring, in this case a nitro group, has reduced the affinity of 1*H*-benzotriazolyl alkyl esters for the silver surface, and subsequently

the compounds appear to be SERRS-invisible for the first time. The nitro group seemingly does not affect enzyme selectivity. With all 3 isomers from the reaction now being suitable precursors for the SERRS analyses, the yield and efficiency of the reaction is significantly increased.

Experimental

Preparation of solutions for SERRS analysis

We prepared 0.001% wt/vol poly(L-lysine) in distilled water for use as an aggregating agent. Enzyme solutions were prepared at 0.1 mg ml^{-1} in water. Stock solutions of the substrate were prepared in acetonitrile at a concentration of 10^{-3} M, and subsequent dilutions were made using distilled water to a concentration of 1×10^{-6} M.

Raman instrumentation

A Renishaw Mark III probe system with a Spectra Physics 362C, 15 mW, argon ion laser was used for the collection of spectra using an excitation wavelength of 514.5 nm.

Reaction monitoring

All reactions were carried out in disposable plastic cuvettes (300–800 nm transmission range) and analysis was done *in situ*. Silver colloid (500 µl), de-ionised water (500 µl) and poly(L-lysine) (20 µl of 0.001%) were added to a cuvette and allowed to aggregate for 15 min. Enzyme solution (100 µl) and substrate solution (100 µl) were then added to the aggregated colloid, the contents were mixed and the spectra accumulated. A spectral acquisition time of 1 s was used. Further details on experimental conditions can be obtained from our previous publication.[1]

Chemical synthesis

Unless otherwise stated, all chemical precursors were purchased from Sigma. Chloromethyl chlorosulfate was purchased from Acros. The precursor dyes for **2**, **3** and **4** were prepared in accordance with previously published work.[1,32,33]

^{1}H NMR and ^{13}C NMR were recorded on a Bruker DPX 400 MHZ spectrometer with the appropriate solvent peak as a reference. *J* values are quoted in Hertz. Elemental analyses were performed as by the University service using a Perkin–Elmer 240 elemental analyser. Mass spectrometry data was provided by the EPSRC Mass Spectrometry Service Centre, Swansea.

(*S*)-Chloromethyl 3-phenylbutanoate 1. (*S*)-3-Phenylbutyric acid (0.335 g, 2 mmol), sodium bicarbonate (0.840 g, 10 mmol) and tetra-*n*-butylammonium hydrogen sulfate were dissolved in water (20 ml). Dichloromethane (20 ml) was added and the mixture left to stir vigorously at 0 °C for 15 min, at which point chloromethylchlorosulfate (0.5 g, 3 mmol) was added, with continuous overnight stirring at room temperature. The organic phase was separated, washed with brine, dried over sodium sulfate before filtration and removal of the solvent by evaporation. The residue was purified by column chromatography, eluting with 20% ethyl acetate in hexane to afford the title compound as a colourless oil (0.4 g, 94%). (Found: C, 61.88; H, 6.15; $C_{11}H_{13}ClO_2$ requires C, 62.12; H, 6.16); δ_H (400 MHz; $CDCl_3$) 1.33 (3H, d, *J* 6.9, CH_3), 2.61 (1H, d, *J* 6.9, CH), 2.71 (1H, d, *J* 6.9, CH), 3.31 (1H, sept, *J* 6.9, CH), 5.64 (1H, d, *J* 6.8, CH), 5.68 (1H, d, *J* 6.8, CH), 7.20–7.33 (5H, m, ArH); δ_C (100 MHz; $CDCl_3$) 21.9, 36.5, 68.7, 126.9, 128.8, 145.3, 170.5; *m/z* 230.0945 ([M + NH_4]$^+$ $C_{11}H_{17}ClNO_2$ requires 230.0942); $[a]_D^{20}$ +62.2 (*c* 1, MeCN)

3-(*S*)-Phenylbutyric acid 5-(4-dimethylaminonaphthalen-1-ylazo)benzotriazol-2-ylmethyl ester 2. [4-(3*H*-Benzotriazol-5-ylazo)naphthalen-1-yl]dimethylamine (0.250 g, 0.8 mmol) was treated with 1.038 M NaOH solution (0.77 ml, 0.8 mmol). The water was removed at reduced pressure and the residue dissolved in anhydrous acetone (10 ml). (*S*)-Chloromethyl 3-phenylbutanoate (0.170 g, 0.8 mmol) was then added, and the reaction mixture left to stir for 2 h at room temperature. The solvent was then removed at reduced pressure and the residue purified by column chromatography, eluting with 20% ethyl acetate in hexane. The 2*H* isomer elutes more rapidly than the other isomers. The title compound was furnished as a red oil (0.12 g, 21%), and had spectroscopic data consistent with that already published.[1] (Found: C, 70.92; H, 5.75; N, 17.01;.$C_{29}H_{28}N_6O_2$ requires C, 70.71; H, 5.73; N, 17.06); δ_H (400 MHz; $CDCl_3$) 1.30 (3H, d, *J* 7.0, CH_3), 2.69 (1H, d, *J* 6.7, CH), 2.76 (1H, d, *J* 6.7, CH), 3.09 (6H, s, CH_3), 3.31 (1H, sept, *J* 7.3, CH), 6.53 (1H, d, *J* 9.9, CH), 6.59 (1H, d, *J* 9.9, CH), 6.99 (1H, dd, *J* 7.6, ArH), 7.23–7.11 (6H, m, 6 × ArH), 7.61 (1H, dd, *J* 7.0, ArH), 7.68 (1H, dd, *J* 6.9, ArH), 7.98 (2H, d, *J* 9.0, ArH), 8.25 (1H, d, *J* 7.6, ArH), 8.51 (1H, s, ArH), 9.04 (1H, d, *J* 7.6, ArH); *m/z* 493.2344 ([M + H]$^+$. $C_{29}H_{29}N_6O_2$ requires 493.2347); $[a]_D^{20}$ +60.2 (*c* 1, MeCN).

3-(*S*)-Phenylbutyric acid 5-amino-4-(naphthalen-1-ylazo)-benzotriazol-2-ylmethyl ester 3. 5-Amino-4-(naphthalen-1-ylazo)benzotriazole (0.100 g, 0.34 mmol) was treated with 1.038 M NaOH solution (0.33 ml, 0.34 mmol). The water was removed at reduced pressure and the residue dissolved in anhydrous acetone (10 ml). (*S*)-Chloromethyl 3-phenylbutanoate (0.070 g, 0.34 mmol) was then added, and the reaction mixture left to stir for 2 h at room temperature. The solvent was then removed at reduced pressure and the residue purified by column chromatography, eluting with 20% ethyl acetate in hexane. The 2*H* isomer elutes more rapidly than the other isomers. The title compound was furnished as a red oil (0.04 g, 23%). (Found: C, 68.88; H, 5.39;.$C_{27}H_{24}N_6O_2$ requires C, 69.81; H, 5.21); δ_H (400 MHz; $CDCl_3$) 1.30 (3H, d, *J* 7.7, CH_3), 2.67 (1H, d, *J* 7.2, CH), 2.72 (1H, d, *J* 7.2, CH), 3.30 (1H, sept, *J* 7.2, CH), 6.50 (1H, d, *J* 10.1, CH), 6.56 (1H, d, *J* 10.1, CH), 7.00 (1H, d, *J* 7.6, ArH), 7.23–7.11 (6H, m, ArH), 7.59 (1H, dd, *J* 7.4, ArH), 7.64 (1H, dd, *J* 7.4, ArH), 7.77 (1H, d, *J* 9.20, ArH), 7.90–7.95 (3H, m, ArH), 8.70 (1H, d, *J* 7.8, ArH); δ_C (100 MHz; acetone) 22.0, 37.0, 42.7, 75.4, 112.0, 123.6, 124.2, 124.4, 126.7, 127.0, 127.4, 128.8, 129.1, 130.0, 131.3, 135.4, 141.4, 144.0, 146.3, 149.7, 170.9; *m/z* 465.2027 ([M + H]$^+$. $C_{27}H_{25}N_6O_2$ requires 465.2034); $[a]_D^{20}$ +61.0 (*c* 1, MeCN).

3-(*S*)-Phenylbutyric acid 6-(4-dimethylamino-naphthalen-1-ylazo)-5-nitrobenzotriazol-2-ylmethyl ester 4. (*S*)-Chloromethyl 3-phenylbutanoate (0.070 g, 0.33 mmol) was dissolved in anhydrous acetone (5 ml). Sodium iodide (0.05 g, 0.33 mmol) was added and the reaction mixture left to stir for 15 min, after which time a precipitate of sodium chloride was seen to form. The solution was filtered and the filtrate collected, with the solvent

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being removed at reduced pressure. The residue was dissolved in DMF (5 ml), and dimethyl [5-(6-nitro-3*H*-benzotriazolylazo)-naphthalen-1-yl]amine (0.12 g, 0.33 mmol) and potassium carbonate (0.045 g, 0.33 mmol) were added. The reaction mixture was left to stir overnight at room temperature and the solvent was then removed at reduced pressure. The residue was partitioned between diethyl ether (50 ml) and water (50 ml). The organic phase was separated and dried over sodium sulfate before filtration and removal of the solvent by evaporation. The residue was purified by column chromatography, eluting with 0–20% ethyl acetate in hexane to afford the title compound as a dark red solid (0.050 g, 19%). (Found: C, 64.38; H, 5.05;.$C_{29}H_{27}N_7O_4$ requires C, 64.79; H, 5.05); δ_H (400 MHz; $CDCl_3$) 1.23 (3H, d, *J* 7.0, CH_3), 2.71 (2H, m, CH_2), 3.16 (6H, s, CH_3), 3.31 (1H, sept, *J* 7.4, CH), 6.53 (1H, d, *J* 11.3, CH), 6.58 (1H, d, *J* 11.4, CH), 7.10–6.99 (5H, m, 5 × ArH), 7.10 (1H, d, *J* 8.0, ArH), 7.61 (1H, dd, *J* 7.3, ArH), 7.71 (1H, dd, *J* 7.3, ArH), 7.96 (1H, s, ArH), 8.00 (1H, d, *J* 8.6, ArH), 8.24 (1H, d, *J* 8.5, ArH), 8.55 (1H, d, *J* 7.1, ArH), 9.03 (1H, dd, *J* 8.5, ArH); δ_C (100 MHz; acetone) 22.4, 37.3, 42.4, 44.8, 68.6, 68.8, 113.7, 116.5, 124.2, 125.9, 126.2, 126.9, 127.1, 128.3, 128.5, 128.9, 132.6, 134.2, 134.4, 134.7, 142.7, 143.8, 144.5, 145.6, 146.2, 146.7, 147.0, 148.8, 157.1, 157.5, 171.9; *m/z* 538.2199 ($[M + H]^+$. $C_{29}H_{29}N_7O_4$ requires 538.2197); $[a]_D^{20}$ +61.5 (*c* 1, MeCN).

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