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Two-colour screening in combinatorial chemistry: prospecting for enantioselectivity in a library of steroid-based receptors

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ABSTRACT

The screening of resin-bound combinatorial libraries with pairs of dye-tagged substrates is a powerful strategy for discovering selective receptors. However, implementation has been hampered by a lack of complementary but chemically similar dyes. We now show that the well-established Disperse Red 1 and the recently-introduced Bristol Blue 1 can be used in parallel to synthesise 'pseudoenantiomeric' analogues of *N*-acetyl-α-amino acids and of the anti-inflammatory drug Naproxen. A steroid-based receptor library has been prepared and screened with these substrates. Preliminary results suggest that some members may be highly enantioselective receptors for *N*-acetyl-α-amino acids.

1. Introduction

The design of selective receptors for complex molecules is still a difficult challenge for supramolecular chemistry. Ideally one might aspire to a fully rational approach, employing computer based design guided by the quantitative modelling of recognition processes. However barriers are imposed by the flexibility of most target molecules, the difficulty of accounting for solvation and the need to consider entropy as well as enthalpy in binding calculations. Where these problems are insurmountable, the 'semi-rational' approach of combinatorial chemistry provides a useful alternative. As shown by Still and others,[1] it is possible to prepare very large libraries of receptor-like molecules using split-and-mix solid phase synthesis. The library members are mixed in a single vessel, but are spatially segregated in that each resin bead carries only one member. Visual screening for binding, employing dye-tagged substrates, allows the whole library to be tested in a single operation. By exploring many structures at once, the method can reveal design solutions which may not have been predicted.

The common variant of Still's procedure employs a single dye-tagged substrate, and is suitable for identifying strong receptors for one particular guest. In many cases, however, it is selectivity rather than binding strength which is most important. For these situations Still developed a two-colour assay, originally employed to optimise selectivity between two peptide substrates.[1g] The two substrates are labelled with complementary chromophores and the library exposed to the mixture. Beads are now selected according to the *purity* (not intensity) of the colour they accumulate. Although this protocol has been successfully demonstrated, difficulties with the chromophores have hampered further development. Of the two dyes employed by Still, Disperse Red 1 (**1**) (Fig. 1) has proved widely useful but Disperse Blue 3 (**2**) is problematic. Dye **2** is hard to obtain pure and lacks chemical stability, being vulnerable to oxidation, reduction and deprotonation. Furthermore, it is not a good structural match for **1**, increasing the risk of false hits due to dye-based selectivity.

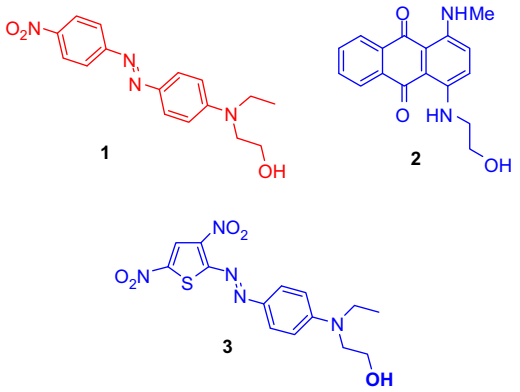

Figure 1. Dyes used for substrate tagging in combinatorial chemistry: Disperse Red 1 (**1**), Disperse Blue 3 (**2**) and Bristol Blue 1 (**3**).

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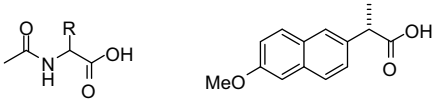
In response to this problem, we have reported the synthesis and preliminary assessment of 'Bristol Blue 1' (**3**), an alternative partner for **1** in the two-colour screening system.[2] **3** is chemically and structurally similar to **1** while being complementary in colour. Our long term goal is to discover enantioselective receptors[3] through the screening of libraries with substrates tagged with these chromophores. We now show that **1** and **3** can be employed to prepare 'pseudoenantiomeric' pairs of carboxylate substrates, related to *N*-acetyl amino acids **4** and to the anti-inflammatory drug *S*-Naproxen (*S*-**5**) (Fig. 2). We also describe the synthesis of a library of steroid-based receptors, and the use of the coloured substrates in preliminary screens of the library. The results suggest that the protocol does indeed have potential for the discovery of enantioselective receptors.



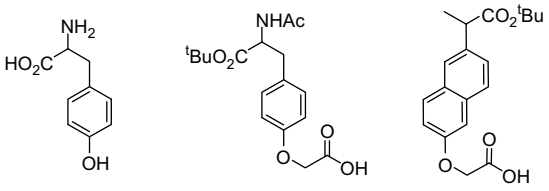
4 *S*-**5**

Figure 2. Substrates for enantioselective recognition: *N*-acetyl amino acids **4** and *S*-Naproxen (*S*-**5**).

2. Results and discussion

2.1. Design of dye labelled substrates

The dye-labelled substrates were intended to be representative of *N*-acetylamino acids **4**, and of Naproxen **5**, such that receptors discovered through two-colour screening would also be effective for the unlabelled prototypes. It was therefore desirable to avoid disturbance of the key recognition features of **4** and **5**, placing the labels remotely from the chiral centers. In the case of **4**, this could be achieved by starting from D- and L-tyrosine **6** and linking the chromophores to the phenolic hydroxyl groups. In the case of the Naproxen enantiomers **5**, the methyl ethers could potentially be cleaved to give naphtholic hydroxyl groups which could be exploited similarly. To make the connections between dyes and substrates, we decided to convert the Ar–OH groups to Ar–OCH_2-CO_2H, and then to esterify with **1** or **3**. Our initial targets were therefore the carboxylic acids **7** (from **4**) and **8** (from **5**) (Fig. 3). The *tert*-butyl esters in **7** and **8** would be compatible with the esterification conditions, and then removable with acid to give the dye-tagged carboxylate substrates.

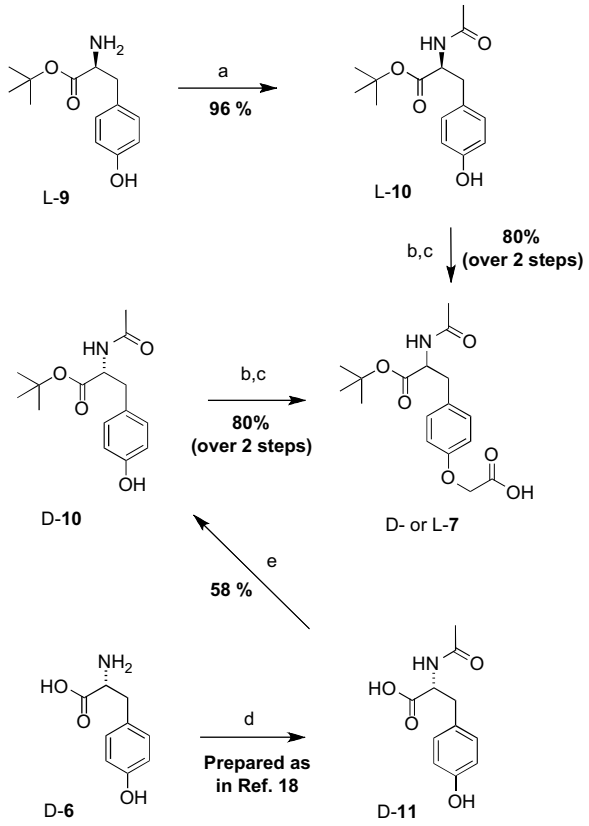


6 **7** **8**

Figure 3. Tyrosine (**6**) and intermediates **7** and **8**.

2.2. Synthesis of *N*-acetyltyrosine derivatives L-7 and D-7

Intermediate L-**7** was prepared from commercially available L-tyrosine *tert*-butyl ester (L-**9**), as shown in Scheme 1. Treatment of L-**9** with acetyl choride, to give acetamide L-**10**, was followed by alkylation with bromobenzyl acetate and debenzylation with H_2/Pd(C) to give L-**7**. Its enantiomer D-**7** was prepared using



Scheme 1. Synthesis of *N*-acetyl amino acid derivatives **7** for coupling to chromophores. Reagents and conditions: (a) AcCl, Et_3N, DCM, 0 °C to rt; (b) bromobenzyl acetate, Cs_2CO_3, DMF, rt; (c) Pd(C)/H_2 (1 atm), THF, rt; (d) Ac_2O, H_2O, 80 °C; (e) *tert*-butyl bromide, K_2CO_3, $Bu_4N^+Br^-$, DMA, 55 °C.

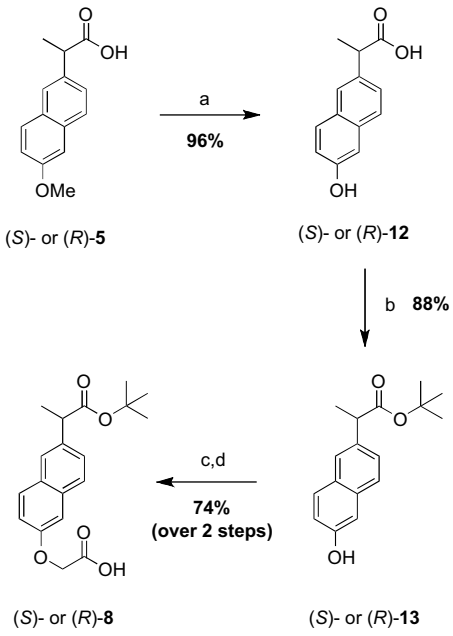
a slightly different route, from D-tyrosine (D-**6**). D-**6** was acetylated using acetic anhydride in water to give acetamide D-**11** in good yield and high purity.[4] *tert*-Butyl ester D-**10** was then formed by treatment of D-**11** with *tert*-butyl bromide (Scheme 1).[5] D-**10** was then converted to D-**7** using the same procedure as for the enantiomeric series.

2.3. Synthesis of Naproxen derivatives (*R*)- and (*S*)-8

Both enantiomers of the Naproxen-derived intermediate **8** were prepared as shown in Scheme 2. Either (*R*)- or (*S*)-Naproxen **5** was heated under reflux in concentrated hydrobromic acid to give the corresponding hydroxy derivative **12**,[6] which was then protected as *tert*-butyl ester by treatment with TFAA followed by *tert*-butanol then ammonium hydroxide.[7] The resulting ester **13** was then O-alkylated with bromobenzyl acetate/K_2CO_3 in MeCN. Finally, hydrogenolysis of the benzyl ester provided carboxylic acid **8**.

2.4. Synthesis of dye tagged pseudoenantiomeric carboxylates

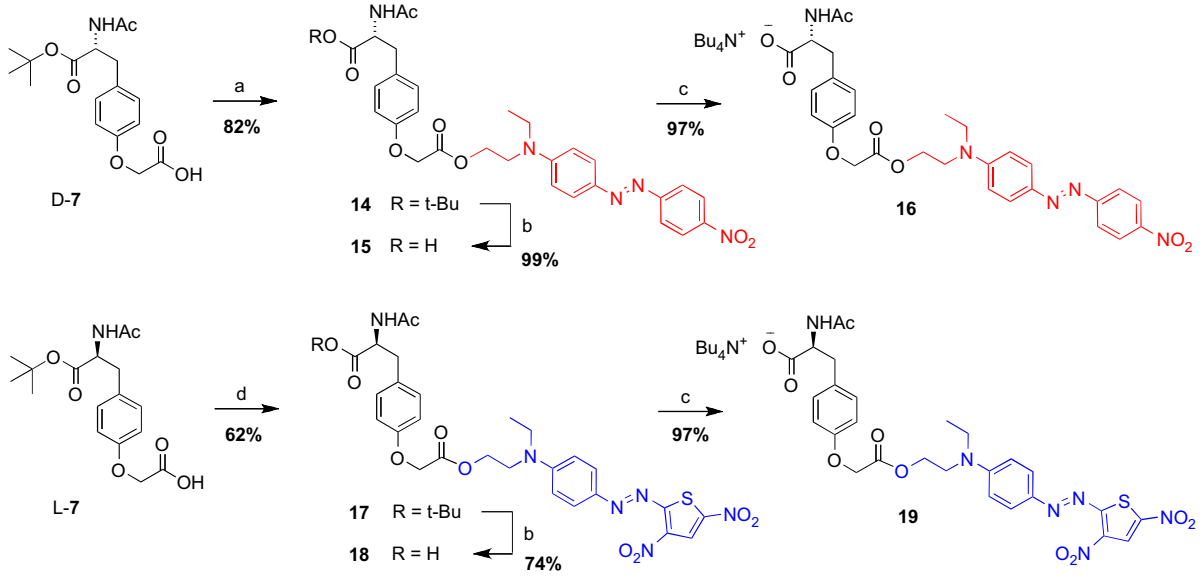
Intermediates D-**7** and L-**7** were attached to chromophores **1** and **3** respectively, via ester linkages formed using 1,1′-carbonyldiimidazole-mediated couplings (Scheme 3). The coupled products **14** and **17** were deprotected with TFA to give acids **15** and **18**. Treatment of the acids with tetrabutylammonium hydroxide gave the lipophilic salts **16** and **19**, suitable for the screening experiments. A similar sequence applied to Naproxen-derived acids (*R*)-**8** and (*S*)-**8** gave salts **22** and **25** (Scheme 4).



Scheme 2. Synthesis of Naproxen derivative **8** for coupling to chromophores. Reagents and conditions: (a) 48% (w/w) HBr, reflux, 2 h; (b) (i) $(CF_3CO)_2O$, dry THF, 0 °C, 4 h, (ii) *tert*-butanol, 0 °C to rt, 16 h, (iii) NH_4OH, 0 °C to rt, 30 min; (c) bromobenzyl acetate, K_2CO_3, dry MeCN, rt, 40 h; (d) H_2/Pd(C), $CHCl_3$, rt, 2 h.

electron poor aromatic urea at the *C*7 position was chosen as the primary binding motif, ureas being well established as carboxylate binding units.[12] Both the *C*3 and *C*12 positions of the steroid were to be appended with peptidic moieties, enclosing the substrate and creating diversity. It was decided to attach the steroid to the resin via a robust amide linkage, with a view to analysing single beads by Edman degradation after selection experiments. Although this method would not give a single definitive anwer in most cases, because the two chains would be cleaved simultaneously to reveal two amino acids at each stage of the process, any analysis would imply a maximum of four different structures. These could be resynthesised for confirmatory testing using parallel solid phase synthesis.[13]

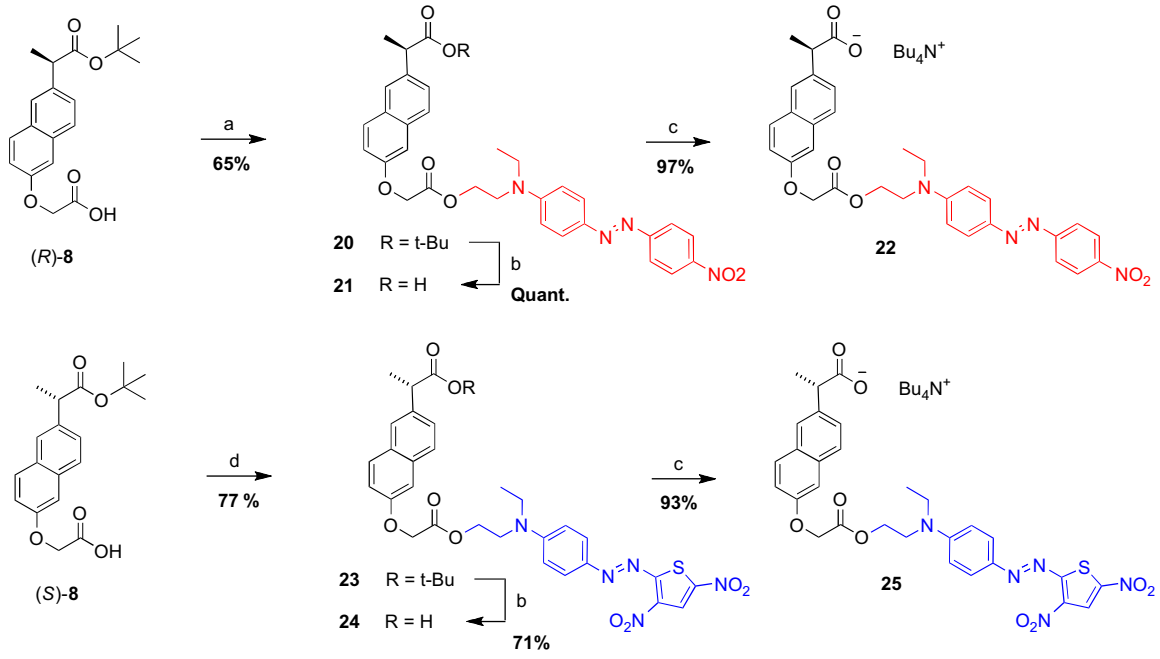
The starting point for library synthesis was the differentially protected triaminosteroid **27**.[9a] As shown in Scheme 5, this was converted to urea **28** which was then hydrolysed to carboxylic acid **29**. Attachment of **29** to high loading Tentagel™ aminomethyl resin gave polymer-bound intermediate **30**. The NF31 test[14] was employed to ensure that the amido coupling had proceeded to high conversion. The test indicated nearly complete coupling between carboxylic acid **29** and the resin, only a few free amine functionalities being detected on the polymer. These few amino groups were capped with acetyl functions by treatment with a large excess of acetic anhydride in CH_2Cl_2/pyridine. The *C*12 *o*-Ns group was removed, to afford the corresponding free amine derivative **31**. 'Split-and-mix' synthesis[15] was then used to introduce dipeptide



Scheme 3. Synthesis of tyrosine-based pseudoenantiomeric substrates **16** and **19**. Reagents and conditions: (a) 1,1′-carbonyldiimidazole, dry DCM, −10 °C, 90 min, then **1**, dry DCM, 0 °C to rt, 16 h; (b) TFA/DCM, 0 °C to rt, 6 h; (c) Bu_4NOH (1.0 M in MeOH), $CHCl_3/H_2O$ (2:1), rt, 30 min; (d) 1,1′-carbonyldiimidazole, dry DCM, −10 °C 90 min, then **3**, dry DCM, 0 °C to rt, 3 days.

2.5. Design and synthesis of receptor library

The general structure chosen for the library is shown in Figure 4. The design was based on our previous experience with cholic acid **26** as a scaffold for supramolecular[8] and combinatorial[9] chemistry. In particular, we have shown that 'cholapod' receptors derived from **26** can bind carboxylates with quite good enantioselectivities (er up to 10:1).[8c,10] The tripodal[11] architecture can surround a substrate, maximising the number of interactions. The steroidal skeleton preorganises the binding functionality imposing a specific orientation on the interactions. The *C*24 of cholic acid provides a convenient anchor point, allowing attachment to a solid support. An 'legs' at position 12. Twelve common *N*-Fmoc protected amino acids were employed, with acid-removable side-chain protection (side-chain protecting groups are given in brackets); Ala, Arg (Pbf), Asp (*O*-*t*-Bu), Cys (Trt), Gly, His (Trt), Lys (Boc), Phe, Pro, Ser (*O*-*t*-Bu), Trp (Boc) and Val. Resin-bound intermediate **31** was divided into 12 portions of approximately equal weight. Each portion was coupled to one of the above amino acids using HATU, HOBt and DIPEA in dry DMF.[16] At this point, the loading of the steroid onto the resin was evaluated by applying the 'Fmoc test'[17] to a sample of resin from the Gly coupling (glycine being the most likely to react quantitatively). An approximate loading of 0.29 mmol of steroid per gram of resin was inferred. The resin portions were recombined



Scheme 4. Synthesis of Naproxen-based pseudoenantiomeric substrates **22** and **25**. Reagents and conditions: (a) 1,1′-carbonyldiimidazole, dry DCM, 0 °C, 1 h, then **1**, dry DCM, 24 h, 0 °C to rt; (b) TFA/DCM, 0 °C to rt, 3 h (red derivative) or 10 h (blue derivative); (c) Bu_4NOH (1.0 M in MeOH), $CHCl_3/H_2O$ (2:1), 0 °C, 10 min; (d) 1,1′-carbonyldiimidazole, dry DCM, 0 °C, 1 h, then **3**, dry DCM, 0 °C to rt, 3 days.

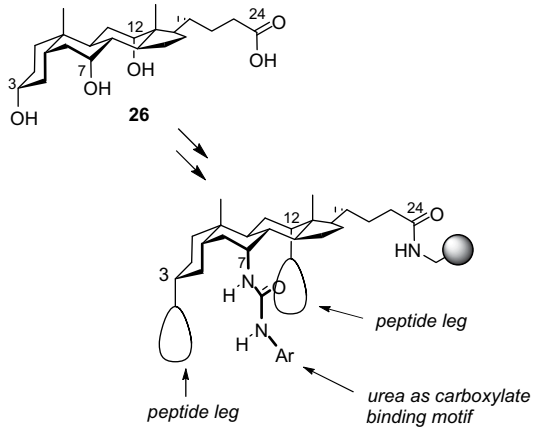


Figure 4. Design of library, and its relationship with cholic acid **6**.

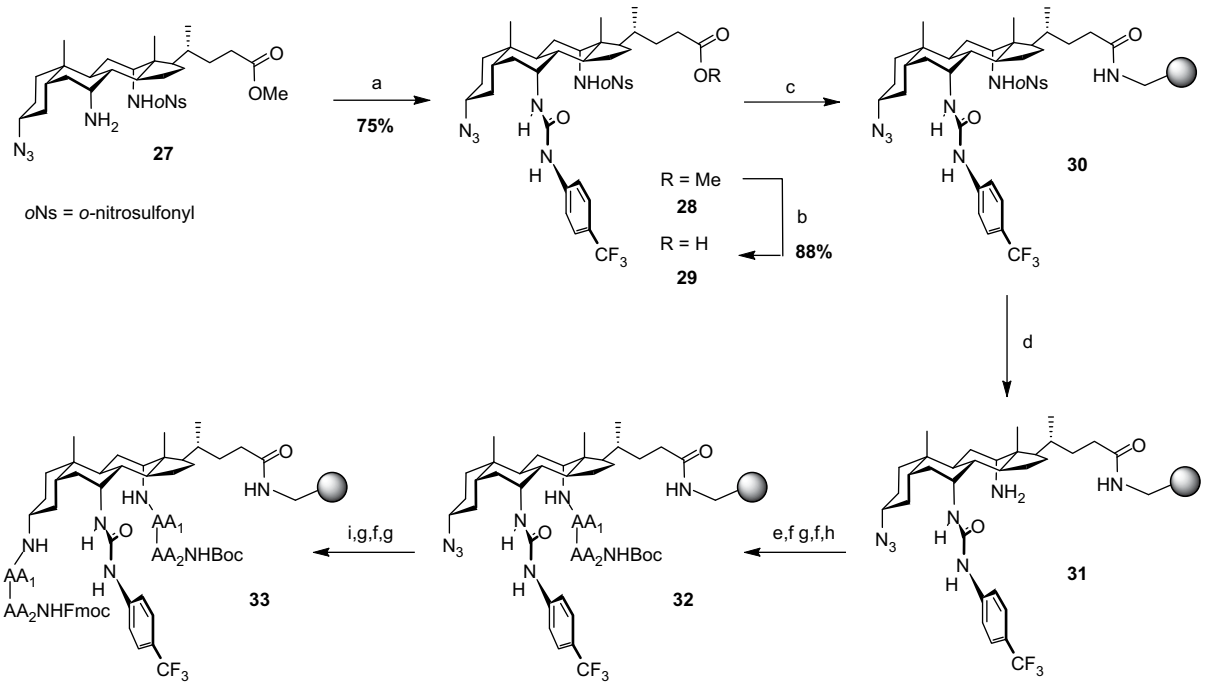
after coupling and the Fmoc group was cleaved by treating the beads with 20% piperidine in DMF. Re-splitting, a second amino acid coupling (HOBt, HBTU, DIPEA in dry DMF), recombination, Fmoc cleavage and Boc protection finished the synthesis of the first peptide chain at *C*12 to afford **32**. The azide group at *C*3 was then reduced with a large excess of PMe_3 in THF, followed by addition of water, to give the corresponding primary amine. Linkage of the first amino acid to the *C*3 amine (HOBt, HBTU, DIPEA in dry DMF), Fmoc cleavage and linkage of a second amino acid completed the synthesis of library **33** (Scheme 5). Library **33** possesses 12^4=20,736 members and is, to our knowledge, the first example of a combinatorial library prepared from an orthogonally protected triamino cholanoate derivative.[18]

2.6. Preliminary screening of steroid-based library 33

As a preliminary test of our strategy, the steroid-based receptor library **33** was screened against the tyrosine-derived pseudoenantiomeric mixture **16**+**19**, and against the Naproxen-derived mixture **22**+**25**. Solvent choice is an important parameter for this type of experiment. Binding must be strong enough that substrates accumulate in the beads, but not so strong that all beads become coloured. It is also important to ensure that complexation is reversible, so that substrate molecules can seek out the most effective receptors. Library **33** was designed to operate principally through hydrogen bonding, so that non-polar solvent systems were indicated. However, the use of chloroform, or less polar solvent systems, seemed to give non-specific irreversible binding. The problem was solved by the addition of small amounts of alcohols, which moderated the binding strength and promoted exchange between bound and unbound substrates.

A typical experiment involved (a) exposure of library to a pseudoenantiomeric substrate mixture in $CHCl_3$/*t*-BuOH (12:1) for 1 h, (b) separation of the beads by filtration, (c) washing with $CHCl_3$ to remove unbound substrate, and (d) suspension of the library in hexane for microscopic examination and bead picking. Studies with **22**+**25** were disappointing. Binding was weak, even with low levels of alcohol in the solvent, and there was little variation between beads. However, the results for the tyrosine-derived pseudoenantiomers were more encouraging. When a representative portion of library **33** was equilibrated with a 1:1 mixture of **16** and **19** in $CHCl_3$/*t*-BuOH (12:1), many of the beads acquired strong colours (Fig. 5). As shown in Figure 5 more blue beads were observed than red, implying that the library was generally biassed towards **19**. This could result from a preference for the blue chromophore, but it is reasonable to suppose that, at least in some cases, the blue beads are L-selective. This sense of selectivity is consistent with earlier results on steroid-based carboxylates receptors, where a range of guanidinium- and urea-substituted systems have all shown L-selectivity with *N*-acetyl-α-aminocarboxylates.[10] Washing of the beads with methanol rendered them almost colourless, proving that binding was due to non-covalent interactions.

The aim of library screening is to identify the best (in this case the most selective) receptors among the many present. The above experiment does not serve this purpose well, as most of the beads are perceived as 'hits'. However, discrimination can be improved by the simple modification of biassing the substrate mixture. In the present



Scheme 5. Synthesis of library of receptors **33**. Reagents and conditions: (a) *p*-(trifluoromethyl)phenyl isocyanate, NEt_3, DMAP, dry THF, reflux, 6 h; (b) NaOH, $MeOH/H_2O$, rt, 3 days; (c) NovaSyn® TG HL aminomethyl resin, TBTU, DMAP, DMF, rt, overnight, then Ac_2O, pyridine, DCM, rt, overnight; (d) PhSH, Cs_2CO_3, dry DMF, 55 °C, 4 days; (e) *N*-Fmoc-α-amino acid derivative, HATU, HOBt, DIPEA, dry DMF, rt, overnight; (f) piperidine, DMF, rt, 30 min; (g) *N*-Fmoc-α-amino acid derivative, HBTU, HOBt, DIPEA, dry DMF, rt, overnight; (h) $(Boc)_2O$, DCM, rt, overnight; (i) PMe_3 (1.0 M in THF), THF, rt, 3 h, then H_2O, rt, 2 h.

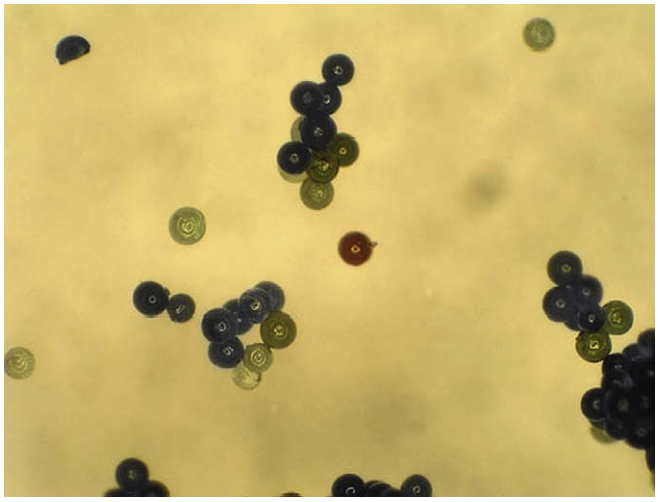


Figure 5. Micrograph of a portion of library **33** after equilibration with a 1:1 mixture of red carboxylate **16**+blue carboxylate **19** for 1 h in $CHCl_3$/*t*-BuOH (12:1). Before viewing the beads were filtered, washed briefly with chloroform and suspended in pure hexane. Most beads appear pale (implying weak binding) or deep blue.

case, the addition of extra red substrate challenges the library such that only the most selective members appear blue. The results of two such experiments, employing 2:1 and 5:1 ratios of red/blue substrates, are shown in Figure 6. Even in the latter case, with a five-fold excess of red substrate, a few beads appeared clearly blue among a majority of red and greenish (unselective) beads. These blue beads were separated from the rest, and set aside for future studies.

3. Conclusions

Combinatorial chemistry with two-colour screening offers hope for the discovery of selective receptors, even in cases where rational

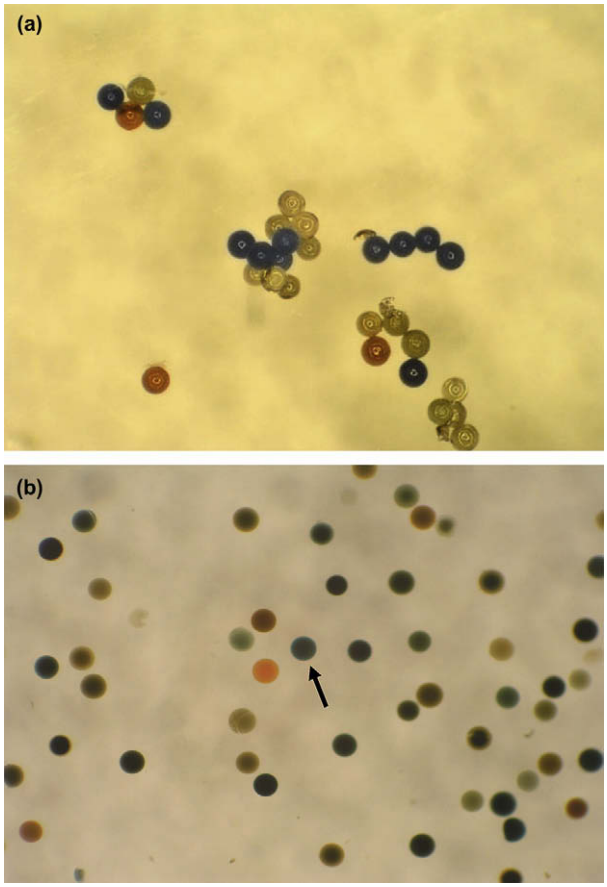


Figure 6. Micrographs of portions of library **33** after equilibration with unbalanced mixtures of **16** (red)+**19** (blue). Experimental procedure as for Figure 5. (a) Red/blue=2:1. Beads are mainly pale or deep blue, as for Figure 5. (b) Red/blue=5:1. Most beads appear pale or shades of green/brown, but a few (e.g., arrow) are clearly perceived as blue.

design is especially difficult. However there are experimental issues which need to be addressed, including the design and synthesis of substrates suitable for screening. In this paper, we have shown that the similar but complementary dyes **1** and **3** can make a valuable contribution. Using parallel reaction sequences they have been attached to substrates of real interest, without disturbing the substrates' key recognition features. The resulting pseudo-enantiomers have been subjected to preliminary testing against a novel steroid-based receptor library. Our observations suggest that library members can vary greatly in their binding properties, and that some distinguish effectively between the substrates. For an individual member, we cannot say for certain that selectivity is based on substrate configuration, rather than the nature of the dye units. However, by picking and studying a number of selective beads, we give ourselves several chances of finding enantioselectivity. Moreover, in principle the issue can be resolved by re-screening the picked beads with substrates in which dyes have been swapped between chiral centers (in other words, using the enantiomers of **16** and **19**). Beads which now select for the opposite colour (red, in this case) should be enantioselective and worthy of detailed study. In future work we will aim to realise this programme and emerge with highly selective receptors.

4. Experimental

4.1. General methods

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise stated. Methanol was distilled over calcium chloride, magnesium and iodine. DMF was obtained dry from Aldrich. THF and DCM were obtained dry from an Anhydrous Engineering Solvent Purification System (AESPS). Analytical TLC was carried out on DC-Alufolien Kieselgel 60F_{254} 0.2 mm plates (Merck) and compounds were visualised by UV fluorescence, 5% phosphomolybdic acid in ethanol, ninhydrin solution or by charring over a Bunsen burner flame. Flash chromatography of reaction products was carried out using Silica 60A, particle size 35–70 μm (Fischer Scientific). IR spectra were recorded on a Perkin–Elmer Spectrum One spectrometer. Melting points were obtained using Gallenkamp melting point blocks and are quoted as uncorrected values. ^{1}H NMR and ^{13}C NMR spectra were recorded on Jeol Delta/GX270 or Jeol Delta/GX400 spectrometers, using deuterated solvents and referenced internally to the residual solvent peak or TMS (δ_H=0.00 ppm, δ_C=0.0 ppm) signal. Coupling constants (*J*-values) are given in Hz. The DEPT 135° technique was used to assign (CH_2) signals. Chemical shifts are reported as follows: value (number of protons, description of absorption, coupling constant(s) where applicable, assignment).

4.2. *N*-Acetyl-L-tyrosine *tert*-butyl ester L-10

L-Tyrosine *tert*-butyl ester (L-**9**) (4.81 g, 20.3 mmol) and triethylamine (2.85 g, 3.95 mL, 28.4 mmol) were dissolved in DCM (100 mL). The solution was vigorously stirred and cooled in an ice–salt bath to 0 °C. Acetyl chloride (1.04 mL, 22.3 mmol) was added dropwise over 15 min. The mixture was then stirred for 20 min at 0 °C, allowed to warm to room temperature slowly and left stirring overnight. DCM was removed by evaporation under reduced pressure, and the oily residue was extracted with EtOAc (3×50 mL). The combined organic layers were washed with water (50 mL) then dried ($MgSO_4$), filtered and evaporated under reduced pressure to give the crude product as a clear oil. Purification by flash column chromatography (hexanes/EtOAc, 3:1) gave L-**10** (5.43 g, 96%) as a colourless oil. $[\alpha]_D$ −0.9 (*c* 0.09, DCM); ^{1}H NMR (400 MHz, D_2O, 298 K, TMS): δ=1.44 (9H, s, C(CH_3)$_3$), 1.98 (3H, s, CH_3), 2.96–3.03 (2H, m, CH_2), 4.74 (1H, m, C*H*), 6.20 (1H, d, *J*=8.3 Hz, N*H*), 6.74 (2H, d, *J*=8.3 Hz, Ar*H*), 6.98 (2H, d, *J*=8.3 Hz, Ar*H*), 7.64 (1H, br s, phenolic O*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=23.1 (CH_3), 27.9 ((CH_3)$_3$C), 37.4 (CH_2), 53.7 (*C*H), 82.6 ((CH_3)$_3$*C*), 115.4 (Ar*C*H), 127.4 (Ar*C*), 130.4 (Ar*C*H), 155.4 (Ar*C*), 170.1 (*C*ONH), 171.1 (*C*O$_2$C(CH_3)$_3$); MS (ESI$^+$): *m*/*z*= 302 [M+Na]$^+$; HRMS (ESI$^+$): *m*/*z* calcd for [$C_{15}H_{21}NO_4$+Na]$^+$ 302.1363, found 302.1367.

4.3. *N*-Acetyl-D-tyrosine *tert*-butyl ester D-10

N-Acetyl-D-tyrosine D-**11**[19] (1.00 g; 4.48 mmol), K_2CO_3 (16.0 g, 116.5 mmol) and $Bu_4N^+Cl^-$ (1.02 g, 4.48 mmol) were suspended in dimethylacetamide (50 mL) with vigorous stirring. *tert*-Butyl bromide (33 mL, 215 mmol) was added to the stirred suspension and the reaction heated to 55 °C and left to stir overnight. The reaction mixture was then poured onto 1.0 M $KHSO_4$ in water (150 mL) and extracted with ethyl acetate (3×100 mL). The combined organic layers were evaporated under reduced pressure leaving a yellow oil. Residual dimethylacetamide was removed by dissolving the oily residue in DCM (100 mL) and washing with ice-water (3×ca. 100 mL). The DCM layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure to give a yellow oil. Purification by flash column chromatography (hexanes/EtOAc, 3:1) gave D-**10** (1.25 g, 58%) as a clear pale yellow oil. $[\alpha]_D$ +0.9 (*c* 0.09, DCM). Other data as for L-**10**.

4.4. *N*-Acetyl-*O*-carboxymethyl-L-tyrosine *tert*-butyl ester L-7

Note: The D enantiomer was prepared in the same fashion.

tert-Butyl ester L-**10** (2.00 g, 7.17 mmol) and Cs_2CO_3 (7.01 g, 21.5 mmol) were dissolved in DMF (75 mL) and stirred vigorously for 15 min. 2-Bromobenzyl acetate (4.5 mL, 28.7 mmol) was added to the pale yellow solution and the mixture was left to stir overnight at room temperature. DMF was removed by evaporation under reduced pressure. The oily residue was dissolved in EtOAc (100 mL) and washed with ice-water (3×ca. 100 mL). The organic layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure giving the crude product as an oil. Purification by flash column chromatography on silica gel using hexanes/ethyl acetate (1:2) as eluent gave *N*-acetyl-*O*-benzyloxycarbonylmethyl-L-tyrosine *tert*-butyl ester as an oily foam (5.95 g, 82%). $[\alpha]_D$ +0.8 (*c* 0.04, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=1.41 (9H, s, C(CH_3)$_3$), 2.00 (3H, s, CH_3), 3.03 (2H, m, CH_2), 4.58 (2H, s, CH_2), 4.70 (1H, m, *H*), 5.28 (2H, s, CH_2), 6.09 (1H, d, *J*=6.4 Hz, N*H*), 6.81 (2H, d, *J*=8.3 Hz, Ar*H*), 7.06 (2H, d, *J*=8.3 Hz, Ar*H*), 7.35 (4H, m, Ar*H*), 8.00 (1H, s, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, TMS): δ=20.7 (CH_3), 27.3 (C(CH_3)$_3$), 37.3 (CH_2), 53.8 (*C*H), 65.1 (CH_2), 66.4 (CH_2), 79.1 (*C*(CH_3)$_3$), 114.2 (Ar*C*), 121.1 (Ar*C*), 128.0 (Ar*C*), 128.3 (Ar*C*), 130.0 (Ar*C*), 134.1 (Ar*C*), 135.3 (Ar*C*), 149.2 (Ar*C*), 168.4 (*C*=O), 170.6 (*C*=O).

To the foregoing diester (1.80 g, 4.21 mmol) dissolved in THF (20 mL) was added activated (heated in vacuo) Pd/C (Degussa, 10% w/w, 180 mg). The mixture was stirred under an atmosphere of H_2 (1 atm), until TLC indicated that the reaction had proceeded to completion. Residual catalyst was removed by filtration through Celite and washing with THF (3×50 mL). The filtrate was then evaporated to dryness under reduced pressure giving acid L-**7** (1.40 g, 98%) as a colourless oil. $[\alpha]_D$ +9.42 (*c* 0.05, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=1.42 (9H, s, C(CH_3)$_3$), 2.00 (3H, s, CH_3), 2.90–2.97 (2H, m, CH_2), 4.53 (2H, s, CH_2), 4.74 (1H, m, α-*H*), 6.09 (1H, d, *J*=7.8 Hz, N*H*), 6.85 (2H, d, *J*=8.3 Hz, Ar*H*), 7.07 (2H, d, *J*=8.3 Hz, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, TMS): δ=23.2 (CH_3), 28.0 (C(CH_3)$_3$), 37.2 (CH_2), 53.7 (*C*H), 67.0 (OCH_2CO_2H), 82.4 (*C*(CH_3)$_3$), 114.7 (Ar*C*H), 128.6 (Ar*C*H), 128.7 (Ar*C*), 130.6 (Ar*C*), 169.5 (*C*=O), 169.2 (*C*=O), (*C*=O); MS (CI$^+$): *m*/*z* (%)=338 [M]$^+$ (7), 278 (59), 228 (5); HRMS (ES$^+$): *m*/*z* calcd for [$C_{17}H_{23}NO_6$+Na]$^+$ 360.1418, found 360.1413.

The enantiomeric purity of L-**7** and D-**7** was confirmed by HPLC analysis using a Daicel Chiralcel OD-H column (250×4.6 mm) eluting with 0.1% TFA in hexane/isopropanol (9:1) at a flow rate of 1.0 mL min^{-1} with the UV detector set at 254 nm. Retention times: L-**7**, 13.97 min; D-**7**, 16.97 min.

4.5. D-Tyrosine-derived red *tert*-butyl ester 14

1,1′-Carbonyldiimidazole (442 mg, 2.73 mmol) dissolved in dry DCM (5 mL) under nitrogen was cooled to 0 °C in an ice–salt bath. To this solution was added acid D-**7** (478 mg, 1.42 mmol) in dry DCM (7 mL) over ca. 5 min. The resultant pale yellowish solution was then stirred for 1 h at 0 °C. Disperse Red 1 **1** (391 mg, 1.09 mmol) was added as a solid to the mixture, while maintaining an outward flow of nitrogen. The mixture was then stirred at 0 °C for a further 30 min and at room temperature for 36 h. The residue was diluted with DCM (50 mL) and washed with water (50 mL). The water layer was extracted with DCM (3×30 mL) and the combined organic layers were dried ($MgSO_4$), filtered and evaporated giving the crude product as a red solid. Purification by flash chromatography on silica gel using hexanes/ethyl acetate (1:1) as eluent, followed by precipitation from $CHCl_3$/hexanes gave **14** as a red amorphous solid (565 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=1.25 (3H, t, *J*=6.8 Hz, NCH_2CH_3), 1.40 (9H, s, $C(CH_3)_3$), 2.00 (3H, s, CH_3), 3.04 (2H, m, CH_2), 3.51 (2H, q, *J*=6.8 Hz, NCH_2CH_3), 3.73 (3H, t, *J*=6.4 Hz, NCH_2CH_2O), 4.44 (3H, t, *J*=6.4 Hz, NCH_2CH_2O), 4.60 (2H, s, OCH_2CO_2R), 4.72 (1H, m, α-*H*), 5.91 (1H, d, *J*=7.6 Hz, N*H*), 6.80 (4H, m, Ar*H*), 7.08 (2H, m, Ar*H*), 7.92 (4H, m, Ar*H*), 8.33 (2H, m, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, TMS): δ=12.4 (CH_3), 23.4 (CH_3), 28.0 ($C(CH_3)_3$), 37.3 (CH_2), 46.3 (CH_2), 49.0 (CH_2), 61.9 (CH_2), 65.4 (CH_2), 82.4 ($C(CH_3)_3$), 112.8 (ArC), 114.6 (ArC), 124.9 (ArC), 125.0 (ArC), 129.9 (ArC), 130.6 (ArC), 137.1 (ArC), 144.8 (ArC), 145.3 (ArC), 154.3 (ArC), 156.8 (ArC), 168.9 (C=O), 169.4 (C=O), 170.9 (C=O); MS (ES^+): *m*/*z* (%)=633 (5). The material was contaminated with a small amount of **1**, which was removed in the next step.

4.6. D-Tyrosine-derived red acid 15

Ester **14** (831 mg, 1.31 mmol) was dissolved in DCM (2 mL) and cooled in an ice–salt bath. TFA (13 mL) was added dropwise with stirring to the red solution. After 15 min the mixture was allowed to warm to room temperature. After stirring for a further 3 h, the solvent was removed by evaporation. The resultant purple residue was dissolved in DCM (100 mL), the solution was washed with brine (100 mL), and the aqueous layer was extracted with DCM (3×100 mL). The combined organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give a red residue. Purification by flash column chromatography on silica gel, eluting with ethyl acetate followed by 2% acetic acid in ethyl acetate, gave acid **15** (745 mg; 1.29 mmol; 99%) as a red solid. Melting point ($CHCl_3$/Hexane)=160.2–163.1 °C; IR: ν_{max} (solid)=3300, 3450, 2971, 1738, 1600, 1509, 1371 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=1.24 (3H, t, *J*=6.8 Hz, NCH_2CH_3), 1.99 (3H, s, acetyl CH_3), 3.04 (2H, m, CH_2Ar), 3.50 (2H, q, *J*=6.8 Hz, NCH_2CH_3), 3.71 (3H, t, *J*=5.1 Hz, NCH_2CH_2O), 4.44 (3H, t, *J*=5.1 Hz, NCH_2CH_2O), 4.56 (2H, s, OCH_2CO_2R), 4.77 (1H, m, α-*H*), 6.72 (2H, d, *J*=8.4 Hz, Ar*H*), 6.80 (2H, d, *J*=9.2 Hz, Ar*H*), 7.10 (2H, m, Ar*H*), 7.23 (1H, d, *J*=7.9 Hz, N*H*), 7.91 (4H, m, Ar*H*), 8.31 (2H, d, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, TMS): δ=12.7 (CH_3), 23.0 (CH_3), 38.1 (CH_2), 46.6 (CH_2), 49.3 (CH_2), 61.7 (CH_2), 66.1 (CH_2), 113.8 (ArCH), 115.0 (ArCH), 124.0 (ArCH), 126.5 (ArCH), 128.7 (ArC), 129.6 (ArCH), 130.4 (ArC), 136.3 (ArC), 146.3 (ArC), 147.6 (ArC), 155.2 (ArC), 156.5 (ArC), 168.7 (C=O), 169.2 (C=O), 172.0 (C=O); elemental analysis: calcd for $C_{29}H_{31}N_5O_8$: C 59.38, H 5.50, N 11.94; found C 58.87, H 5.23, N 12.31.

4.7. D-Tyrosine-derived red carboxylate salt 16

To red acid **15** (55 mg, 0.095 mmol) dissolved in $CHCl_3$ (2 mL) was added a solution of $NaHCO_3$ (64 mg, 0.76 mmol) and $Bu_4N^+ \cdot HSO_4^-$ (130 mg, 0.38 mmol) in deionised water (1.8 mL). After 5 min of vigorous stirring, the bi-phasic solution was poured into a separating funnel containing $CHCl_3$ (15 mL) and deionised water (15 mL). The organic layer was separated, dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure giving the Bu_4N^+ salt **16** (76 mg, 97%) as a red amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=0.81 (3H, m, NCH_2CH_3), 0.87 (12H, m, $Bu_4N^+CH_3$), 1.17 (8H, m, $Bu_4N^+CH_2$), 1.35 (8H, m, $Bu_4N^+CH_2$), 1.83 (3H, s, CH_3), 3.03–3.25 (10H, m, $Bu_4N^+CH_2N$ and benzylic CH_2), 3.41 (2H, q, *J*=7.3 Hz, NCH_2CH_3), 3.62 (2H, t, *J*=6.4 Hz, NCH_2CH_2O), 4.33 (3H, m, NCH_2CH_2O and α-H), 4.48 (2H, s, OCH_2CO), 6.65 (2H, d, *J*=8.3 Hz, Ar*H*), 6.71 (2H, d, *J*=8.8 Hz, Ar*H*), 6.81 (1H, d, *J*=8.3 Hz, N*H*), 7.08 (2H, d, *J*=8.3 Hz, Ar*H*), 7.83 (4H, m, Ar*H*), 8.23 (2H, d, *J*=8.3 Hz, Ar*H*); MS (ES^+): *m*/*z* (%)=578 (5) $[M-(NC_{16}H_{36})+2H]^+$, 242 (41) $[NC_{16}H_{36}]^+$; HRMS (ES^+): Mass calcd for $[C_{45}H_{66}N_6O_6-(C_{16}H_{36}N)+2H]^+$=578.2245, found 578.2256.

4.8. L-Tyrosine-derived blue *tert*-butyl ester 17

1,1′-Carbonyldiimidazole (196 mg, 1.0 mmol) was dissolved in DCM (5 mL) under nitrogen and cooled to 0 °C in an ice–salt bath. A solution of acid **7** (219 mg, 0.65 mmol) in DCM (3 mL) was then added with vigorous stirring at 0 °C. The solution was stirred for 2 h at 0 °C, after which blue azo dye **3** (183 mg, 0.50 mmol) was added as a solution in DCM (7 mL). The solution was stirred for a further 30 min at 0 °C, then for 6 h at room temperature, before partioning between DCM (30 mL) and water (30 mL). The water layer was separated and extracted with DCM (3×30 mL) the combined organic layers were then dried (Na_2SO_4), filtered and evaporated under reduced pressure to leave a blue residue. Purification by flash column chromatography on silica gel using hexane/ethyl acetate (1:2) gave **17** (212 mg, 62%) as a blue amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=1.28 (3H, t, *J*=7.08 Hz, CH_3CH_2N), 1.39 (9H, s, $C(CH_3)_3$), 1.96 (3H, s, $NHCOCH_3$), 3.04 (2H, m, CH_2Ph), 3.55 (2H, q, *J*=7.08 Hz, CH_3CH_2N), 3.78 (2H, t, *J*=6.0 Hz, OCH_2CH_2N), 4.47 (2H, t, *J*=6.0 Hz, OCH_2CH_2N), 4.58 (2H, s, OCH_2CO), 4.69 (1H, m, NHCHCO), 5.90 (1H, d, *J*=7.6 Hz, N*H*), 6.75 (2H, d, *J*=8.6 Hz, Ar*H*), 6.82 (2H, d, *J*=8.6 Hz, Ar*H*), 7.04 (2H, d, *J*=8.8 Hz, Ar*H*), 7.94 (2H, d, *J*=8.8 Hz, Ar*H*), 8.35 (1H, s, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.4 (CH_3), 23.3 (CH_3), 28.0 ($(CH_3)_3C$), 37.2 (CH_2), 46.2 (CH_2), 48.9 (CH), 53.6 (CH_2), 61.8 (CH_2), 65.3 (CH_2), 82.4 ($(CH_3)_3C$), 114.4 (ArCH), 124.9 (ArCH), 129.8 (ArC), 130.7 (ArCH), 137.11 (ArC), 144.0 (ArC), 154.2 (ArC), 156.6 (ArC), 162.2 (ArC), 168.9 (CO_2), 169.4 (CONH), 171.0 (CO_2); MS (ESI^+): *m*/*z*=685 $[M+H]^+$, 707 $[M+Na]^+$; HRMS (ESI^+): *m*/*z* calcd for $[C_{31}H_{37}N_6O_{10}S+H]^+$ 685.2286, found 685.2269 and $[C_{31}H_{37}N_6O_{10}S+Na]^+$ 707.2106, found 707.2084.

4.9. L-Tyrosine-derived blue acid 18

tert-Butyl ester **22** (250 mg, 0.365 mmol) was dissolved in DCM (1 mL) and the blue solution was cooled to 0 °C in an ice–salt bath with vigorous stirring. Dropwise addition of TFA (7 mL) at 0 °C gave a purple solution which was left to stir in the ice–salt bath for 15 min. Stirring was continued for a further 8 h at room temperature. Evaporation gave a metallic-purple coloured residue which was dissolved in DCM (50 mL) and washed with brine (3×50 mL). The blue organic layer was dried (Na_2SO_4) and evaporated to give a blue foam. This material was dry-loaded onto silica gel and subjected to gravity column chromatography on silica gel using DCM/MeOH/acetic acid (92:5:3) as eluent. The blue acid **18** (169 mg, 74%) was obtained as a blue amorphous solid. IR: ν_{max} (solid state)=2938, 1737, 1598, 1543, 1511, 1388 cm^{-1}; ^{1}H NMR (400 MHz,

$CDCl_3$, 298 K, TMS): δ=1.25 (3H, t, J=8.5 Hz, CH_3CH$_2$N), 1.98 (3H, s, NHCOCH_3), 3.11 (2H, dd, J=14.1, 5.9 Hz, CH_2), 3.56 (2H, q, J=7.1 Hz, CH$_3$CH_2N), 3.80 (2H, t, J=6.0 Hz, OCH$_2$CH_2N), 4.48 (2H, m, OCH_2CH$_2$N), 4.59 (2H, s, OCH_2CO), 4.78 (1H, m, NHCHCO), 6.01 (1H, d, J=7.6 Hz, NH), 6.72 (2H, d, J=8.6 Hz, ArH), 6.84 (2H, d, J=8.6 Hz, ArH), 7.04 (2H, d, J=9.3 Hz, ArH), 7.93 (2H, d, J=9.3 Hz, ArCH), 8.34 (1H, s, ArCH); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.3 (CH_3), 23.0 (CH_3), 36.3 (CH_2), 46.0 (CH_2), 48.8 (CH), 53.6 (CH_2), 61.9 (CH_2), 65.1 (CH_2), 114.6 (ArC), 125.0 (ArCH), 129.1 (ArC), 130.5 (ArC), 137.1 (ArC), 144.0 (ArC), 154.5 (ArC), 156.7 (ArC), 162.6 (ArC), 168.9 (C=O), 170.7 (C=O); MS (ESI$^+$): m/z=629 [M+H]$^+$, 651 [M+Na]$^+$; HRMS (ESI$^+$): m/z calcd for [$C_{27}H_{28}N_6O_{10}S$+H]$^+$ 629.1660, found 629.1649 and [$C_{27}H_{28}N_6O_{10}S$+Na]$^+$ 651.1480, found 651.1463.

4.10. L-Tyrosine-derived blue carboxylate salt 19

The procedure described above for **16** was employed to convert acid **18** into salt **19**. IR: ν_{max} (solid state)=2936, 1737, 1598, 1543, 1511, 1388 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ=0.99 (12H, t, J=8.5 Hz, Bu$_4$N$^+$CH_3), 1.25 (3H, t, J=8.5 Hz, CH$_3$CH$_2$N), 1.41 (8H, m, Bu$_4$N$^+$CH_2), 1.65 (8H, m, Bu$_4$N$^+$CH_2), 1.98 (3H, s, CH_3), 3.13 (2H, dd, J 14.2, 5.9, CH_2Ph), 3.24 (8H, t, J=8.5 Hz, Bu$_4$N$^+$CH_2), 3.55 (2H, q, J=7.1 Hz, CH$_3$CH_2N), 3.80 (2H, t, J=6.1 Hz, OCH$_2$CH_2N), 4.44 (2H, t, J=6.1 Hz, OCH_2CH$_2$N), 4.59 (3H, m), 6.73 (2H, d, J=8.6 Hz, ArH), 6.84 (2H, d, J=8.6 Hz, ArH), 7.02 (1H, d, J=7.80 Hz, NH), 7.12 (2H, d, J=8.5 Hz, ArH), 7.96 (2H, d, J=8.5 Hz, ArCH), 8.37 (1H, s, ArCH); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.4 (CH_3), 13.6 (CH_3), 21.2 (CH_2), 23.1 (CH_3), 23.9 (CH_2), 31.9 (CH_2), 46.4 (CH_2), 48.8 (CH), 58.9 (CH_2), 61.8 (CH_2), 62.5 (CH_2), 65.1 (CH_2), 114.6 (ArCH), 124.9 (ArCH), 129.1 (ArC), 130.5 (ArCH), 137.1 (ArC), 144.0 (ArC), 154.5 (ArC), 156.7 (ArC), 162.6 (ArC), 168.9 (CO$_2$), 170.7 (CONH); MS (ESI$^+$): m/z=629 [M+H]$^+$, 651 [M+Na]$^+$ from the tyrosine carboxylate and 242.28 [M]$^+$ counterion salt; HRMS (ESI$^+$): m/z calcd for [$C_{27}H_{28}N_6O_{10}S$+H]$^+$ 629.1660, found 629.1649 and [$C_{27}H_{28}N_6O_{10}S$+Na]$^+$ 651.1480, found 651.1463 and [$C_{16}H_{36}N$]$^+$ 242.2842, found 242.2854.

4.11. (*S*)-2-(6-Hydroxy-naphthalen-2-yl)-propionic acid (*S*)-12[6]

Note: The (R)-enantiomer was prepared in the same fashion.

(*S*)-Naproxen **5** (3.0 g, 13.0 mmol) was suspended in HBr (48% w/w in water, 120 mL) and heated at reflux for 2 h. The mixture was allowed to warm to room temperature and filtered. The solid was washed with chilled water to afford the title product (*S*)-**12** (2.71 g, 96%) as a white solid. $[\alpha]_D^{20}$ +56.1 (*c* 0.5, acetone); IR (solid state): ν_{max}=3215, 1698, 1128 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ=1.46 (3H, d, J=7.1 Hz, CH_3), 3.80 (1H, q, J=7.1 Hz, CH), 7.08–7.13 (2H, m, ArH), 7.36 (1H, dd, J=8.4, 1.5 Hz, ArH), 7.65–7.69 (2H, m, ArH), 7.76 (1H, d, J=8.4 Hz, ArH), 9.72 (1H, s, ArOH), 12.32 (1H, br s, CO$_2H$); ^{13}C NMR (100 MHz, DMSO-d_6): δ=19.3 (CH_3), 45.4 (CH), 109.3 (ArC), 119.6 (ArC), 126.4 (ArC), 126.9 (ArC), 127.0 (ArC), 127.0 (ArC), 128.5 (ArC), 129.9 (ArC), 134.4 (ArC), 156.0 (ArC), 176.3 (CO$_2$H) (lit.[20] δ=18.4, 44.54, 108.4, 118.7, 125.5, 126.09, 126.13, 127.6, 129.1, 133.5, 135.4, 155.1, 175.5).

4.12. (*S*)-2-(6-Hydroxy-naphthalen-2-yl)-propionic acid *tert*-butyl ester (*S*)-13

Note: The (R)-enantiomer was prepared in the same fashion.

The carboxylic acid (*S*)-**12** (2.39 g, 11.05 mmol) was dissolved in dry THF (110 mL) under a nitrogen atmosphere. The solution was cooled to 0 °C, then trifluoroacetic anhydride (13.93 g, 9.42 mL, 66.32 mmol) was added dropwise and the mixture was further stirred for 4 h maintaining the temperature below 5 °C. *tert*-Butanol (32 mL) was added dropwise and the resulting mixture was then allowed to rise to room temperature and was vigorously stirred overnight. The reaction was cooled again to ice-bath temperature and NH_4OH (35% in water, 6 mL) was added dropwise. When the addition was finished the mixture was allowed to rise to room temperature again and was finally stirred for a further 30 min before the volatiles were evaporated under vacuum. The crude residue was triturated with boiling DCM and the crystalline solid formed was removed by filtration. The filtrate was washed with saturated aqueous $NaHCO_3$ and dried over $MgSO_4$, then the solvent was evaporated under reduced pressure to give the *tert*-butyl ester (*S*)-**13** (2.66 g, 88%) as a white solid. An analytical sample was prepared by crystallising (*S*)-**13** from hot benzene/hexane. Melting point (benzene/hexane)=140–141 °C; $[\alpha]_D^{20}$ +24.9 (*c* 0.5, $CHCl_3$); IR: ν_{max} (solid state)=3373, 2985, 1696 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.41 (9H, s, C(CH_3)$_3$), 1.52 (3H, d, J=7.2 Hz, CHCH_3), 3.74 (1H, q, J=7.1 Hz, CHCH$_3$), 7.04–7.09 (2H, m, ArH), 7.38 (1H, dd, J=8.5, 1.6 Hz, ArH), 7.59 (1H, d, J=8.6 Hz, ArH), 7.63 (1H, s, ArH), 7.66 (1H, d, J=8.8 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ=18.8 (CH_3), 28.4 (C(CH_3)$_3$), 46.8 (CH), 81.0 (C(CH$_3$)$_3$), 109.7 (ArCH), 118.2 (ArCH), 126.2 (ArCH), 126.9 (ArCH), 126.9 (ArCH), 129.3 (ArC), 134.0 (ArCH), 136.6 (ArC), 153.7 (ArC), 175.3 (CO$_2$); MS (EI$^+$): m/z (%)=272 (25) [M]$^+$, 171 (100) [M−CO$_2$C(CH$_3$)$_3$]$^+$; HRMS (EI$^+$): m/z calcd for [$C_{17}H_{20}O_3$]$^+$ 272.1412, found 272.1409.

4.13. (*S*)-2-(6-Benzyloxycarbonylmethoxy-naphthalen-2-yl)-propionic acid *tert*-butyl ester

Note: The (R)-enantiomer was prepared in the same fashion.

The ester (*S*)-**13** (2.50 g, 9.18 mmol) and K_2CO_3 (1.29 g, 9.19 mmol) were suspended in dry acetonitrile (90 mL) under a nitrogen atmosphere. Benzyl 2-bromoacetate (2.74 g, 1.89 mL, 11.96 mmol) was added and the reaction mixture was stirred at room temperature for 40 h. The solvent was then evaporated under reduced pressure. The residue was dissolved in DCM, washed with water, dried ($MgSO_4$) and the organic solvent was evaporated. Flash chromatography (hexane/EtOAc, 8:1) afforded the title product (2.87 g, 74%) as a colourless oil. $[\alpha]_D^{20}$ +9.2 (*c* 0.5, $CHCl_3$); IR: ν_{max} (oil)=2978, 2934, 1754, 1720, 1605 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.39 (9H, s, C(CH_3)$_3$), 1.52 (3H, d, J=7.1 Hz, CHCH_3), 3.74 (1H, q, J=7.1 Hz, CHCH$_3$), 4.77 (2H, s, CH_2), 5.26 (2H, s, CH_2), 7.01 (1H, d, J=2.4 Hz, ArH), 7.21 (1H, dd, J=8.9, 2.5 Hz, ArH), 7.26–7.42 (6H, m, ArH), 7.62 (1H, d, J=8.5 Hz, ArH), 7.66 (1H, s, ArH), 7.73 (1H, d, J=9.0 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ=18.9 (CH_3), 28.3 (C(CH_3)$_3$), 46.8 (CH), 65.9 (CH_2), 67.4 (CH_2), 80.9 (C(CH$_3$)$_3$), 107.5 (ArCH), 119.0 (ArCH), 126.1 (ArCH), 126.9 (ArCH), 127.4 (ArCH), 128.8 (ArC), 129.0 (ArCH), 130.0 (ArCH), 133.6 (ArC), 135.6 (ArC), 137.3 (ArC), 156.1 (ArC), 169.0 (CO$_2$), 174.2 (CO$_2$); MS (EI$^+$) m/z (%)=420 (45) [M]$^+$, 364 (18) [M−C(CH$_3$)$_3$+H]$^+$, 319 (100) [M−CO$_2$C(CH$_3$)$_3$]$^+$; HRMS (EI$^+$): m/z calcd for [$C_{26}H_{28}O_5$]$^+$ 420.1937, found 420.1937.

4.14. (*S*)-2-(6-Carboxymethoxy-naphthalen-2-yl)-propionic acid *tert*-butyl ester (*S*)-8

Note: The (R)-enantiomer was prepared in the same fashion.

The above benzylic ester (1.08 g, 2.57 mmol) and Pd/C (Degussa type, 10% w/w Pd content, 50% of water content) (220 mg) were suspended in chloroform (25 mL) and vigorously stirred under 1 atm of hydrogen for 2 h. The mixture was filtered through a plug of Celite, washing with chloroform. The filtrate was evaporated under reduced pressure to obtain the carboxylic acid (*S*)-**9** (846 mg, quantitative yield) as an off-white solid. For analytical purposes a sample was crystallised from dichloromethane/hexane obtaining (*S*)-**8** as white plates. Melting point (DCM/hexane)=117–118 °C; $[\alpha]_D^{20}$ 17.5 (*c* 0.5, $CHCl_3$); IR: ν_{max} (solid state)=3055, 2978, 2904, 1748, 1720, 1607, 1505 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.39 (9H, s, C(CH_3)$_3$), 1.52 (3H, d, J=7.1 Hz, CHCH_3), 3.75 (1H, q, J=7.1 Hz, CHCH$_3$), 4.78 (2H, s, CH_2), 7.08 (1H, d, J=2.4 Hz, ArH), 7.21 (1H, dd,

J=8.9, 2.5 Hz, Ar*H*), 7.42 (1H, dd, *J*=8.4, 1.4 Hz, Ar*H*), 7.68 (2H, d+s, *J*=9.4 Hz, Ar*H*), 7.74 (1H, d, *J*=9.0 Hz, Ar*H*), 9.95 (s, 1H, CO_2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=18.8 (CH_3), 28.3 ($C(CH_3)_3$), 46.8 (*C*H), 65.3 (CH_2), 81.1 ($C(CH_3)_3$), 107.5 (Ar*C*H), 118.8 (Ar*C*H), 126.2 (Ar*C*H), 127.0 (Ar*C*H), 127.4 (Ar*C*H), 130.0 (Ar*C*), 130.1 (Ar*C*H), 133.6 (Ar*C*), 137.5 (Ar*C*), 155.7 (Ar*C*), 173.9 (CO_2), 174.4 (CO_2); MS (EI^+) *m*/*z* (%)=330 (23) $[M]^+$, 274 (8) $[M-C(CH_3)_3+H]^+$, 229 (100) $[M-CO_2C(CH_3)_3]^+$; HRMS (EI^+): *m*/*z* calcd for $[C_{19}H_{22}O_5]^+$ 330.1467, found 330.1467. Anal. found: C, 69.00; H, 6.79%. $C_{19}H_{22}O_5$ requires C, 69.07; H, 6.71%.

4.15. (*R*)-Naproxen-derived red *tert*-butyl ester 20

Acid derivative (*R*)-**8** (492 mg, 1.49 mmol) and 1,1′-carbonyldiimidazole (241 mg, 1.49 mmol) were dissolved in dry DCM (15 mL) at 0 °C and the mixture was stirred for 1 h under a nitrogen atmosphere. Disperse Red 1 **1** (347 mg, 1.10 mmol) dissolved in dry DCM (1 mL) was added. The mixture was allowed to rise to room temperature then further stirred for 24 h. The mixture was then diluted with DCM (15 mL) and washed with saturated aqueous $NaHCO_3$. The organic phase was dried ($MgSO_4$) and the solvent evaporated under reduced pressure. Flash chromatography (hexane/EtOAc, 4:1) afforded the title compound **20** (447 mg, 65%) as an amorphous red solid. An analytical sample was crystallised from dichloromethane/hexanes as red platelets. Melting point (DCM/hexane) >96–97 °C (decomposed); IR: ν_{max} (solid state)=2971, 1757, 1728, 1603, 1519, 1509 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ=1.20 (3H, t, *J*=6.9 Hz, CH_2CH_3), 1.39 (9H, s, $C(CH_3)_3$), 1.51 (3H, d, *J*=7.1 Hz, $CHCH_3$), 3.44 (2H, q, *J*=7.1 Hz, CH_2CH_3), 3.69–3.77 (3H, m, $CHCH_3$+CH_2), 4.45 (2H, t, *J*=6.1 Hz, CH_2), 4.73 (2H, s, CH_2), 6.77 (2H, d, *J*=9.2 Hz, Ar*H*), 7.01 (1H, d, *J*=2.4 Hz, Ar*H*), 7.18 (1H, dd, *J*=8.9, 2.5 Hz, Ar*H*), 7.40 (1H, dd, *J*=8.5, 1.4 Hz, Ar*H*), 7.64 (1H, d, *J*=8.9 Hz, Ar*H*), 7.65 (1H, s, Ar*H*), 7.73 (1H, d, *J*=9.0 Hz, Ar*H*), 7.87–7.93 (4H, m, Ar*H*), 8.32 (2H, d, *J*=9.0 Hz, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.6 (CH_3), 18.9 (CH_3), 28.3 ($C(CH_3)_3$), 42.0 (CH_2), 46.0 (CH_2), 46.8 (*C*H), 62.6 (CH_2), 65.7 (CH_2), 84.8 ($C(CH_3)_3$), 108.2 (Ar*C*H), 111.9 (Ar*C*H), 118.9 (Ar*C*H), 123.1 (Ar*C*H), 125.0 (Ar*C*H), 126.2 (Ar*C*H), 126.6 (Ar*C*H), 127.0 (Ar*C*H), 127.4 (Ar*C*H), 130.1 (Ar*C*H), 133.6 (Ar*C*), 142.1 (Ar*C*), 176, 157.1 (Ar*C*), 157.6 (Ar*C*), 164.8 (CO_2), 176.1 (CO_2); MS (ESI^+) *m*/*z* (%)=649 (11) $[M+Na]^+$, 627 (44) $[M+H]^+$, 571 (65) $[M-C(CH_3)_3+H]^+$; HRMS (ESI^+): *m*/*z* calcd for $[C_{38}H_{38}N_4O_7+H]^+$ 627.2813, found 627.2819. Anal. found: C, 67.11; H, 6.37; N, 8.92%. $C_{35}H_{38}N_4O_7$ requires C, 67.08; H, 6.11; N, 8.94%.

4.16. (*R*)-Naproxen-derived red acid 21

The *tert*-butyl ester **20** (870 mg, 1.39 mmol) was dissolved in DCM (4 mL) and the solution was cooled to 0 °C before TFA (4 mL) was added dropwise with vigorous stirring. The mixture was stirred for 1 h at 0 °C and then allowed to warm to room temperature before further stirring for 3 h. The volatiles were evaporated, and the crude residue was dissolved in chloroform and washed with water. The aqueous layer from the washings was extracted several times with chloroform until no red colouration remained in the aqueous phase. The combined organic layers were dried ($MgSO_4$) and the solvent evaporated under reduced pressure to give the carboxylic acid **21** (785 mg, quantitative yield) as a red solid. An analytical sample was prepared by crystallisation from dichloromethane/hexane. Melting point (DCM/hexane)=152–153 °C; IR: ν_{max} (solid state)=3054, 2908, 1737, 1704, 1600, 1587, 1508 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ=1.20 (3H, t, *J*=7.0 Hz, CH_2CH_3), 1.58 (3H, d, *J*=6.8 Hz, $CHCH_3$), 3.43 (2H, q, *J*=7.0 Hz, CH_2CH_3), 3.70 (2H, t, *J*=6.0 Hz, CH_2), 3.87 (1H, m, $CHCH_3$), 4.44 (2H, t. *J*=6.0 Hz, CH_2), 4.72 (2H, s, CH_2), 6.74 (2H, d, *J*=8.9 Hz, Ar*H*), 6.99 (1H, d, *J*=2.0 Hz, Ar*H*), 7.18 (1H, dd, *J*=8.8, 1.8 Hz, Ar*H*), 7.40 (1H, d, *J*=8.4 Hz, Ar*H*), 7.63 (1H, d, *J*=8.3 Hz, Ar*H*), 7.68 (1H, s, Ar*H*), 7.71 (1H, d, *J*=8.9 Hz, Ar*H*), 7.86 (2H, d, *J*=8.9 Hz, Ar*H*), 7.91 (2H, d, *J*=8.9 Hz, Ar*H*), 8.32 (2H, d, *J*=8.7 Hz, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.5 (CH_3), 18.5 (CH_3), 45.5 (*C*H), 45.9 (CH_2), 48.9 (CH_2), 62.6 (CH_2), 65.7 (CH_2), 107.4 (Ar*C*H), 111.9 (Ar*C*H), 119.1 (Ar*C*H), 123.0 (Ar*C*H), 125.0 (Ar*C*H), 126.5 (Ar*C*H), 126.6 (Ar*C*H), 126.8 (Ar*C*H), 127.7 (Ar*C*H), 129.1 (Ar*C*), 129.8 (Ar*C*), 130.1 (Ar*C*H), 133.8 (Ar*C*), 136.0 (Ar*C*), 144.3 (Ar*C*), 151.5 (Ar*C*), 156.1 (Ar*C*), 157.0 (Ar*C*), 169.2 (CO_2), 179.9 (CO_2H); MS (ES^+) *m*/*z* (%)=593 (60) $[M+Na]^+$, 571 (100) $[M+H]^+$; HRMS (ESI^+): *m*/*z* calcd for $[C_{31}H_{30}N_4O_7+H]^+$ 571.2187 found, 571.2190. Anal. found: C, 60.99; H, 5.22; N, 8.77%. $C_{31}H_{30}N_4O_7\cdot 2H_2O$ requires C, 61.38; H, 5.65; N, 9.24%. The enantiopurity of the carboxylic acid **21** was analysed by chiral HPLC on an (*S*,*S*)-Whelk O1 analytical column, eluting with $EtOH/CHCl_3/CF_3CO_2H$ (80:20:0.1) (flow rate=1 mL/min, UV detection at λ=254 nm). A small sample of the corresponding *S* enantiomer was prepared and analysed to confirm that the separation was possible. The enantiomeric excess of **21** was found to be 99%. Retention times: **21**, 8.8 min; (*S*)-**21**, 12.5 min.

4.17. (*R*)-Naproxen-derived red carboxylate salt 22

The carboxylic acid **21** (785 mg, 1.39 mmol) was dissolved in chloroform (15 mL) and cooled to 0 °C. Tetrabutylammonium hydroxide (1.0 M in methanol, 1.44 mL, 1.44 mmol) was diluted in 15 mL of chloroform and was added dropwise with vigorous stirring. The mixture was stirred for a further 5 min before the solvent was evaporated under reduced pressure. The residue was redissolved in ethyl acetate (50 mL) and washed with water (10 mL). The organic phase was dried ($MgSO_4$) and the solvent evaporated to give **22** (1.09 g, 97%) as a brownish-red amorphous solid. ^1H NMR (400 MHz, $CDCl_3$): δ=0.90 (12H, t, *J*=7.3 Hz, CH_3), 1.20 (3H, t, *J*=7.1 Hz, CH_2CH_3), 1.25–1.34 (8H, m, CH_2), 1.38–1.48 (8H, m, CH_2), 1.54 (3H, d, *J*=7.0 Hz, $CHCH_3$), 3.05–3.09 (8H, m, CH_2), 3.51 (2H, q, *J*=7.1 Hz, CH_2CH_3), 3.57 (2H, t, *J*=6.0 Hz, CH_2), 3.73 (1H, q, *J*=7.1 Hz, $CHCH_3$), 4.70 (4H, s+m, 2×CH_2), 6.79 (2H, d, *J*=9.3 Hz, Ar*H*), 6.99 (1H, d, *J*=2.4 Hz, Ar*H*), 7.13 (1H, dd, *J*=9.0, 2.6 Hz, Ar*H*), 7.57 (1H, d, *J*=8.5 Hz, Ar*H*), 7.67 (1H, d, *J*=8.9 Hz, Ar*H*), 7.68 (1H, dd, *J*=8.5, 1.6 Hz, Ar*H*), 7.78 (1H, s, Ar*H*), 7.85 (2H, d, *J*=9.2 Hz, Ar*H*), 7.89 (2H, d, *J*=8.9 Hz, Ar*H*), 8.30 (2H, d, *J*=9.1 Hz, Ar*H*); MS (ESI^+): *m*/*z* (%)=571 (100) $[M+H]^+$, 242 (47) $[NBu_4]^+$.

4.18. (*S*)-Naproxen-derived blue *tert*-butyl ester 23

The carboxylic acid derivative **8** (600 mg, 1.82 mmol) and carbonyl diimidazole (300 mg, 1.86 mmol) were dissolved in dry DCM (20 mL) at 0 °C and the mixture was stirred for 1 h under nitrogen atmosphere. Bristol Blue 1 **3** (347 mg, 1.14 mmol) dissolved in dry DCM (2 mL) was added dropwise. The resulting mixture was allowed to rise to room temperature and was further stirred for 3 days. The volatiles were evaporated and the crude solid was dissolved in EtOAc (50 mL). The solution was washed with a saturated aqueous $NaHCO_3$ solution (50 mL), the organic phase was dried ($MgSO_4$) and the solvent evaporated under reduced pressure. Flash chromatography (hexane/EtOAc, 3:2) afforded the title compound **23** (596 mg, 77%) as a deep-blue amorphous solid. IR: ν_{max} (solid state)=2978, 1762, 1724, 1599, 1545 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ=1.23 (3H, t, *J*=7.1 Hz, CH_2CH_3), 1.39 (9H, s, $C(CH_3)_3$), 1.52 (3H, d, *J*=7.1 Hz, $CHCH_3$), 3.49 (2H, q, *J*=7.1 Hz, CH_2CH_3), 3.74 (1H, q, *J*=7.1 Hz, $CHCH_3$), 3.78 (2H, t, *J*=5.9 Hz, CH_2), 4.48 (2H, t, *J*=5.9 Hz, CH_2), 4.74 (2H, s, CH_2), 6.78 (2H, d, *J*=9.3 Hz, Ar*H*), 6.99 (1H, d, *J*=2.4 Hz, Ar*H*), 7.16 (1H, dd, *J*=8.9, 2.5 Hz, Ar*H*), 7.41 (1H, dd, *J*=8.5, 1.4 Hz, Ar*H*), 7.63 (1H, d, *J*=8.5 Hz, Ar*H*), 7.66 (1H, s, Ar*H*), 7.73 (1H, d, *J*=8.9 Hz, Ar*H*), 7.92 (2H, d, *J*=8.8 Hz, Ar*H*), 8.35 (1H, s, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.6 (CH_3), 18.8 (CH_3), 28.3 ($C(CH_3)_3$), 46.6 (CH_2), 46.7 (*C*H), 49.3 (CH_2), 62.3 (CH_2), 65.7 (CH_2), 80.9 ($C(CH_3)_3$), 107.1 (Ar*C*H), 113.0 (Ar*C*H), 118.8 (Ar*C*H), 125.3 (Ar*C*H), 126.1 (Ar*C*H), 127.0 (Ar*C*H), 127.3 (Ar*C*H), 129.9 (Ar*C*), 130.1 (Ar*C*H),

133.5 (ArC), 137.5 (ArC), 144.4 (ArC), 154.5 (ArC), 155.8 (ArC), 169.2 (CO_2), 174.1 (CO_2); MS (EI^+) m/z (%)=677 (68) $[M]^+$; HRMS (ESI^+): m/z calcd for $[C_{33}H_{35}N_5O_9S+Na]^+$ 700.2053, found 700.2048.

4.19. (*S*)-Naproxen-derived blue acid 24

The *tert*-butyl ester **23** (560 mg, 0.83 mmol) was dissolved in DCM (8 mL) and the solution was cooled to 0 °C before TFA (6 mL) was added dropwise with vigorous stirring. The mixture was stirred for 1 h at 0 °C and then allowed to warm to room temperature before further stirring for 10 h. The volatiles were evaporated and the crude residue was redissolved in chloroform and washed with water. The aqueous layer from the washings was extracted several times with chloroform until no blue colouration remained in the aqueous phase. The combined organic layers were dried ($MgSO_4$) and the solvent evaporated under reduced pressure. Flash chromatography (DCM/EtOAc, 6:1 to DCM/EtOAc, 2:1) afforded the carboxylic acid **24** (364 mg, 71%) as a deep-blue amorphous solid. IR: ν_{max} (solid state)=3110, 3058, 2976, 2932, 1758, 1736, 1704, 1596, 1542 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ=1.21 (3H, t, J=7.1 Hz, CH_2CH_3), 1.57 (3H, d, J=7.1 Hz, $CHCH_3$), 3.44 (2H, q, J=7.1 Hz, CH_2CH_3), 3.75 (2H, t, J=5.8 Hz, CH_2), 3.86 (1H, q, J=7.1 Hz, $CHCH_3$), 4.46 (2H, t, J=5.8 Hz, CH_2), 4.72 (2H, s, CH_2), 6.71 (2H, d, J=9.3 Hz, Ar*H*), 6.95 (1H, d, J=2.4 Hz, Ar*H*), 7.13 (1H, dd, J=8.9, 2.5 Hz, Ar*H*), 7.34 (1H, dd, J=8.6, 1.5 Hz, Ar*H*), 7.59 (1H, d, J=8.5 Hz, Ar*H*), 7.65 (1H, br s, Ar*H*), 7.68 (1H, d, J=9.0 Hz, Ar*H*), 7.85 (2H, d, J=8.9 Hz, Ar*H*), 8.28 (1H, s, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=12.6 (CH_3), 18.5 (CH_3), 45.4 (CH_2), 46.5 (*C*H), 49.2 (CH_2), 62.4 (CH_2), 65.6 (CH_2), 107.1 (ArCH), 113.0 (ArCH), 119.0 (ArCH), 125.3 (ArCH), 126.9 (ArCH), 127.0 (ArCH), 127.7 (ArCH), 130.2 (ArCH), 130.6 (ArC), 133.7 (ArC), 143.6 (ArC), 154.5 (ArC), 169.8 (CO_2), 175.3 (CO_2); MS (ESI^+) m/z (%)=644 (76) $[M+Na]^+$, 622 (65) $[M+H]^+$; HRMS (ESI^+): m/z calcd for $[C_{29}H_{27}N_5O_9S+H]^+$ 622.1602, found 622.1605.

4.20. (*S*)-Naproxen-derived blue carboxylate salt 25

The carboxylic acid **24** (374 mg, 0.60 mmol) was dissolved in chloroform (7 mL) and cooled to 0 °C. Tetrabutylammonium hydroxide (1.0 M in methanol, 0.63 mL, 0.63 mmol) was diluted in chloroform (7 mL) and was added dropwise, with vigorous stirring. The mixture was stirred for a further 5 min before the solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc (30 mL) and washed with water (8 mL). The organic layer was dried ($MgSO_4$) and the solvent was evaporated to give the title compound **25** (480 mg, 93%) as a dark blue amorphous solid. ^1H NMR (400 MHz, $CDCl_3$): δ=0.91 (12H, t, J=7.3 Hz, CH_3), 1.21 (3H, t, J=7.1 Hz, CH_2CH_3), 1.25–1.34 (8H, m, CH_2), 1.38–1.48 (8H, m, CH_2), 1.55 (3H, d, J=7.2 Hz, $CHCH_3$), 3.05–3.09 (8H, m, CH_2), 3.46 (2H, q, J=7.1 Hz, CH_2CH_3), 3.73 (2H, t, J=5.8 Hz, CH_2), 3.84 (1H, q, J=7.1 Hz, $CHCH_3$), 4.46 (2H, t, J=5.8 Hz, CH_2), 4.72 (2H, s, CH_2), 6.71 (2H, d, J=9.3 Hz, Ar*H*), 6.95 (1H, d, J=2.4 Hz, Ar*H*), 7.13 (1H, dd, J=8.9, 2.5 Hz, Ar*H*), 7.34 (1H, dd, J=8.6, 1.5 Hz, Ar*H*), 7.59 (1H, d, J=8.5 Hz, Ar*H*), 7.65 (1H, br s, Ar*H*), 7.68 (1H, d, J=9.0 Hz, Ar*H*), 7.85 (2H, d, J=8.9 Hz, Ar*H*), 8.28 (1H, s, Ar*H*); MS (ESI^+): m/z (%)=622 (71) $[M+H]^+$, 242 (49) $[NBu_4]^+$.

4.21. Methyl 3α-azido-7α-[(4-trifluoromethylphenyl)-aminocarbonylamino]-12α-[(2-nitrobenzenesulfonyl)-amino]-5β-cholan-24-oate 28

To a solution of the steroidal derivative **27**[9a] (2.37 g, 3.76 mmol) and DMAP (229 mg, 1.88 mmol), in dry THF (40 mL), were added triethylamine (378 mg, 0.52 mL, 3.74 mmol) and *p*-trifluoromethylphenyl isocyanate (838 mg, 0.64 mL, 4.48 mmol) at room temperature with vigorous stirring. The reaction mixture was heated at reflux for 6 h before the solvent was evaporated under reduced pressure. The residue was redissolved in dichloromethane, washed with aqueous HCl (1 M) and dried ($MgSO_4$). The organic solvent was evaporated under reduced pressure. Flash chromatography (hexane/EtOAc, 2:1) afforded **28** (2.29 g, 75%) as a pale yellowish amorphous solid; IR: ν_{max} (solid state)=3422, 3286, 2956, 2870, 2090, 1697, 1535, 1159 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ=0.82 (3H, d, J=5.6 Hz, 21-CH_3), 0.83 (3H, s, 18-CH_3), 0.86 (3H, s, 19-CH_3), 3.09 (1H, m, 3β-*H*), 3.62 (3H, s, CO_2CH_3), 3.82 (1H, d, J=9.6 Hz, 7β-*H*), 4.02 (1H, br s, 12β-*H*), 5.17 (1H, d, J=7.8 Hz, N*H*SO_2), 6.47 (1H, br s, CH–N*H*C(O)), 7.39 (1H, s, NHC(O)N*H*Ar), 7.53 (2H, d, J=8.7 Hz, Ar*H*), 7.62 (2H, d, J=8.7 Hz, Ar*H*), 7.78–7.86 (2H, m, Ar*H*), 7.93–7.95 (1H, m, Ar*H*), 8.16–8.19 (1H, m, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ=14.6 (CH_3), 23.1 (CH_3), 23.5 (CH_3), 26.2 (CH_2), 26.9 (CH_2), 29.6 (*C*H), 32.0 (CH_2), 32.5 (CH_2), 35.2 (CH_2), 35.4 (CH_2), 35.6 (CH_2), 37.0 (*C*H), 42.0 (*C*H), 44.4 (*C*H), 46.9 (*C*H), 49.3 (CH), 51.7 (CO_2CH_3), 57.7 (*C*H), 61.2 (*C*H), 118.4 (ArCH), 125.8 (ArCH), 126.5 (ArCH), 126.5 (ArC), 131.0 (ArCH), 133.4 (ArCH), 134.5 (ArCH), 143.4 (ArC), 154.4 (NH*C*(O)NH), 174.7 (CO_2CH_3); MS (ESI^+): m/z (%)=840 (49) $[M+Na]^+$, 818 (87) $[M+H]^+$; HRMS (ESI^+): m/z calcd for $[C_{39}H_{50}F_3N_7O_7S+NH_4]^+$ 835.3783, found 835.3787.

4.22. 3α-Azido-7α-[(4-trifluoromethylphenyl)-aminocarbonylamino]-12α-[(2-nitrobenzenesulfonyl)-amino]-5β-cholan-24-oic acid 29

Steroid **28** (2.29 g, 2.80 mmol) was dissolved in methanol (23 mL). A solution of NaOH (448 mg, 11.20 mmol) in deionised water (7 mL) was added and the mixture was stirred at room temperature for three days. The reaction was quenched with aqueous HCl (2 M, 10 mL). The mixture was evaporated under reduced pressure, redissolved in ethyl acetate, washed with water and dried ($MgSO_4$). The solvent was removed under vacuum. Flash chromatography (DCM/MeOH, 98:2) gave the carboxylic acid **29** (1.73 g, 88%) as a white solid. IR: ν_{max} (solid state)=3293, 2939, 2869, 2091, 1684, 1537, 1159 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ=0.76 (d, J=6.5 Hz, 3H, 21-H_3), 0.79 (s, 3H, 18-H_3), 0.86 (s, 3H, 19-H_3), 3.08 (br s, 1H, 3b-*H*), 3.83 (m, 1H, 7b-*H*), 3.96 (br s, 1H, 12b-*H*), 5.39 (b s, 1H, N*H*SO_2R), 6.38 (br s, 1H, CH–N*H*C(O)), 7.52 (d, J=8.6 Hz, 2H, Ar*H*), 7.59 (d, J=8.6 Hz, 2H, Ar*H*), 7.74–7.92 (m, 3H, Ar*H*), 8.15 (d, J=7.8 Hz, 1H, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD 5:1): δ=13.4 (CH_3), 17.2 (CH_3), 22.7 (CH_3), 23.1 (CH_2), 26.0 (CH_2), 26.4 (CH_2), 27.1 (CH_2), 28.4 (*C*H), 30.5 (CH_2), 31.0 (CH_2), 31.8 (CH_2), 34.6 (*C*), 34.9 (CH_2), 35.1 (CH_2), 35.2 (*C*H), 36.7 (*C*H), 41.4 (*C*H), 44.1 (*C*H), 45.8 (*C*), 46.1 (*C*H), 47.4 (*C*H), 57.9 (*C*H), 61.0 (*C*H), 117.5 (ArCH), 123.4 (q, J=32.3 Hz, Ar*C*–CF_3), 124.6 (q, J=216.0 Hz, *C*F_3), 125.1 (ArCH), 126.0 (q, J=3.8 Hz, F_3C–C=*C*H), 130.7 (ArCH), 133.0 (ArCH), 133.7 (ArCH), 135.0 (ArC), 143.0 (ArC), 147.9 (ArC), 155.1 (NH*C*O), 177.0 (CO_2H); MS (ESI^+): m/z (%)=826 (100) $[M+Na]^+$, 804 (26) $[M+H]^+$, 615 (27) $[M-CF_3-(C_6H_4)-NHCO]^+$; HRMS (ESI^+): m/z calcd for $[C_{38}H_{48}F_3N_7O_7S+NH_4]^+$ 821.3626, found 821.3627. Anal. found: C, 54.12; H, 6.26; N, 11.35%. $C_{38}H_{48}F_3N_7O_7S\cdot 2H_2O$ requires C, 54.34; H, 6.24; N, 11.67%.

4.23. Protocols in solid phase

Reactions on solid phase were carried out in polypropylene 1, 2, 5 or 25 mL solid phase extraction tubes (SPET) purchased from Supelco. SPETs (therefore resins) were agitated by a Stuart SB2 solid phase rotator. After reaction, beads were dried on a Supelco Visiprep solid phase extraction vacuum manifold. For observation after screening experiments, beads were placed on Petri dishes and viewed through an optical Olympus ST-PZ microscope equipped with an Olympus U-PMTVC and C3040-ADL camera adaptors. Micrographs such as those shown in Figures 5 and 6 were taken with

an Olympus C-5050 ZOOM digital camera and processed with standard computer software.

4.24. Typical NF31 test for amino groups on solid phase

A solution of the NF31 dye[13] in acetonitrile (ca. 1 M, 0.5 mL) was added to a collection of approximately 20 beads in a small sample vial and heated to reflux and left to cool. The heating/cooling cycle was repeated, then the beads were left to settle and the solvent carefully removed with the aid of a pipette. The beads were washed with acetonitrile (3×1 mL) and DCM (3×1 mL), the solvent being carefully removed by pipette each time, before being analysed under the microscope. Intense red beads indicate the presence of amino groups (positive result).

4.25. 3α-Azido-7α-[(4-trifluoromethylphenyl)-aminocarbonylamino]-12α-[(2-nitrobenzenesulfonyl)-amino]-5β-cholan-24-oic acid loaded onto Tentagel (30)

Steroidal derivative **29** (1.60 g, 1.99 mmol), TBTU (636 mg, 1.99 mmol), DMAP (725 mg, 5.94 mmol) were dissolved in DMF (11 mL). This mixture was then added to high loading NovaSyn® TG HL (0.44 mmol g^{-1})[21] amino resin (3.00 g, 1.32 mmol) in a solid phase extraction tube (SPET) and agitated overnight. The resin was then drained, washed with DMF (3×10 mL), MeOH (3×10 mL) and DCM (3×10 mL) and dried. Testing with NF31 (see above) gave a negative result. The resin was then agitated overnight with acetic anhydride (1 mL), pyridine (1 mL) and DCM (10 mL) to cap any remaining amine groups, giving resin-bound steroid **30**. The characteristic signal for the *C*3 azide at ν_{max}=2092 cm^{-1} in the IR spectrum confirmed the presence of the steroid.

4.26. *C*12 *o*-Ns deprotection on solid phase (30→31)

Resin **30** (3.50 g, ≤1.32 mmol), Cs_2CO_3 (3.87 g, 11.88 mmol) and thiophenol (2.62 g, 2.44 mL, 23.76 mmol) were suspended in dry DMF (25 mL) into a two-necked flask. Nitrogen gas was bubbled through the mixture (via syringe needle) for agitating purposes while it was heated at 60 °C for 3 days. DMF lost by evaporation was replaced as necessary. The resin was removed by filtration, washed with water (3×10 mL), DMF (3×10 mL), MeOH (3×10 mL) and finally DCM (3×10 mL) and dried. This resin was subjected again to the same reaction conditions (Cs_2CO_3 and thiophenol in hot DMF) to ensure complete cleavage of the *C*12 *o*-Ns group. NF31 test showed preponderance of brightly red beads as expected from the presence of the free amino function.

4.27. Standard procedure for peptide synthesis on solid phase (as carried out for library 33)

The bulk resin was divided into twelve equal portions in twelve SPET tubes. Each *N*-Fmoc-α-amino acid derivative (3 equiv) was placed in a sample vial to which was added 1 mL of solution A and 1 mL of solution B. Solution A consisted of HATU (12×3 equiv) and HOBt (12×2.9 equiv) dissolved in DMF (12 mL). Solution B consisted of DIPEA (12×6 equiv) in DMF (12 mL). Each tube was gently shaken to ensure complete dissolution. After being left to stand for 1 min, each amino acid coupling solution was added to a portion of resin (1 equi loading) with DMF (4 mL) and agitated overnight. Each portion of resin was then filtered, washed with DMF (3×10 mL), MeOH (3×10 mL) and DCM (3×10 mL) and a few beads of each portion subjected to NF31 test as shown above. A negative test indicated that the reaction was complete. Any red colouration indicated that the reaction needed to be repeated.

4.28. Standard procedure for estimation of first amino acid attachment

Completely dry Fmoc-containing resin (~3 mg, weighed accurately) was placed in a 10 mm quartz UV cuvette. Freshly prepared 20% piperidine in DMF (3 mL, measured accurately) was added to the cuvette and to another cuvette as reference. The resin was carefully agitated for 5 min with the aid of a Pasteur pipette. The cuvettes were placed in a spectrophotometer and the absorbance at 301 nm read. The level of loading on the resin can be estimated from the following equation:

Fmoc loading (mmol g^{-1})=(Abs_{sample}−Abs_{ref})/(1.65×weight of resin in mg). In the present case the loading was calculated as 0.29 mmol of steroid per gram of resin.

4.29. Standard procedure for removal of Fmoc protecting groups

The twelve resin portions employed for peptide synthesis were combined and washed with MeOH (3×10 mL) and DMF (3×10 mL) and agitated with 20% piperidine in DMF (10 mL) for 30 min. This protocol was repeated twice on the same beads to ensure complete removal of the Fmoc group. Care was taken to vent the sample to avoid pressure build-up of CO_2 in the tube. The beads were then filtered and washed with DMF (3×10 mL), MeOH (3×10 mL) and DCM (3×10 mL). The NF31 test produced intense red beads, confirming deprotection.

4.30. Standard procedure for Boc-protecting the peptide podand at *C*12

Following Fmoc removal of the second amino acid on the *C*12 position, the resin was agitated overnight with di-*tert*-butyl dicarbonate (4 equiv) in DCM (12 mL). The resin was then drained and washed with DCM (3×10 mL), MeOH (3×10 mL) and DCM again (3×10 mL). NF31 test on a few beads indicated the reaction was complete.

4.31. Standard procedure for reducing the azide at *C*3 position

A solution of 1.0 M of trimethylphosphine in THF (5 mL) was diluted with THF (5 mL) and added to the resin, which was left open to the atmosphere to avoid build-up of nitrogen gas. This was gently agitated every 20 min until no more nitrogen was seen to be evolving (~3 h). Deionised water (4 mL) was added and the tube agitated for 2 h. The resin was then washed with THF (3×10 mL), MeOH (3×10 mL) and finally DCM (3×10 mL) before being air-dried. NF31 test on a few beads gave an intense red colour indicating that the reaction was successful. IR of the resin showed the absence of the azide peak at ν=2091 cm^{-1}.

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