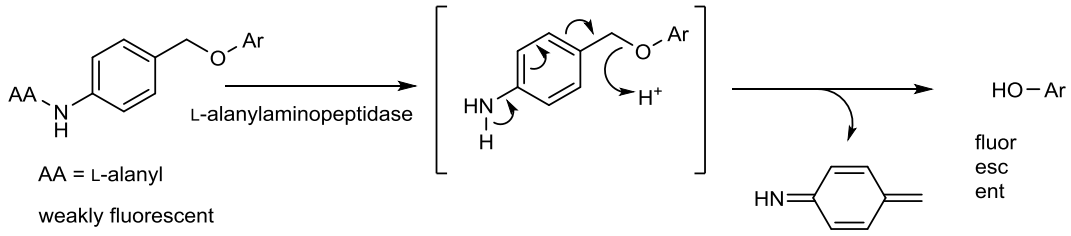

Detection of L-alanylaminopeptidase activity in microorganisms using fluorogenic self-immolative enzyme substrates

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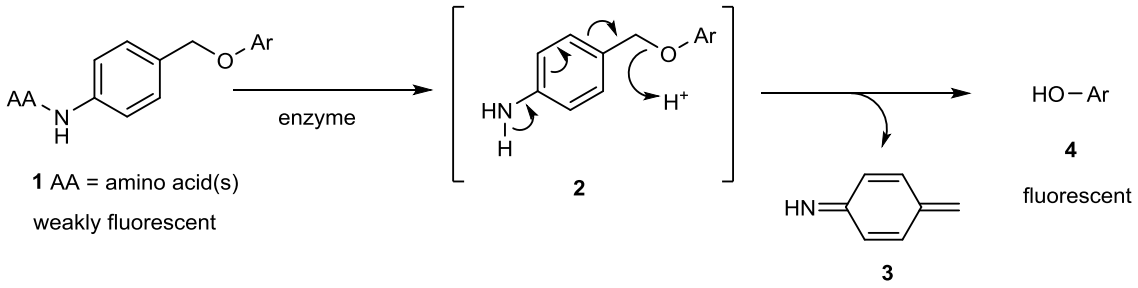
Abstract: A series of fluorogenic enzymatic substrates that incorporate a self-immolative spacer were synthesised for the purpose of identifying L-alanylaminopeptidase activity in microorganisms in agar media. These substrates resulted in the generation of fluorescent microorganism colonies with Gram-negative microorganisms.

Keywords: fluorogenic enzyme substrates, L-alanylaminopeptidase detection, microorganisms.

1. Introduction

Molecules that contain self-immolative spacers have found widespread applications in areas such as drug delivery, prodrug systems, chemical sensors and enzyme sensors.[1] In one aspect of self-immolative spacer design, fluorescent substrates of general structure **1** have been constructed around a *para*-aminobenzyl alcohol (PABA) core for the purpose of enabling the detection of protease enzyme activity as outlined in Scheme 1. This system is designed so that the presence of a protease enzyme results in the release of a fluorescent phenolic derivative **4**. Thus, the weakly fluorescent substrates **1** are hydrolytically cleaved by an appropriate enzyme producing the *O*-arylated-4-aminobenzyl alcohol intermediate **2** which subsequently undergoes fragmentation with concomitant liberation of the imine **3** and the fluorescent phenolic derivative **4**. Renard, Romieu and co-workers described the preparation of two fluorescent substrates based on the general structure **1**; a sensor **5** for the detection of penicillin G acylase[2,3] and a probe **6** for determining Caspase-3 activity (Figure 1).[2] Structurally related chemiluminescent substrates for detecting penicillin G acylase and Caspase-3 activities have also been described.[4] A fluorogenic assay based on structure **7** for

monitoring the activity of the autophagy-initiating enzyme ATG4B has recently been reported (Figure 1).[5]



Scheme 1. Fluorogenic self-immolative spacers for protease detection.

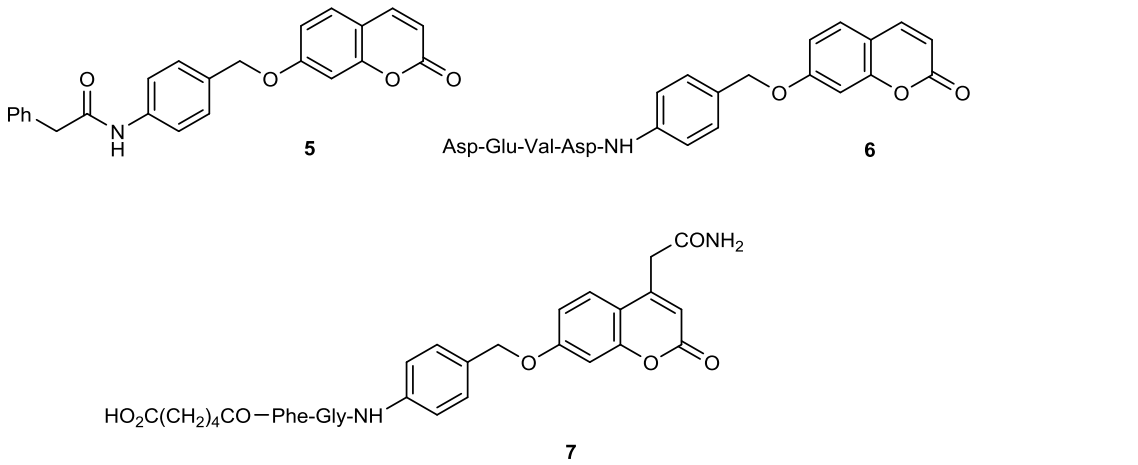
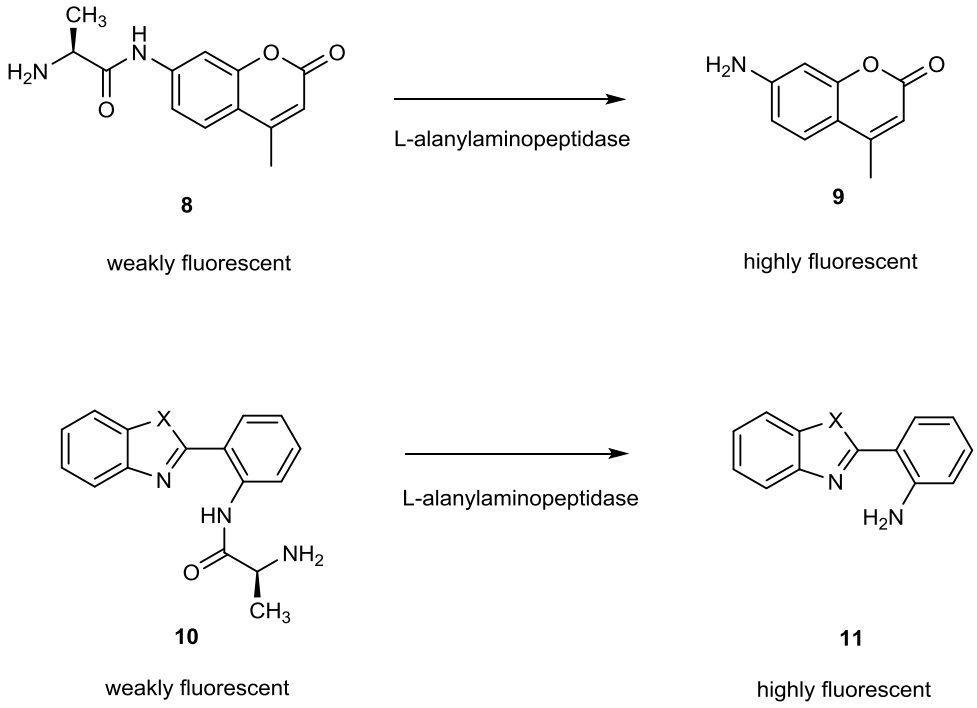


Figure 1. PABA-based fluorogenic self-immolative spacers for protease detection.



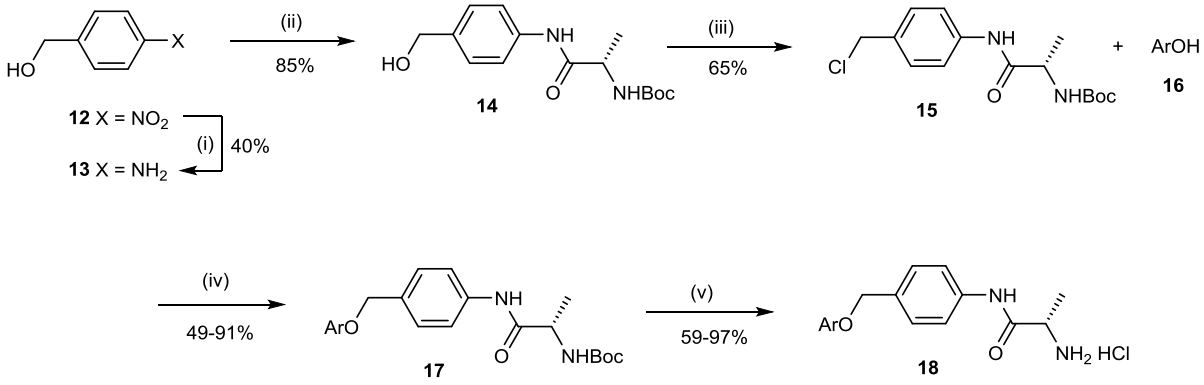
Scheme 2. Fluorogenic L-alanylaminopeptidase substrates.

The identification of specific types of enzyme activity in microorganisms has proved tremendously useful in diagnostic microbiology.[6] Of particular relevance to this paper is the detection of L-alanylaminopeptidase activity which has enabled the differentiation between Gram-positive and Gram-negative microorganisms.[7,8] This enzyme is widely distributed in Gram-negative microorganisms whereas, in contrast, it is generally absent or less abundant in most Gram-positive microorganisms. Fluorogenic substrates that have been used for the detection of L-alanylaminopeptidase activity include the commercially available coumarin derivative **8** which liberates the highly fluorescent 7-amino-4-methylcoumarin **9** in the presence of an L-alanylaminopeptidase enzyme (Scheme 2). We have previously described the synthesis and evaluation of a series of fluorogenic substrates **10** (X = S, O) that produced the corresponding fluorescent 2-(2-aminophenyl)benzoxazole **11** (X = O) and 2-(2-aminophenyl)benzothiazoles **11** (X = S) in the presence of L-alanylaminopeptidase.[9] Fluorogenic L-alanylaminopeptidase substrates derived from 2-(4-aminophenyl)benzothiazoles were also prepared and evaluated.[10]

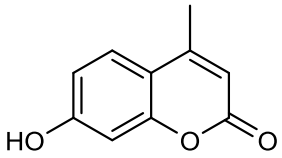
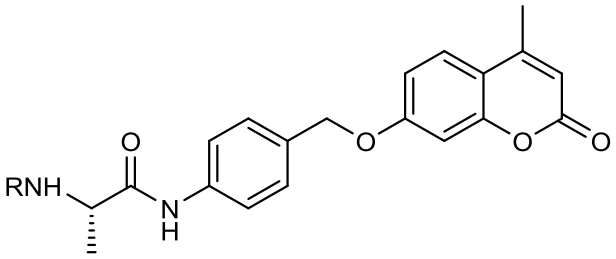
2. Synthesis of substrates

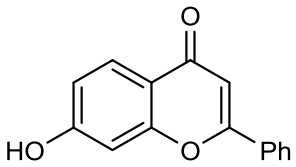
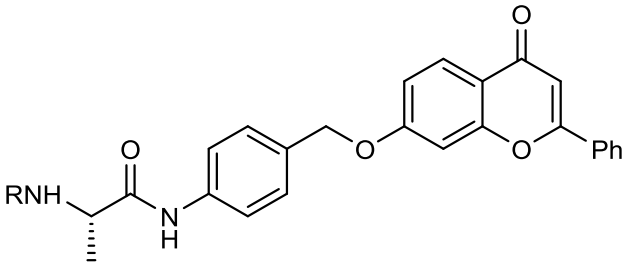
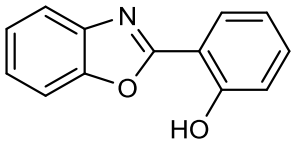
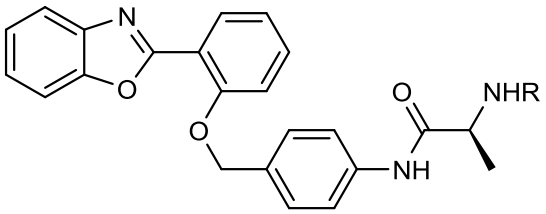
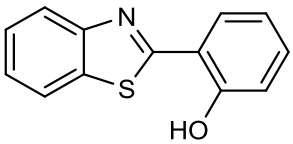
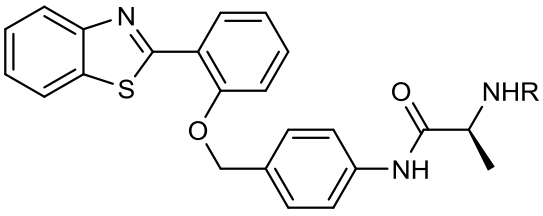
The substrates depicted in Scheme 2 all liberate fluorescent heterocyclic amine derivatives. In view of the availability and structural diversity of fluorescent phenols, we wished to develop substrates that would enable the detection of L-alanylaminopeptidase activity in microorganisms such that a fluorescent phenolic derivative is produced. A potential benefit of these proposed substrates would be that fluorogenic phenols with specific properties, e.g. tailored excitation/emission wavelengths, could be selected from an extensive pool of known molecules hence extending the availability of fluorophores that might be incorporated into aminopeptidase substrates. Thus, in this paper we describe the synthesis and evaluation of a series of novel self-immolative spacer substrates **18** (Table 1) for the purpose of detecting L-alanylaminopeptidase activity in microorganisms.

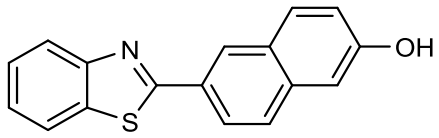
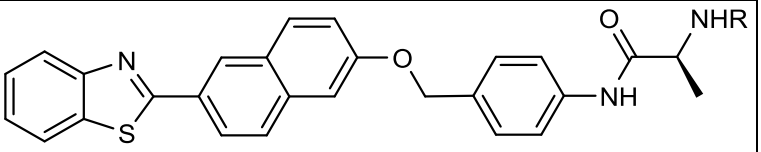
The synthetic route chosen for the preparation of the substrates **18** is shown in Scheme 3. Commercially available 4-aminobenzyl alcohol **13** was found to be relatively unstable to storage and hence it was prepared immediately before use by reduction of the readily available and inexpensive 4-nitrobenzyl alcohol **12**. A mixed anhydride condensation of amine **13** with Boc-protected L-alanine furnished compound **14** which was reacted with methanesulphonyl chloride giving the benzylic chloride **15** directly, presumably via displacement of the mesylate group in the initially formed mesylate derivative by chloride. Compound **15** was then reacted with an appropriate heterocyclic phenol **16** under basic conditions affording the Boc-protected derivatives **17a-e**. Treatment of these compounds with hydrogen chloride produced the required substrates **18a-e** respectively as their hydrochloride salts.



Scheme 3. Synthesis of substrates **18**. Reagents and conditions: (i) $Cu(II)(acac)_2$, $NaBH_4$, EtOH, rt; (ii) Boc-L-alanine, $^{i}BuOCOCl$, *N*-methylmorpholine, THF, -5 °C, 2 h then rt 20 h; (iii) $MeSO_2Cl$, Et_3N, CH_2Cl_2, 0 °C, 2 h then rt 18 h; (iv) Cs_2CO_3, DMF, 80-90 °C, 3-12 h; (v) EtOAc/HCl, rt, 1-2 h.

ArOH **16**	Boc-protected amines **17** and substrates **18**
 **16a**	 **17a** R = Boc 91% **18a** R = H. HCl 90%

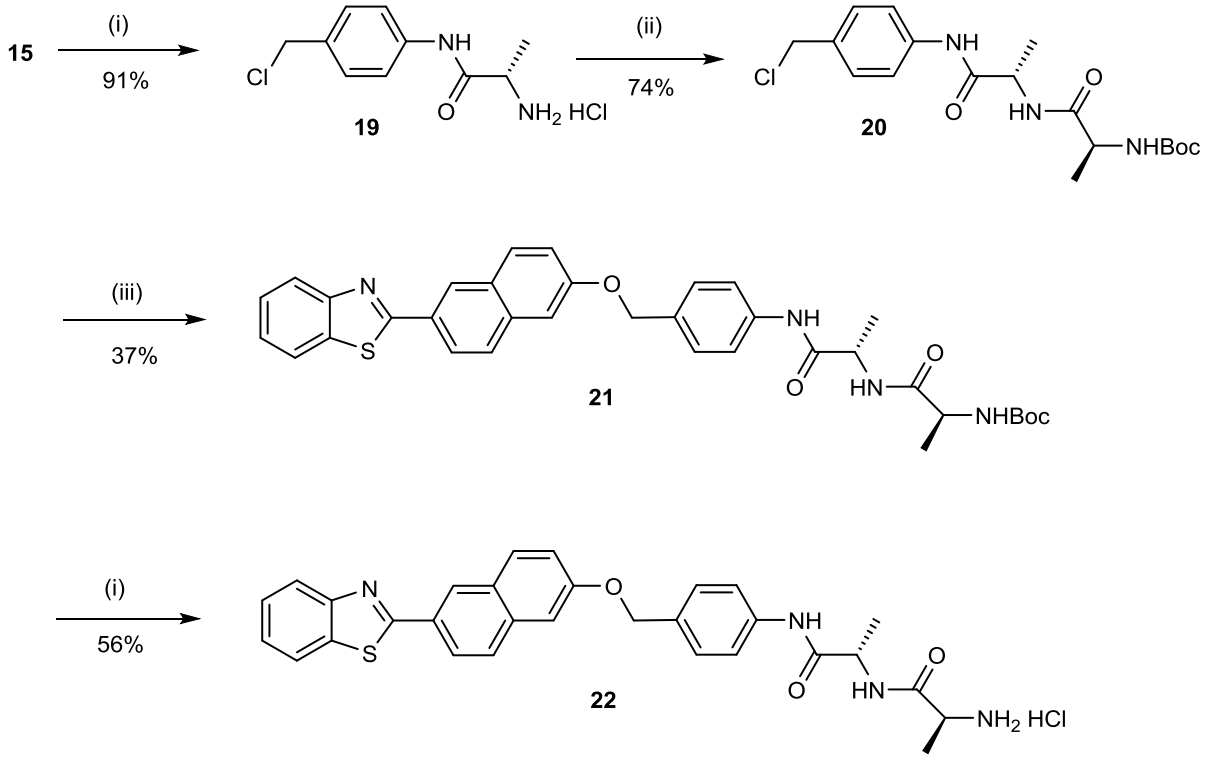
 **16b**[a]	 **17b** R = Boc 49% **18b** R = H. HCl 95%
 **16c**	 **17c** R = Boc 26% **18c** R = H. HCl 83%
 **16d**	 **17d** R = Boc 84% **18d** R = H. HCl 97%

 **16e**	 **17e** R = Boc 67% **18e** R = H. HCl 59%

[a] 3-Hydroxyflavone similarly gave a Boc-protected amine. However, attempted removal of the Boc-group resulted in fragmentation and formation of 3-hydroxyflavone.

Table 1. Structures and yields of self-immolative spacer substrates **18** and their Boc-protected precursors **17**

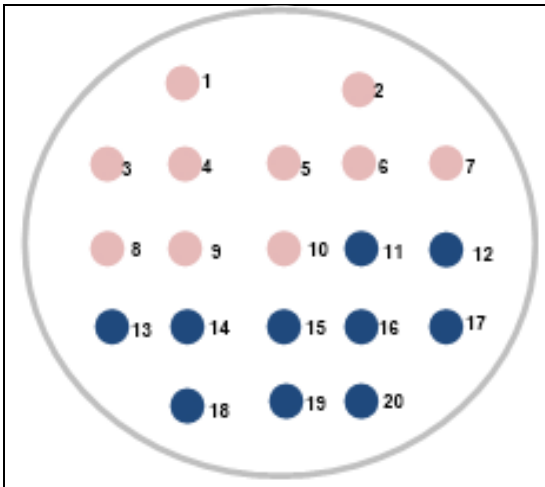
Based upon previous studies, di-L-alanyl aminopeptidase substrates were found to be less inhibitory to Gram-positive bacteria.[7] Thus, the substrate **22** was also prepared as part of this study in order to assess its scope of activity (Scheme 4). Removal of the Boc-group in compound **15** under acidic conditions gave compound **19** which was then subjected to a mixed anhydride coupling reaction with Boc-L-alanine affording the protected di-L-alanyl derivative **20**. The reaction of compound **20** with 6-(1,3-benzothiazol-2-yl)naphthalene-2-ol **16e** under basic conditions yielded compound **21** from which the required substrate **22** was obtained by treatment with hydrogen chloride (Scheme 4).



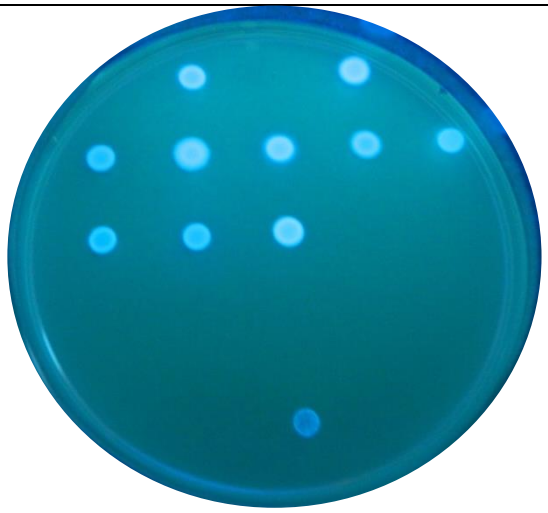
Scheme 4. Synthesis of the substrate **22**. Reagents and conditions: (i) EtOAc/HCl, rt, 1-2 h; (ii) Boc-L-alanine, ^{i}BuOCOCl, *N*-methylmorpholine, DMF , -5 ^{o}C, 2 h then rt 20 h; (iii) **16e**, Cs_2CO_3, DMF, 80-90 ^{o}C, 12 h.

3. Evaluation of substrates

Each substrate was evaluated in Columbia agar media (37 ^{o}C in air for 18 hours) on a single plate against 20 clinically important microorganisms, including 10 Gram-negative bacteria, 8 Gram-positive bacteria and 2 yeasts (substrate concentration 100 mgL^{-1}). The growth of the microorganisms was compared to control plates in which no substrate was present. The Gram-negative microorganisms all grew well on the control plates whereas the Gram-positive microorganisms and the yeasts showed only moderate growth. Figure 2 depicts the arrangement of microorganisms on the agar plates and shows a representative example of an agar plate produced by incorporation of substrate **18e** into the media.



Arrangement of microorganisms on plates[a]



Substrate **18e**

[a] Microorganisms are numbered in the sequence shown in the Tables. Pink spots represent Gram-negative bacteria, blue spots represent Gram-positive bacteria and the yeast species.

Figure 2. Representative Columbia agar plate depicting fluorescence generated by various microorganisms with substrate **18e** when viewed under UV illumination (360 nm).

The coumarin substrate **18a** gave intense, blue fluorescent colonies with most of the Gram-negative bacteria and moderately intense, blue fluorescent colonies with four of the Gram-positive bacteria (*S. pyogenes*, *L. monoctogenes*, *E. faecium* and *E. faecalis*) and also with the yeast species, *C. albicans* (Table 2). There was some diffusion of the fluorescence from the colonies into the surrounding media with this substrate and this could be a potential disadvantage when investigating polymicrobial cultures obtained from pathological specimens because the diffusion of fluorescence through the agar media into surrounding colonies may not allow a clear differentiation of species that demonstrate enzyme activity. The 7-hydroxyflavone derived substrate **18b** produced moderately intense, yellow fluorescent colonies with most of the Gram-negative bacteria (Table 2). Growth of the majority of the Gram-positive bacteria was inhibited by this substrate and hence no

fluorescence was observed. Growth of the yeast species, *C. glabrata*, was also inhibited by this substrate whereas *C. albicans* did show moderate growth but only produced very weak fluorescence.

		18a		18b	
	Microorganism / Reference[a]	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**
	Gram-negative microorganisms				
1	*Escherichia coli* NCTC 10418	++	++ blue	++	+ yellow
2	*Klebsiella pneumoniae* NCTC 9528	++	++ blue	++	+ yellow
3	*Providencia rettgeri* NCTC 7475	++	+ blue	++	+ yellow
4	*Enterobacter cloacae* NCTC 11936	++	++ blue	++	+ yellow
5	*Serratia marcescens* NCTC 10211	++	++ blue	++	+ yellow
6	*Salmonella typhimurium* NCTC 74	++	++ blue	++	+ yellow
7	*Pseudomonas aeruginosa* NCTC 10662	++	+ blue	++	-
8	*Yersinia enterocolitica* NCTC 11176	++	++ blue	++	+ yellow
9	*Burkholderia cepacia* NCTC 10743	++	++ blue	++	+ yellow
10	*Acinetobacter baumannii* NCTC 12156	++	++ blue	++	+ yellow
	Gram-positive microorganisms				
11	*Streptococcus pyogenes* NCTC 8306	+	+ blue	-	-
12	*Staphylococcus aureus* (MRSA) NCTC 11939	+	-	-	-
13	*Staphylococcus aureus* (MSSA) NCTC 6571	+/-	-	-	-
14	*Staphylococcus epidermidis* NCTC 11047	-	-	-	-
15	*Listeria monocytogenes* NCTC 11994	+	+ blue	Tr.	-
16	*Enterococcus faecium* NCTC 7171	+	+ blue	-	-
17	*Enterococcus faecalis* NCTC 775	+	+ blue	+	-
18	*Bacillus subtilis* NCTC 9372	Tr.	-	-	-
	Yeasts				
19	*Candida albicans* ATCC 90028	+	+ blue	+	Tr. yellow
20	*Candida glabrata* NCPF 3943	+	-	Tr.	-

[a]NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

[b] ++ strong growth, + moderate growth, +/- weak growth, Tr. trace of growth.

[c] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence, Tr. trace of fluorescence.

Table 2. Evaluation of substrates **18a** and **18b**.

The substrates **18c-e** all produced highly fluorescent colonies with the panel of Gram-negative microorganisms (Table 3). Thus, the benzothiazole derivative **18d** gave intensely yellow fluorescent colonies with all of the Gram-negative bacteria. This substrate was inhibitory towards the Gram-positive bacteria and no growth was apparent and hence no fluorescent colonies were produced. The yeast species, *C. albicans*, grew moderately well and produced blue-fluorescent colonies. The substrates **18c** and **18e** both gave similar results to substrate **18d**, except that the colonies of Gram-negative bacteria were associated with an intense green fluorescence (substrate **18c**, data not shown) and a strong blue fluorescence (substrate **18e**). The observed fluorescence with substrates **18c-e** was restricted to the microorganism colonies and this is an advantage over our previously described substrates **10** in which noticeable diffusion of the fluorophore into the surrounding media was apparent.[9] Additionally, some diffusion of the fluorophore is also observed with the aminocoumarin-derived substrate **8** in agar media. In accord with expectation, the di-L-alanyl substrate **22** was less inhibitory towards most of the selection of Gram-positive microorganisms compared to the mono-L-alanyl substrate **18e** and consequently blue fluorescent colonies were produced with growing Gram-positive microorganisms and also with both yeasts. It is interesting to

note that a chromogenic L-alanylaminopeptidase substrate based on a 9-(4-aminophenyl)-10-methylacridinium core was non-inhibitory to five of a panel of ten Gram-positive microorganisms and did not undergo hydrolysis even when microorganism growth occurred.[7] The di-L-alanyl-analogue was even less inhibitory, allowing growth of nine of the same panel of Gram-positive microorganisms. This contrasts with our work described here and elsewhere,[9] in which the L-alanyl fluorogenic substrates were often inhibitory to most Gram-positive microorganisms. This difference in detection profile between these two sets of fluorogenic and chromogenic substrates might be attributed to a variety of factors, including for example, the greater sensitivity of fluorogenic substrates, the higher degree of toxicity of the fluorogenic substrates to Gram-positive microorganisms, the degree of permeation of the substrates into the cell and structural differences between the substrates; the chromogenic substrates are quaternised heterocycles whereas the fluorogenic substrates are not.

		18d		**18e**		**22**	
	Microorganism / Reference[a]	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**
	Gram-negative microorganisms						
1	*Escherichia coli* NCTC 10418	++	++ yellow	++	++ blue	++	++ blue
2	*Klebsiella pneumoniae* NCTC 9528	++	++ yellow	++	++ blue	++	++ blue
3	*Providencia rettgeri* NCTC 7475	++	++ yellow	++	++ blue	++	++ blue
4	*Enterobacter cloacae* NCTC 11936	++	++ yellow	++	++ blue	++	++ blue
5	*Serratia marcescens* NCTC 10211	++	++ yellow	++	++ blue	++	++ blue
6	*Salmonella typhimurium* NCTC 74	++	++ yellow	++	++ blue	++	++ blue
7	*Pseudomonas aeruginosa* NCTC 10662	++	++ yellow	++	++ blue	++	++ blue
8	*Yersinia enterocolitica* NCTC 11176	++	++ yellow	++	++ blue	++	++ blue
9	*Burkholderia cepacia* NCTC 10743	++	++ yellow	++	++ blue	++	++ blue
10	*Acinetobacter baumannii* NCTC 12156	++	++ yellow	++	++ blue	++	++ blue
	Gram-positive microorganisms						
11	*Streptococcus*	-	-	-	-	Tr.	-

	pyogenes NCTC 8306						
12	*Staphylococcus aureus* (MRSA) NCTC 11939	-	-	-	-	+	++ blue
13	*Staphylococcus aureus* (MSSA) NCTC 6571	-	-	-	-	+	++ blue
14	*Staphylococcus epidermidis* NCTC 11047	-	-	-	-	-	-
15	*Listeria monocytogenes* NCTC 11994	-	-	-	-	+	+ blue
16	*Enterococcus faecium* NCTC 7171	-	-	-	-	+	+ blue
17	*Enterococcus faecalis* NCTC 775	-	-	-	-	+	+ blue
18	*Bacillus subtilis* NCTC 9372	-	-	-	-	-	-
	Yeasts						
19	*Candida albicans* ATCC 90028	+	++ blue	+	++ blue	+	++ blue
20	*Candida glabrata* NCPF 3943	-	-	-	-	+	++ blue

[a]NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

[b] ++ strong growth, + moderate growth, +/- weak growth, Tr. trace of growth.

[c] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence, Tr. trace of fluorescence.

Table 3. Evaluation of substrates **18d, 18e** and **22**

4. Conclusions

In conclusion, the substrates **18c-18e** all produced highly fluorescent colonies with the panel of Gram-negative microorganisms. This has been attributed to the combination of two synergistic effects; the wide distribution of L-alanylaminopeptidase in Gram-negative microorganisms and the inhibitory effect of these substrates against Gram-positive microorganisms. Some Gram-positive microorganisms gave fluorescent colonies with substrates that were not inhibitory to their growth (substrates **18a** and **22**).

5. Experimental

NMR spectra were recorded on a JEOL ECS400 Delta spectrometer at frequencies of 400 MHz for ^{1}H-NMR spectra and 101 MHz for ^{13}C-NMR spectra. All chemical shifts are quoted in ppm relative to TMS as an internal standard. The multiplicity of the signals is expressed as follows; s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet and m = multiplet, or in combinations (*e.g.* td = triplet of doublets). High resolution mass spectra (HRMS) were obtained using a Finnigan MAT 900 XLT high resolution double focussing mass spectrometer or a Thermo Scientific LTQ Orbitrap XL mass spectrometer using nanoelectrospray ionisation. Low resolution mass spectrometry (LRMS)

was performed via direct injection of dilute methanolic solutions (containing 0.1% formic acid) into a Thermo Finnigan LCQ Advantage MS detector using electrospray ionisation. Infra-red spectra were obtained via a diamond anvil sample cell using a Perkin Elmer 1000FT-IR spectrometer. Melting points are reported uncorrected as determined on a Stanford Research Systems MPA161 melting point apparatus. Thin layer chromatography was performed on Merck plastic foil plates pre-coated with silica gel 60 F_{254}. Merck silica gel 60 was used for column chromatography.

The preparation of agar plates followed the procedure described previously.[10]

5.1 4-Aminobenzyl alcohol (13)[11]

A mixture of $Cu(acac)_2$ (0.35 g, 1.31 mmol) and $NaBH_4$ (0.25 g, 6.53 mmol) in EtOH (70 mL) was stirred (1 h) under N_2. A solution of 4-nitrobenzyl alcohol (1.00 g, 6.53 mmol) in EtOH (70 mL) was added followed by $NaBH_4$ (0.50 g, 12.06 mmol). The mixture was stirred (2 h), distilled water (120 mL) was added and the mixture was filtered. The filtrate volume was reduced by evaporation of most of the EtOH and the remaining solution was extracted with CH_2Cl_2 (2 x 50 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated to yield a brown viscous oil which solidified upon standing yielding compound **13** (0.32 g, 40%). This product was used directly without further purification. ^{1}H-NMR (400 MHz d_6-DMSO) δ 6.91 (2H, d, *J* = 8.2 Hz, Ar-H), 6.46 (2H, d, *J* = 8.2 Hz, Ar-H), 4.88 (2H, bs, NH), 4.75 (1H, t, *J* = 5.4 Hz, OH), 4.24 (2H, d, *J* = 5.4 Hz, CH_2).

5.2 6-(1,3-Benzothiazol-2-yl)naphthalen-2-ol (16e)

A homogeneous mixture of 2-(6-methoxynaphthalen-2-yl)benzothiazole[12] (2.0 g, 6.86 mmol) and pyridine hydrobromide (20.0 g, 125 mmol) was heated (4 h) at 190 °C < T < 200 °C . The reaction was allowed to cool to room temperature and water (30 mL) was added. The mixture was poured into warm water (100 mL) and the precipitate was collected. The resulting solid was washed well with water and dried in a desiccator giving compound **16e** as grey crystals (1.88 g, 99%), mp 251-253°C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.15 (1H, broad s, OH), 8.56 (1H, m, Ar-H), 8.16 (1H, d, *J* = 7.8 Hz, Ar-H), 8.13-7.97 (3H, m, Ar-H), 7.86 (1H, d, *J* = 8.7 Hz, Ar-H), 7.56 (1H, td, *J* = 7.8 and 1.0 Hz, Ar-H), 7.46 (1H, td, *J* = 7.8 and 1.0 Hz, Ar-H), 7.23-7.16 (2H, m, Ar-H); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 168.3 (Ar-C) , 154.2 (Ar-C), 147.4 (Ar-C), 136.7 (Ar-C), 134.9 (Ar-C), 131.3 (Ar-C), 127.9 (Ar-C), 127.8 (Ar-C), 127.8 (Ar-C), 127.6 (Ar-C), 127.2 (Ar-C), 125.8 (Ar-C), 124.7 (Ar-C), 123.1 (Ar-C), 122.8 (Ar-C), 120.3 (Ar-C), 109.4 (Ar-C); IR ν_{max} 3400-3200 (OH), 1635, 1496, 1296, 1260, 1190, 1156, 923, 867, 794, 758, 721 cm^{-1}; HRMS calcd for $C_{17}H_{12}NOS$ $[M+H]^+$ 278.0634; found 278.0637.

5.3 *tert*-Butyl {(2*S*)-1-[4-(Hydroxymethyl)anilino]-1-oxopropan-2-yl}carbamate (14)

Amine **13** (0.74 g, 6.00 mmol) was dissolved in dry THF (40 mL) and the resulting solution was cooled to -5 °C in an ice/salt bath. In a separate flask, to a stirred solution of Boc-L-alanine (1.20 g, 6.30 mmol) in dry THF (40 mL) was added *N*-methylmorpholine (0.61 g, 6.00 mmol) and the mixture was cooled to -5 °C. Isobutyl chloroformate (0.82 g, 6.00 mmol) was then added, and after stirring for 90 seconds at -5 °C, the solution of amine **13** was added drop-wise. The resulting mixture was stirred at -5 °C (1 h) and then at room temperature overnight. The solvent was evaporated and the resulting crude product was dissolved in CH_2Cl_2 and washed successively with 0.1 M aq. citric acid solution, 10% aq. $NaHCO_3$ solution and water. The organic layer was dried ($MgSO_4$) and evaporated. The resulting oil crystallised upon standing giving compound **14** (1.50 g, 85%) as light orange crystals, mp

128-129 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 9.84 (1H, broad s, NH), 7.50 (2H, d, *J* = 8.3 Hz, H-2), 7.19 (2H, d, *J* = 8.3 Hz, H-3) 7.03 (1H, d, *J* = 7.3 Hz, NH), 5.07 (1H, t, *J* = 5.5 Hz, OH), 4.39 (2H, d, *J* = 5.5 Hz, CH_2), 4.02-4.10 (1H, m, CH), 1.34 (9H, s, 3 x CH_3), 1.21 (3H, d, *J* = 7.3 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 172.2 (C=O), 155.7 (C=O), 138.2 (Ar-C), 137.8 (Ar-C), 127.3 (2 x Ar-C), 119.4 (2 x Ar-C), 78.5 ($\underline{C}(CH_3)_3$), 63.1 ($\underline{C}H_2OH$), 50.9 ($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 18.6 ($CH\underline{C}H_3$); IR ν_{max} 3360 (broad), 3337, 3330, 2987, 1690 (C=O), 1670 (C=O), 1519, 1316, 1245, 1167, 1001, 834 cm^{-1}; HRMS calcd for $C_{15}H_{23}N_2O_4$ 295.1652 $[M+H]^+$; found 295.1656.

5.4 *tert*-Butyl {(2*S*)-1-[4-(Chloromethyl)anilino]-1-oxopropan-2-yl}carbamate (15)

To a stirred solution of compound **14** (1.50 g, 5.10 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C under N_2 was added Et_3N (1.55 g, 15.30 mmol) followed by the drop-wise addition of methanesulphonyl chloride (1.76 g, 15.30 mmol). The reaction mixture was stirred at 0 °C (2 h) and then at room temperature (18 h). The solvent was then evaporated and EtOAc (60 mL) was added to the residue. The solution was washed successively with 5% aq. citric acid solution (100 mL), 5% aq. $NaHCO_3$ solution (100 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$) and evaporated. The crude product was purified by column chromatography (SiO_2, CH_2Cl_2) giving compound **15** as an off-white powder (1.04 g, 65%), mp 142-143 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.03 (1H, broad s, NH), 7.60 (2H, d, *J* = 8.7 Hz, H-3), 7.37 (2H, d, *J* = 8.7 Hz, H-2), 7.11 (1H, d, *J* = 6.9 Hz, NH), 4.72 (2H, s, CH_2), 4.10 (1H, m, CH), 1.38 (9H, s, 3 x CH_3), 1.25 (3H, d, *J* = 7.3 Hz, CH_3); ^{13}C-NMR (101MHz, d_6-DMSO) δ 172.6 (C=O), 155.7 (C=O), 139.7 (Ar-C), 132.8 (Ar-C), 130.0 (2 x Ar-C), 119.7 (2 x Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 51.0 ($\underline{C}HCH_3$), 46.7 ($\underline{C}H_2Cl$), 28.7 ($C(\underline{C}H_3)_3$), 18.5 ($CH\underline{C}H_3$); IR ν_{max} 3337 (broad, NH), 2986, 1671(C=O), 1514, 1318, 1245, 1158, 666 cm^{-1}; HRMS calcd for $C_{15}H_{22}{}^{35}ClN_2O_3$ $[M+H]^+$ 313.1313; found 313.1315.

5.5 (2*S*)-2-Amino-*N*-[4-(chloromethyl)phenyl]propanamide Hydrochloride (19)

Compound **15** (3.00 g) in saturated anhydrous EtOAc/HCl (35 mL) was stirred (1 h) at room temperature. The solvent was evaporated giving compound **19** (2.30 g, 91%) as a dark, sticky solid which was used without further purification. ^{1}H-NMR (400 MHz, d_6-DMSO) δ 11.13 (1H, broad s, NH), 8.46 (3H, broad s, NH_3^+), 7.69 (2H, d, *J* = 8.4 Hz, H-3), 7.42 (2H, d, *J* = 8.4 Hz, H-4), 4.74 (2H, s, CH_2), 4.18-4.08 (1H, m, CH), 1.49 (3H, d, *J* = 6.87 Hz, CH_3); ^{13}C-NMR (101MHz, d_6-DMSO) δ 168.9 (C=O), 139.0 (Ar-C), 133.5 (Ar-C), 130.1 (2 x Ar-C), 119.9 (2 x Ar-C), 49.4 ($\underline{C}HCH_3$), 46.6 (CH_2Cl), 17.8 ($CH\underline{C}H_3$); IR ν_{max} 3650-2350 (broad, NH_3^+ and NH), 1681 (C=O stretch), 1607, 1547, 1511, 1416, 1307, 1257, 1193, 1109, 824 cm$^{-1.}$

5.6 *tert*-Butyl *N*-[(1*S*)-1-{[(1*S*)-1-{[4-(Chloromethyl)phenyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamate (20)

A mixture of compound **19** (1.15 g, 5.00 mmol) and *N*-methylmorpholine (0.56 g, 5.50 mmol) in dry DMF (20 mL) was cooled to -5 °C in an ice/salt bath. In a separate flask, to a stirred solution of Boc-L-alanine (0.99 g, 5.25 mmol), in dry DMF (20 mL) was added *N*-methylmorpholine (0.51 g, 5.00 mmol) and the mixture was cooled to -5 °C. Isobutyl chloroformate (0.68 g, 5.00 mmol) was added to this mixture and after stirring for 90 seconds at -5 °C, the solution of the amine **19** was added. The resulting mixture was stirred at -5 °C (1 h) and then at room temperature overnight. The solvent was evaporated and the residue was dissolved in EtOAc (60 mL), washed successively with 0.1 M aq. citric acid solution (30 mL), 10% aq. $NaHCO_3$ solution (30 mL) and water (30 mL). The organic layer

was dried ($MgSO_4$) and evaporated giving compound **20** as a light brown powder (1.25 g, 74 %), mp 164 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.05 (1H, s, NH), 8.05 (1H, d, *J* = 7.3 Hz, NH), 7.60 (2H, d, *J* = 8.2 Hz, Ar-H), 7.37 (2H, d, *J* = 8.2 Hz, Ar-H), 7.00 (1H, d, *J* = 7.3 Hz, NH), 4.72 (2H, s, CH_2), 4.39 (1H, dt, *J* = 7.3 Hz, CH), 3.99 (1H, dt, *J* = 7.3 Hz, CH), 1.38 (9H, s, 3 x CH_3), 1.31 (3H, d, *J* = 6.9 Hz, CH_3), 1.18 (3H, d, *J* = 6.9 Hz, CH_3);^{13}C-NMR (101 MHz, d_6-DMSO) δ 173.1 (C=O), 171.8 (C=O), 155.7 (C=O), 139.5 (Ar-C), 132.9 (Ar-C), 130.0 (2 x Ar-C), 119.7 (2 x Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 50.2 ($\underline{C}HCH_3$), 49.5 ($\underline{C}HCH_3$), 46.7 ($\underline{C}H_2Cl$), 28.7 ($C(\underline{C}H_3)_3$), 18.7 ($CH\underline{C}H_3$), 18.5 ($CH\underline{C}H_3$); IR ν_{max} 3387 (NH), 3320 (NH), 3258 (NH), 2986, 1689 (C=O), 1649 (C=O), 1517, 1292, 1255, 1166, 1023, 679, 656 cm^{-1}; HRMS calcd for $C_{18}H_{27}{}^{35}ClN_3O_4$ 384.1685 $[M+H]^+$; found 384.1685.

5.7 *tert*-Butyl *N*-[(1S)-1-[(4-{[(4-Methyl-2-oxo-2*H*-chromen-7-yl)oxy]methyl}phenyl)carbamoyl]ethyl]carbamate (17a)

A mixture of 4-methylumbelliferone **16a** (0.14 g, 0.80 mmol), compound **15** (0.30 g, 0.80 mmol) and Cs_2CO_3 (0.26 g, 0.80 mmol) was stirred (3 h) in dry DMF (20 mL) at 80 °C. The mixture was allowed to cool to room temperature and the solvent was evaporated. CH_2Cl_2 (30 mL) was added to the residue. The mixture was filtered, the filtrate was washed successively with 10% aq. $NaHCO_3$ solution (30 mL) and water (30 mL). The organic fraction was dried ($MgSO_4$) and evaporated giving compound **17a** as a grey powder (0.33 g, 91%), mp 180-182 °C which was used without any further purification; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 9.10 (1H, broad s, NH), 7.64 (1H, d, *J* = 8.7 Hz, Ar-H), 7.58 (2H, d, *J* = 8.7 Hz, Ar-H), 7.37 (2H, d, *J* = 8.7 Hz, Ar-H), 7.08-6.95 (3H, m, 2 x Ar-H, NH), 6.17 (1H, d, *J* = 0.9 Hz, Ar-H), 5.11 (2H, s, CH_2), 4.07 (1H, m, CH), 2.35 (3H, s, CH_3), 1.34 (9H, s, 3 x CH_3), 1.21 (3H, d, *J* = 7.3 Hz, CH_3);^{13}C-NMR (101 MHz, d_6-DMSO) δ 172.5 (C=O), 161.9 (C=O), 160.7 (C=O), 155.7 (Ar-C), 155.2 (Ar-C), 153.9 (Ar-C), 139.5 (Ar-C), 131.3 (Ar-C), 129.2 (2 x Ar-C), 127.0 (Ar-C), 119.6 (2 x Ar-C), 113.8 (Ar-C), 113.3 (Ar-C), 111.7 (Ar-C), 102.2 (Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 70.2 (CH_2), 51.0 ($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 18.7 (CH_3), 18.5 (CH_3); IR ν_{max} 3321 (NH), 3315 (NH), 2980, 1716 (C=O), 1666 (C=O), 1614 (C=O), 1517, 1385, 1249, 1155, 1069, 842 cm^{-1}; HRMS calcd for $C_{25}H_{29}N_2O_6$ $[M+H]^+$ 453.2020; found 453.2014.

5.8 *tert*-Butyl *N*-[(1S)-1-[(4-{[(4-Oxo-2-phenyl-4*H*-chromen-7-yl)oxy]methyl}phenyl)carbamoyl]ethyl]carbamate (17b)

A mixture of 7-hydroxyflavone **16b** (0.38 g, 1.60 mmol), compound **15** (0.5 g, 1.60 mmol) and Cs_2CO_3 (0.52 g, 1.60 mmol) was stirred (5 h) in dry DMF (20 mL) at 90°C. The mixture was allowed to cool to room temperature and the solvent was evaporated. EtOAc (30 mL) was added to the residue and the resulting mixture was washed successively with 2.0 M aq. NaOH solution (3 x 30 mL) and water (30 mL). The organic fraction was dried ($MgSO_4$) and evaporated affording compound **17b** as a fluffy yellow powder (0.40 g, 49%), mp 113 °C which was not purified further; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.03 (1H, s, NH), 8.10 (2H, dd, *J* = 7.8 and 1.8 Hz, Ar-H), 7.96 (1H, d, *J* = 9.2 Hz, Ar-H), 7.67 (2H, d, *J* = 8.7 Hz, Ar-H), 7.62-7.56 (3H, m, Ar-H), 7.48-7.41 (3H, m, Ar-H), 7.15-7.08 (2H, m, Ar-H, NH), 6.99 (1H, s, Ar-H), 5.22 (2H, s, CH_2), 4.13 (1H, dt, *J* = 7.1 Hz, CH), 1.39 (9H, s, 3 x CH_3), 1.27 (3H, d, *J* = 7.3 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 176.9 (C=O), 172.5 (C=O), 163.5 (C=O), 162.7 (Ar-C), 47.9 (Ar-C), 155.7 (Ar-C), 139.6 (Ar-C), 132.2 (Ar-C), 131.7 (Ar-C), 131.2 (Ar-C), 129.6 (2 x Ar-C), 129.3 (2 x Ar-C), 126.7 (3 x Ar-C), 119.6 (2 x Ar-C), 117.8 (Ar-C), 115.7 (Ar-C), 107.4 (Ar-C), 102.4 (Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 70.4 (CH_2O), 51.0 ($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 18.5 (CH_3); IR ν_{max} 3350-3260 (broad, NH), 2984, 1681

(C=O), 1628 (C=O), 1600, 1517, 1449, 1366, 1246, 1161, 829, 771 cm^{-1}; LRMS calcd for $C_{30}H_{30}N_2O_6$ $[M+H]^+$ 537.55; found 536.99.

5.9 *tert*-Butyl *N*-[(1*S*)-1-({4-[2-(1,3-Benzoxazol-2-yl)phenoxymethyl]phenyl}carbamoyl)ethyl]carbamate (17c)

A mixture of 2-(2-hydroxyphenyl)benzoxazole **16c** (0.31 g, 1.45 mmol), compound **15** (0.500 g 1.60 mmol) and Cs_2CO_3 (0.781 g, 2.40 mmol) were stirred (5 h) in dry DMF (20 mL) at 90°C. The mixture was then allowed to cool to room temperature and the solvent was evaporated. EtOAc (40 mL) was added to the residue and the resulting mixture was washed successively with saturated aq. $NaHCO_3$ solution (40 mL) and water (40 mL). The organic fraction was dried ($MgSO_4$) and evaporated. The residue was triturated with Et_2O and the resulting material was purified by column chromatography (SiO_2, CH_2Cl_2 changing to CH_2Cl_2-MeOH (98:2)) giving compound **17c** as a light brown powder (0.19 g, 26%), mp 101°C; ^{1}H-NMR (400 MHz d_6-DMSO) δ 9.92 (1H, s, NH), 8.00 (1H, dd, *J* = 7.6 Hz, Ar-H), 7.45-7.59 (5H, m, Ar-H), 7.35-7.41 (2H, m, Ar-H), 7.30 (1H, dd, *J* = 8.7 Hz, Ar-H), 7.11 (1H, t, *J* = 7.8 Hz, Ar-CH), 7.04 (1H, d, *J* = 6.9 Hz, NH), 5.24 (2H, s, CH_2), 4.06 (1H, m, CH), 1.33 (9H, s, 3 x CH_3), 1.20 (3H, d, *J* = 7.3 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 162.0 (C=O), 155.7 (C=O), 150.6 (Ar-C), 141.7 (Ar-C), 139.1 (Ar-C), 133.7 (Ar-C), 132.0 (Ar-C), 131.6 (Ar-C), 128.1 (Ar-C), 127.5 (Ar-C), 125.8 (2 x Ar-C), 125.2 (Ar-C), 121.6 (Ar-C), 120.2 (Ar-C), 119.6 (2 x Ar-C), 119.5 (Ar-C), 116.5 (Ar-C), 115.0 (Ar-C), 111.2 (Ar-C), 78.5 ($\underline{C}(CH_3)_3$), 70.2 (CH_2), 50.9($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 19.3 ($CH\underline{C}H_3$); IR ν_{max} 3306 (broad, NH), 2976, 1669 (C=O), 1602, 1516, 1453, 1414, 1365, 1311, 1245, 1160, 1055, 1018, 822, 749 cm^{-1}; HRMS calcd for $C_{28}H_{30}N_3O_5$ $[M+H]^+$ 488.2180; found 488.2171.

5.10 *tert*-Butyl *N*-[(1*S*)-1-({4-[2-(1,3-Benzothiazol-2-yl)phenoxymethyl]phenyl}carbamoyl)ethyl]carbamate (17d)

A mixture of 2-(2-hydroxyphenyl)benzothiazole **16d** (0.33 g, 1.47 mmol), compound **15** (0.46 g, 1.47 mmol) and Cs_2CO_3 (0.48 g, 1.47 mmol) was stirred (6 h) in dry DMF (20 mL) at 90 °C. The mixture was allowed to cool to room temperature and the solvent was evaporated. EtOAc (30 mL) was added to the residue and the resulting mixture was washed successively with 10% aq. $NaHCO_3$ solution (40 mL) and water (40 mL). The organic fraction was dried ($MgSO_4$) and evaporated. The residue was purified by column chromatography (SiO_2, CH_2Cl_2 changing to CH_2Cl_2-MeOH (98:2)) giving compound **17d** as a white powder (0.27 g, 84%), mp 102 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.04 (1H, s, NH), 8.46 (1H, dd, *J* = 7.8 and 1.8 Hz, Ar-H), 8.12-8.03 (2H, m, Ar-H), 7.67 (2H, d, *J* = 8.7 Hz, Ar-H), 7.57-7.50 (4H, m, Ar-H), 7.44-7.38 (2H, m, Ar-H), 7.17 (1H, t, *J* = 7.1 Hz, Ar-H), 7.12 (1H, *J* = 7.3 Hz, NH), 5.39 (2H, s, CH_2), 4.13 (1H, dt, *J* = 7.1 Hz, CH), 1.39 (9H, s, 3 x CH_3), 1.27 (3H, d, *J* = 6.9 Hz, CH_3);^{13}C-NMR (101 MHz, d_6-DMSO) δ 172.5 (C=O), 162.7 (C=O), 156.4 (Ar-C), 155.7 (Ar-C), 152.0 (Ar-C), 139.6 (Ar-C), 136.0 (Ar-C), 132.8 (Ar-C), 131.2 (Ar-C), 129.5 (2 x Ar-C), 129.4 (Ar-C), 126.8 (Ar-C), 125.4 (Ar-C), 122.9 (Ar-C), 122.4 (Ar-C), 122.0 (Ar-C), 121.7 (Ar-C), 119.6 (2 x Ar-C), 114.4 (Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 70.7 (CH_2O), 51.0 ($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 18.5 (CH_3); IR ν_{max} 3360-3250 (broad, NH), 2982, 1669 (C=O), 1601 (C=O), 1510, 1499, 1244, 1161, 754 cm^{-1}; LRMS calcd for $C_{28}H_{29}N_3O_4SNa$ $[M+Na]^+$ 526.60; found 526.13.

5.11 *tert*-Butyl *N*-[(1S)-1-{[4-({[6-(1,3-Benzothiazol-2-yl)naphthalen-2-yl]oxy}methyl)phenyl]carbamoyl}ethyl]carbamate (17e)

A mixture of **16e** (0.30 g, 1.08 mmol), compound **15** (0.41 g, 1.30 mmol) and Cs_2CO_3 (0.53 g, 1.62 mmol) was stirred (12 h) in dry DMF (20 mL) at 90 °C. The mixture was allowed to cool to room temperature and the solvent was evaporated. EtOAc (60 mL) was added to the residue and the mixture was washed successively with 10% aq. $NaHCO_3$ solution (30 mL) and water (2 x 20 mL). The organic fraction was dried ($MgSO_4$) and evaporated. The residue was purified by column chromatography (eluent; EtOAc, 100%) giving compound **17e** as a pink powder (0.40 g, 67%), mp 204-205 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.04 (1H, broad s, NH), 8.63 (1H, d, *J* = 0.9 Hz, NH), 8.21-8.15 (2H, m, Ar-H), 8.12-8.05 (2H, m, Ar-H), 7.97 (1H, d, *J* = 8.7 Hz, Ar-H), 7.67 (2H, d, *J* = 8.7 Hz, Ar-H), 7.59-7.53 (2H, m, Ar-H), 7.51-7.43 (3H, m Ar-H), 7.33 (1H, dd, *J* = 9.2 and 2.3 Hz, Ar-H), 7.11 (1H, d, *J* = 7.3 Hz, Ar-H), 5.21 (2H, s, CH_2), 4.13 (1H, dt, *J* = 7.3 Hz, CH), 1.39 (9H, s, 3 x CH_3), 1.27 (3H, d, *J* = 7.3 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 172.5 (C=O), 168.1 (C=O), 158.3 (Ar-C), 155.7 (Ar-C), 154.2 (Ar-C), 139.5 (Ar-C), 136.3 (Ar-C), 135.0 (Ar-C), 131.7 (Ar-C), 131.1 (Ar-C), 129.2 (2 x Ar-C), 128.7 (Ar-C), 128.6 (Ar-C), 128.3 (Ar-C), 127.8 (Ar-C), 127.2 (Ar-C), 125.9 (Ar-C), 125.0 (Ar-C), 123.2 (Ar-C), 122.9 (Ar-C), 120.6 (Ar-C), 119.6 (2 x Ar-C), 108.0 (Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 69.9 (CH_2), 51.0 ($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 18.6 (CH_3); IR ν_{max} 3312 (broad, NH), 2980, 1688 (C=O), 1663 (C=O), 1603, 1520, 1248, 1161, 812, 757, 674 cm^{-1}; LRMS calcd for $C_{32}H_{31}N_3O_4SNa$ $[M+Na]^+$ 576.67; found 576.06.

5.12 (2*S*)-2-Amino-*N*-(4-{[(4-methyl-2-oxo-2*H*-chromen-7-yl)oxy]methyl}phenyl)propanamide Hydrochloride (18a)

Compound **17a** (0.26 g, 0.57 mmol) was stirred (2 h) in anhydrous EtOAc/HCl (15 mL) at room temperature. The resulting precipitate was collected and dried giving compound **18a** (0.20 g, 90%) as a white powder, mp 202-204 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.92 (1H, broad s, NH), 8.35 (3H, broad s, NH_3^+), 7.61-7.68 (3H, m, Ar-H), 7.41 (2H, d, *J* = 8.7 Hz, Ar-H), 7.02 (1H, d, *J* = 2.3 Hz, Ar-H), 6.97 (1H, dd, *J* = 8.7 Hz, 2.3 Hz, Ar-H), 6.17 (1H, d, *J* = 0.9 Hz, Ar-H), 5.13 (2H, s, CH_2), 4.01-4.13 (1H, m, CH), 2.35 (3H, s, CH_3), 1.43 (3H, d, *J* = 6.9 Hz, CH_3);^{13}C-NMR (101 MHz, d_6-DMSO) δ 168.9 (C=O), 161.8 (C=O), 160.7 (Ar-C), 155.2 (Ar-C), 154.0 (Ar-C), 138.8 (Ar-C), 132.2 (Ar-C), 129.3 (2 x Ar-C), 127.0 (Ar-C), 119.9 (2 x Ar-C), 113.8 (Ar-C), 113.3 (Ar-C), 111.8 (Ar-C), 102.2 (Ar-C), 70.1 (CH_2), 18.7 (CH_3), 17.8 (CH_3); IR ν_{max} 3600-2550 (broad, NH and NH_3^+), 1682 (C=O), 1606 (C=O), 1514, 1395, 1260, 836 cm^{-1}; HRMS calcd for $C_{20}H_{21}N_2O_4$ $[M]^+$ 353.1496; found 353.1495.

5.13 (2*S*)-2-Amino-*N*-(4-{[(4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy]methyl}phenyl)propanamide Hydrochloride (18b)

Compound **17b** (0.24 g, 0.47 mmol) was stirred (1 h) in anhydrous EtOAc/HCl (10 mL) at room temperature. The resulting precipitate was collected and dried giving compound **18b** as an orange powder (0.20 g, 95%), mp 98-100 °C; ^{1}H-NMR (400 MHz d_6-DMSO) δ 11.05 (1H, s, NH), 8.45 (3H, d, *J* = 3.7 Hz, NH_3^+), 8.10 (2H, dd, *J* = 7.3 and 1.8 Hz, Ar-H), 7.97 (1H, d, *J* = 8.7 Hz, Ar-H), 7.75 (2H, d, *J* = 8.7 Hz, Ar-H), 7.63-7.56 (3H, m, Ar-H), 7.51 (2H, d, *J* = 8.7 Hz, Ar-H), 7.44 (1H, d, *J* = 2.3 Hz, Ar-H), 7.14 (1H, dd, *J* = 8.7 and 2.3 Hz, Ar-H), 6.99 (1H, s, Ar-H), 5.25 (2H, s, CH_2), 4.17-4.10 (1H, m, CH), 1.50 (3H, d, *J* = 6.9 Hz, CH_3);^{13}C-NMR (101 MHz, d_6-DMSO) δ 176.9 (C=O), 168.9 (C=O), 163.4 (Ar-C), 162.7 (Ar-C), 157.9 (Ar-C), 138.9 (Ar-C), 132.2 (Ar-C), 132.0 (Ar-C), 131.7 (Ar-C), 129.63 (2 x Ar-C), 129.3 (2 x Ar-C), 126.7 (-CH=), 119.9 (2 x Ar-C), 117.8 (Ar-C), 115.7 (Ar-C), 107.3 (Ar-C), 102.5 (Ar-C), 70.3 (CH_2), 49.4 ($\underline{C}HCH_3$), 17.8 (CH_3); IR ν_{max} 3600-2250 (broad, NH_3^+ NH), 1693 (C=O), 1614 (C=O), 1515, 1450, 1378, 1246, 1173, 1092, 829, 772, 674 cm^{-1}; HRMS calcd for $C_{25}H_{23}N_2O_4$ $[M]^+$ 415.1652; found 415.1647.

5.14 (2*S*)-2-Amino-*N*-{4-[2-(1,3-Benzoxazol-2-yl)phenoxymethyl]phenyl}propanamide Hydrochloride (18c)

Compound **17c** (0.25 g, 0.51 mmol) was stirred (1 h) in anhydrous EtOAc/HCl (5 mL) at room temperature. The resulting precipitate was collected and dried giving compound **18c** as a light brown solid (0.18 g, 83%), mp 132°C, ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.88 (1H, s, NH), 8.36 (3H, bs, NH_3^+), 8.00 (1H, dd, *J* = 7.2 Hz, Ar-H), 7.63-7.78 (4H, m, Ar-H), 7.50-7.56 (3H, m, Ar-H), 7.35-7.41 (2H, m, Ar-H), 7.30 (1H, dd, *J* = 8.7 Hz, Ar-H), 7.12 (1H, t, *J* = 7.3 Hz, Ar-H), 5.25 (2H, s, CH_2), 4.04 (1H, m, CH), 1.42 (3H, d, *J* = 6.9 Hz, CH_3), ^{13}C-NMR (101 MHz, d_6-DMSO) δ 155.7 (C=O), 150.6 (Ar-C), 141.7 (Ar-C), 139.1 (Ar-C), 133.7 (Ar-C), 132.0 (Ar-C), 131.6 (Ar-C), 128.1 (Ar-C), 127.5 (Ar-C), 127.2 (2 x Ar-C), 125.1 (Ar-C), 121.3 (Ar-C), 121.0 (Ar-C), 119.4 (2 x Ar-C), 119.0 (Ar-C), 116.5 (Ar-C), 115.0 (Ar-C), 111.1 (Ar-C), 70.2 (CH_2), 50.3 ($\underline{C}HCH_3$), 17.7 ($CH\underline{C}H_3$); IR ν_{max} 3300-2025 (broad, NH + NH_3^+), 2976, 1668 (C=O), 1609, 1515, 1455, 1418, 1310, 1139, 994, 797, 751, 722, 704 cm^{-1}; HRMS calcd for $C_{23}H_{22}N_3O_3$ $[M]^+$ 388.1656; found 388.1653.

5.15 (2*S*)-2-Amino-*N*-{4-[2-(1,3-benzothiazol-2-yl)phenoxymethyl]phenyl}propanamide Hydrochloride (18d)

Compound **17d** (0.20 g, 0.40 mmol) was stirred (1 h) in anhydrous EtOAc/HCl (10 mL) at room temperature. The resulting precipitate was collected and dried giving compound **18d** as a yellow powder (0.17 g, 97%), mp 136 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 11.11 (1H, s, NH), 8.55-8.40 (4H, m, Ar-H, NH_3^+), 8.12-8.03 (2H, m, Ar-H), 7.76 (2H, d, *J* = 8.7 Hz, Ar-H), 7.62-7.51 (4H, m, Ar-H), 7.45-7.38 (2H, m, Ar-H), 7.17 (1H, t, *J* = 7.6 Hz, Ar-H), 5.42 (2H, s, CH_2), 4.14 (1H, dt, *J* = 5.7 Hz, CH), 1.50 (3H, d, *J* = 6.9 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 168.9 (C=O), 162.7 (Ar-C), 156.4 (Ar-C), 151.9 (Ar-C), 138.9 (Ar-C), 135.9 (Ar-C), 132.8 (Ar-C), 132.0 (Ar-C), 129.6 (2 x Ar-C), 129.4 (Ar-C), 126.8 (Ar-C), 125.5 (Ar-C), 122.9 (Ar-C), 122.4 (Ar-C), 122.0 (Ar-C), 121.8 (Ar-C), 119.8 (2 x Ar-C), 114.4 (Ar-C), 70.6 (CH_2), 49.4 ($\underline{C}HCH_3$), 17.8 (CH_3); IR ν_{max} 3650-2250 (broad, NH_3^+ and NH peaks), 2982, 1666 (C=O), 1600 (C=O), 1515, 1247, 989, 754 cm^{-1}; HRMS calcd for $C_{23}H_{22}N_3O_2S$ $[M]^+$ 404.1427; found 404.1426.

5.16 (2*S*)-2-Amino-*N*-[4-({[6-(1,3-benzothiazol-2-yl)naphthalen-2-yl]oxy}methyl)phenyl]propanamide Hydrochloride (18e)

Compound **17e** (0.25 g, 0.45 mmol) was stirred (1 h) in anhydrous EtOAc/HCl (12 mL) at room temperature. The resulting precipitate was collected and dried giving compound **18e** as a yellow powder (0.13 g, 59%), mp 200-203 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.30 (1H, broad s, NH), 8.65-8.55 (1H, m, Ar-H), 8.42-8.28 (3H, m, NH_3^+), 8.20-7.95 (6H, m, Ar-H), 7.75-7.35 (6H, m, Ar-H), 7.25-7.18 (1H, m, Ar-H), 5.23 (2H, s, CH_2), 4.35-4.25 (1H, m, CH), 1.43 (3H, d, *J* = 6.9 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 168.1 (C=O), 154.2 (Ar-C), 136.7 (Ar-C), 134.9 (Ar-C), 131.2 (Ar-C), 129.4 (Ar-C), 129.0 (2 x Ar-C), 128.7 (Ar-C), 128.0 (Ar-C), 127.5 (Ar-C), 127.6 (Ar-C), 127.2 (Ar-C), 125.8 (Ar-C), 124.7 (Ar-C), 123.1 (Ar-C), 122.9 (Ar-C), 122.8 (Ar-C), 120.3 (Ar-C), 119.9 (2 x Ar-C), 119.8 (Ar-C), 109.5 (Ar-C), 69.7 (CH_2), 49.4 ($\underline{C}HCH_3$), 17.7 (CH_3); IR ν_{max} 3650-2300 (broad, NH_3^+ and NH), 1690 (C=O), 1606,1512, 1481, 1254, 1198, 818, 755, 686 cm^{-1}; HRMS calcd for $C_{27}H_{23}N_3O_2S$ $[M]^+$ 454.1584; found 454.1581.

5.17 *tert*-Butyl N-[(1*S*)-1-{[(1*S*)-1-{[4-({[6-(1,3-

Benzothiazol-2-yl)naphthalen-2-yl]oxy}methyl)phenyl]carbamoyl}ethyl]carbamoyl}ethyl]carbamate (21)

A mixture of **16e** (0.40 g, 1.44 mmol), compound **20** (0.61 g, 1.58 mmol) and Cs_2CO_3(0.70 g, 2.16 mmol) was stirred (12 h) in dry DMF (25 mL) at 90 °C. The mixture was allowed to cool to room temperature and the solvent was evaporated and water (50 mL) was added to the residue. The mixture was extracted with EtOAc (3 x 25 mL) and the organic fractions were dried ($MgSO_4$) and evaporated. The residue was purified by column chromatography (SiO_2, EtOAc-CH_2Cl_2 (50:50)) giving compound **21** as a yellow powder (0.33 g, 37%), mp 204-205 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.05 (1H, s, NH), 9.97 (1H, m, NH), 8.63 (1H, m, NH), 8.21-8.00 (4H, m, Ar-H), 7.97 (1H, d, *J* = 8.7 Hz, Ar-H), 7.66 (1H, d, *J* = 8.7 Hz, Ar-H), 7.60-7.42 (5H, m, Ar-H), 7.33 (1H, dd, *J* = 8.7 and 2.3 Hz, Ar-H), 7.25 (1H, d, *J* = 8.5 Hz, Ar-H), 7.01 (1H, d, *J* = 7.3 Hz, Ar-H), 5.21 (2H, s, CH_2), 4.45-4.38 (1H, m, CH), 4.04-3.96 (1H, m, CH), 1.38-1.31 (12H, m, CH_3, 3 x CH_3), 1.20 (3H, d, *J* = 7.3 Hz, CH_3); ^{13}C-NMR (101 MHz, d_6-DMSO) δ 173.1 (C=O), 171.8 (C=O), 168.8 (C=O), 158.3 (Ar-C), 155.7 (Ar-C), 154.2 (Ar-C), 139.3 (Ar-C), 136.3 (Ar-C), 135.0 (Ar-C), 131.9 (Ar-C), 131.1 (Ar-C), 129.2 (Ar-C), 128.7 (2 x Ar-C), 128.3 (Ar-C), 127.7 (Ar-C), 127.2 (Ar-C), 125.9 (Ar-C), 125.0 (Ar-C), 123.2 (Ar-C), 122.9 (Ar-C), 120.6 (Ar-C), 119.7 (2 x Ar-C), 119.5 (Ar-C), 108.0 (Ar-C), 78.6 ($\underline{C}(CH_3)_3$), 69.8 (CH_2), 50.2 ($\underline{C}HCH_3$), 49.4 ($\underline{C}HCH_3$), 28.7 ($C(\underline{C}H_3)_3$), 18.8 ($CH\underline{C}H_3$), 18.5 ($CH\underline{C}H_3$); IR ν_{max} 3340-3240 (broad, NH), 2980, 1690 (C=O), 1655 (C=O), 1645 (C=O), 1514, 1249, 1163, 1019, 802, 756 cm^{-1}; HRMS calcd for $C_{35}H_{37}N_4O_5S$ $[M+H]^+$ 625.2479; found 625.2474.

5.18 (2*S*)-2-[(2*S*)-2-Aminopropanamido]-*N*-[4-({[6-(1,3-benzothiazol-2-yl)naphthalen-2-yl]oxy}methyl)phenyl]propanamide Hydrochloride (22)

Compound **21** (0.20 g, 0.32 mmol) was stirred (1 h) in anhydrous EtOAc/HCl (10 mL) at room temperature. The resulting precipitate was collected and dried giving compound **22** (0.10 g, 56%) as an orange powder, mp > 220 °C; ^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.03 (1H, s, NH), 8.80-8.57 (1H, m, NH), 8.28-7.85 (10H, m, NH_3^+, Ar-H), 7.68 (1H, d, *J* = 8.7 Hz, Ar-H), 7.57-7.31 (5H, m, Ar-H), 7.17 (1H, d, *J* = 8.7 Hz, Ar-H), 5.22 (2H, s, CH_2), 4.54-4.42 (1H, m, CH), 3.94-3.81 (1H, m, CH), 1.45-1.27 (6H, m, 2 x CH_3); IR ν_{max} 3650-2400 (broad, NH_3^+ and NH), 1667 (C=O), 1601, 1514, 1446, 1393, 1249, 1179, 818, 757 cm^{-1}; HRMS calcd for $C_{30}H_{29}N_4O_3S$ $[M]^+$ 525.1955; found 525.1943.

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