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Bifunctional Activity Labels for Selection of Filamentous Bacteriophages Displaying Enzymes

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Abstract—Two bifunctional activity labels of β-lactamases or penicillin binding proteins have been prepared. They feature a penicillin sulfone derivative, i.e. a suicide substrate of serine β-lactamases, or a penicillin derivative connected to a biotin moiety through a spacer containing a disulfide bridge. The biotinyl spacer **4** was prepared by coupling biotin to ε-amino-caproic acid, then to cystamine, and purified by transient protection with *t*-Boc. The penicillin sulfone inhibitor **13** was prepared by chemoselective sulfonylation of methoxymethyl 6-aminopenicillinate with pentafluorophenoxy- or benzyloxy-carbonylmethylsulfonyl chloride (**9**), followed by permanganate oxidation. Both direct coupling of the activated ester **13b** and indirect coupling of the acid **13c** obtained by benzyl ester deprotection, afforded the biotinylated sulfone inhibitor **16**. The acid **6** resulting from reaction of the biotinyl spacer **4** with glutaric anhydride was activated as pentafluorophenyl-ester **7** and reacted with 6-aminopenicillanic acid to afford the penicillin binding protein label **18**. Selection of the most active β-lactamase displayed on phage from a mixture containing less active enzymes could be accomplished in three rounds of labeling and affinity chromatography using suicide inhibitor **16**.

Introduction

Recently, the interest of the scientific community for accelerated molecular evolution in the laboratory has grown as a consequence of the introduction of new techniques allowing the construction and the handling of vast combinatorial libraries of random mutants. Peptides and proteins have been displayed on filamentous phages in fusion with the minor (gene 3 product) or the major (gene 8 product) coat proteins; as they retain their binding properties, the phages displaying them can be affinity purified. Linkage between recognition and replication systems allows an extremely efficient amplification of the selected binder phages (for recent reviews, see Refs 1 and 2).

Enzymes, alkaline phosphatase,[3] trypsin[4] and β-lactamase,[5] have also been displayed on filamentous phages and shown to retain their catalytic activity. The large intracellular enzyme β-galactosidase has been displayed as an active tetramer on λ phages.[6] Phage-enzymes have been affinity purified by binding to an immobilized ligand. Accordingly, phage-enzymes with modified binding sites might be selectively enriched from a library by proper choice of the ligand. Selection from combinatorial libraries of phages displaying an antibody which catalyzes the specific hydrolysis of an activated thiol ester substrate has been performed by covalent bond formation. Antibodies with a cysteine suitably located in the complementary-determining loops for nucleophilic catalysis were recruited by disulfide interchange with an α-phenetyl pyridyl disulfide derivative.[7]

Key words—Phage enzyme, activity label, β-lactamase, D,D-carboxypeptidase, enzyme inhibition, biotinyl spacer.

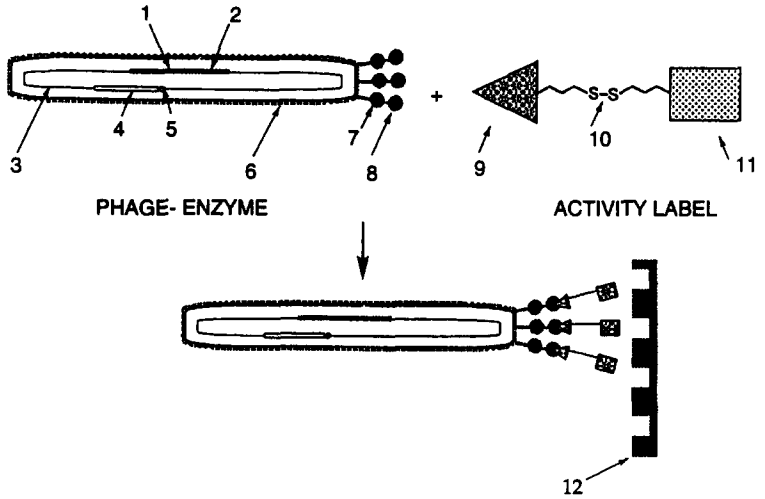
We have adopted the suicide inhibitor concept to select for catalytic activity instead of binding affinity.[5,8] Mixtures of phages displaying active or inactive enzymes are incubated with an activity label that features a mechanism-based inactivator (or suicide inhibitor)[8] of the target-enzyme and a biotin moiety allowing subsequent separation by affinity chromatography[4–6] on a streptavidin support. The two entities are connected through a linker of 15–20 atoms (25–35 Å length) including a disulfide bond which can be reductively cleaved after immobilization of the active phages (Fig. 1).

Biotinylated irreversible inhibitors have also found use in the sensitive detection and affinity purification of enzymes. After labeling, the enzymes can be separated by gel electrophoresis and detected using colorimetric or chemiluminescent signals generated on incubation with streptavidin–peroxidase conjugates.[9,10]

In this article, we describe in detail the synthesis of activity labels for β-lactamases and penicillin binding proteins (PBPs) involved in the biosynthesis of bacterial walls (Scheme 1). We also describe our preliminary results on the selection of the most active β-lactamase from a mixture.

Results and Discussion

The enzymes currently displayed on the surface of the phages are the RTEM β-lactamase and mutants susceptible of exhibiting β-lactamase or D,D-carboxypeptidase related penicillin binding activities (for more information on these enzymes see Ref. 11–13). Thus, the selected inhibitors for the construction of the required active labels belong to the β-lactam family: penicillins[14] are well-known D,D-carboxypeptidase inhibitors, and some related sulfone



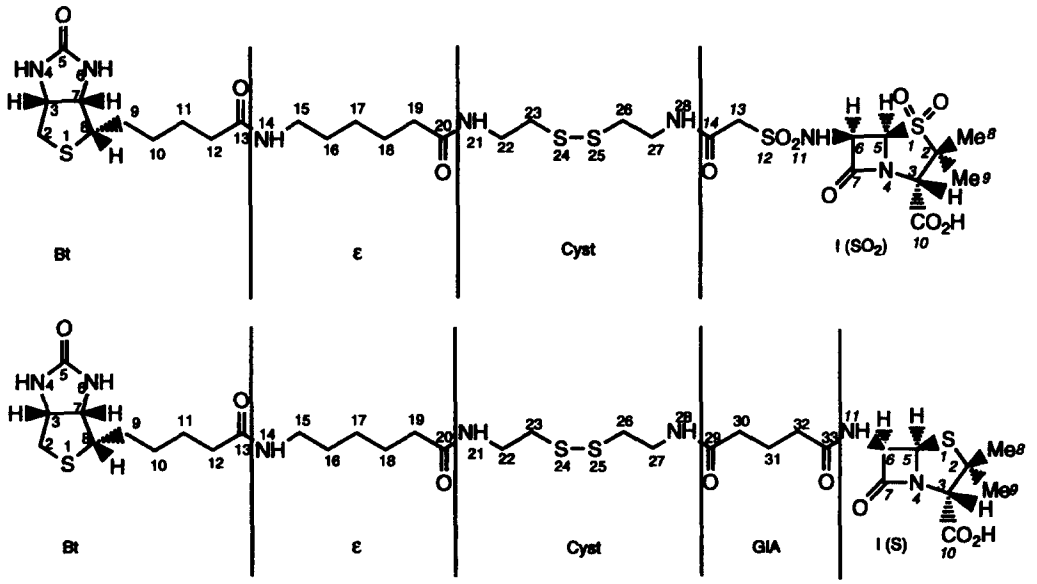
1. Gene encoding the displayed enzyme; 2. phage gene III; 3. ssDNA; 4. Tet gene; 5. origin of replication; 6. coat proteins (gene VIII product); 7. protein III; 8. displayed enzyme; 9. suicide substrate; 10. cleavable bond; 11. ligand (biotin); 12. support (streptavidin).

Figure 1. Principle of selection.

derivatives are powerful β-lactamase inhibitors,[15] i.e. the 6-sulfonylamidopenicillanic acid sulfones.[16] Both classes of inhibitors can be easily prepared from the commercially available (+)-6-aminopenicillanic acid (6-APA).

Our general strategy for the construction of the target-molecules Bt-ε-Cyst-I(SO_2) and Bt-ε-Cyst-GlA-I(S) (Scheme 1) involves the stepwise preparation of the biotinyl spacers (Scheme 2) and the coupling of the sensitive β-lactam inhibitors in the final step.

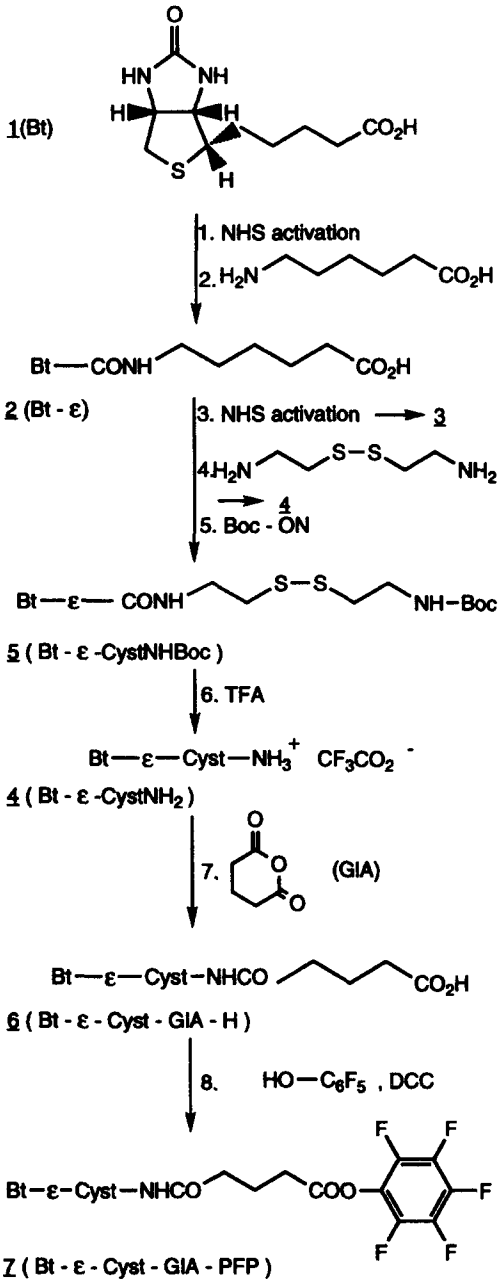
ε-Aminocaproic acid coupling[17] to (*R*)-(+)-biotinyl-*N*-hydroxysuccinimide ester[18] was performed as described in the literature. Further activation of biotinyl-*N*-ε-aminocaproic acid **2** was effected by reaction with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in *N*-methylpyrrolidone for two days at 20 °C. The yield of NHS-ester **3** was significantly improved by addition of one equivalent of *N*,*N*-dimethylaminopyridine.[19] A DMF solution of biotinyl-*N*-ε-aminocaproyl-*N*-hydroxysuccinimide ester **3** was added to an excess of cystamine (free base) in DMF at 0 °C to give the monoacylated derivative **4** as the main product. Formation of bis-acylated cystamine as secondary product was observed by thin-layer chromatography of the crude mixture on a silica gel plate (CH_2Cl_2:MeOH:HOAc, 9:1:1): migration of Bt-ε-Cyst-NH_2 gave an R_f value of 0.1 while the less polar Bt-ε-Cyst-ε-Bt appeared at $R_f = 0.2$. We found that the best way to isolate the desired monoadduct **4** was to first protect the free amine as *t*-butyl carbamate **5** and then to perform the chromatographic separation. Thus, the crude acylation mixture was treated with an excess of 2-(*t*-butoxy-



Bt: Biotin; ε: ε-aminocaproic acid; Cyst: cystamine; GlA: glutaric acid.
I (SO_2): β-lactamase inhibitor (penam sulfone derivative).
I (S): D,D-peptidase inhibitor (penam derivative).

Scheme 1. Activity labels for selection of β-lactamases or D,D-carboxypeptidases displayed on filamentous bacteriophages.

carbonyloxyimino)-2-phenylacetonitrile (BOC-ON)[20] and triethylamine in dioxane-water. Standard work-up and column-chromatography on silicagel (CH_2Cl_2:MeOH, 4:1) furnished pure biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-*t*-butoxycarbonyl **5**. This intermediate was quantitatively deprotected with trifluoroacetic acid[21] to give biotinyl-*N*-ε-aminocaproyl-*N*-cystamine **4** as the trifluoroacetate salt; the yield from the precursor **2** was about 60%. The amine **4** readily reacted with glutaric anhydride in DMF to form biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-glutaryl acid **6** in high yield. This acid was activated for the coupling with enzyme inhibitors by forming the pentafluorophenyl (PFP) ester.[22] Reaction of **6** with pentafluorophenol and dicyclohexylcarbodiimide in DMF for two days at room temperature, gave the ester **7** in 95% yield (Scheme 2).



Scheme 2. Synthesis of biotinyl-spacers.

The selected β-lactamase inhibitor was the novel 6-β-[(hydroxycarbonyl)methylsulfonyl]aminopenicillanic acid sulfone we recently disclosed (Fig. 2).[16] The derived ester and amide of the side-chain also displayed irreversible inhibitory activity against the *Escherichia coli* RTEM β-lactamase.[16] Therefore, anchoring the inhibitor on the biotinyl spacer **4** via an amide bond involving the side-chain carboxyl group will maintain the required activity. The chemoselectivity of the coupling was controlled by protecting the C-3 carboxylic function of the penam moiety. We chose the methoxymethyl (MOM) ester[21] which could be cleaved under mild neutral conditions without degradation of the sensitive β-lactam ring.[16,23]

The C-3 protected inhibitor **13c** (Scheme 3) was previously prepared[16] by catalytic hydrogenation of the precursor **13a** resulting from the sulfonylation of 6-APA methoxymethyl ester **11** with benzyloxycarbonyl-methylsulfonyl chloride **9a** and subsequent sulfide oxidation.

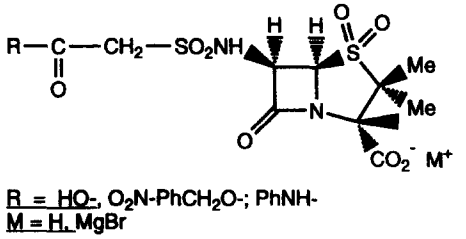
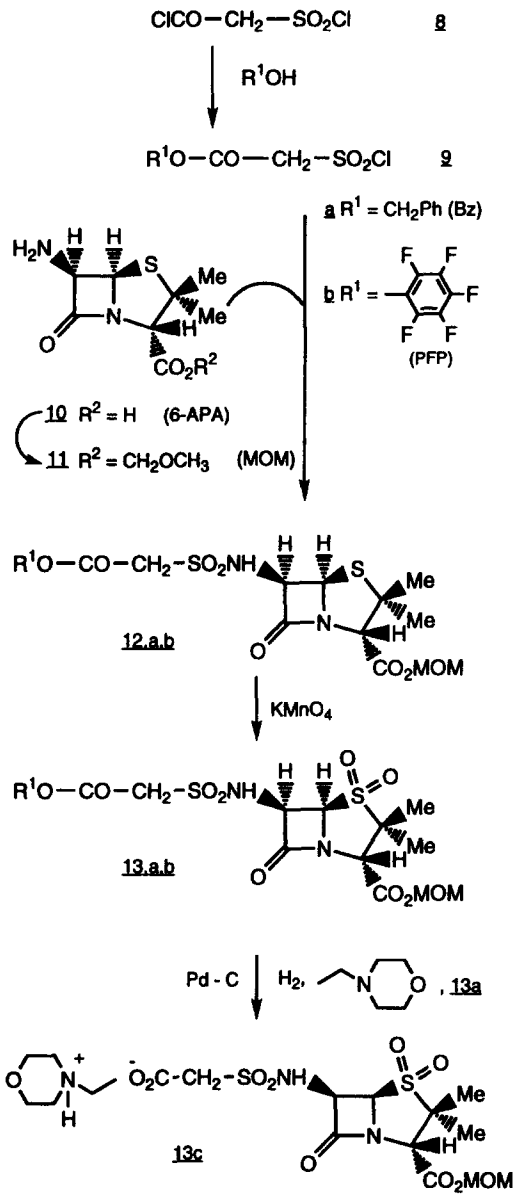


Figure 2. Structure of I(SO_2).

According to the same strategy we synthesized the pentafluorophenyl ester **13b** as an activated form of **13c** (Scheme 3). The pentafluorophenoxycarbonylmethyl-sulfonyl chloride **9b** was quantitatively obtained by heating (70 °C) an equimolar mixture of sulfoacetyl dichloride **8** and pentafluorophenol. The complete selectivity results from the higher electrophilic character of the acid chloride function as compared to the sulfonyl chloride function.[24] Reaction of **9b** with 6-APA methoxymethyl ester **11** and triethylamine, in dichloromethane at low temperature, furnished the crude sulfonamide **12b** which was directly oxidized with potassium permanganate in moist acetic acid. The MOM 6-[(pentafluorophenoxycarbonyl)methylsulfonyl]amino-penicillinate sulfone **13b** was recovered in about 30% yield from **11**. This rather low yield most probably results from the poor chemoselectivity of the coupling step using the bifunctional reagent **9b**. Indeed, in a control experiment, we observed that the reaction of **9b** with one equivalent of *n*-propylamine gave, after hydrolysis, a 2:1 mixture of pentafluorophenyl-(*N*-propylsulfonamido)-acetate and *N*-propyl-(*N*-propylsulfonamido)acetamide (two typical singlets in ^{1}H NMR, at 4.97 ppm and 4.10 ppm respectively).

We first examined the coupling of the CO_2H-ended penam sulfone **13c** with the NH_2-ended biotinyl spacer **4**. We found that *O*-benzotriazol-1-yl-*N*,*N*,*N'*,*N'*-tetramethyl-uronium hexafluorophosphate (HBTU)[25] was the most adapted coupling reagent to our purpose; the reaction was

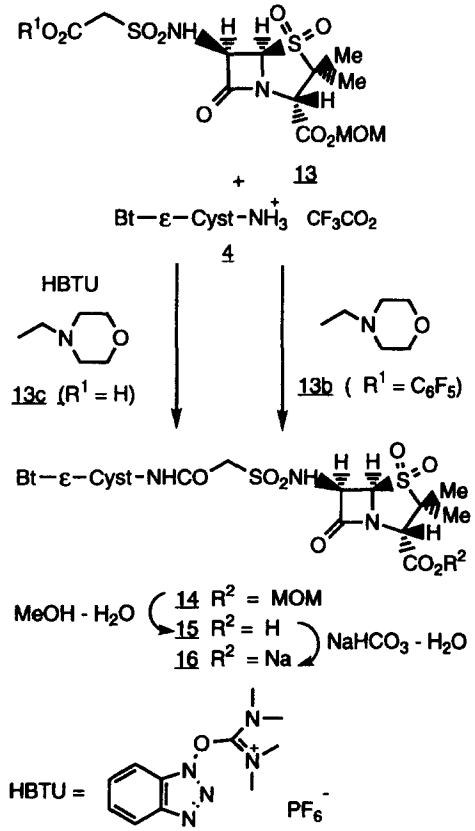


Scheme 3. Synthesis of β-lactamase inhibitors.

performed in 2 h, in DMF at room temperature, in the presence of *N*-ethylmorpholine (Scheme 4). The amide **14**, recovered in 52% yield after standard work-up, was fully characterized by IR, ^1H and ^{13}C NMR spectroscopic data (see Experimental). Treatment of the biotinyl spacer **4** with the PFP-activated inhibitor **13b** also produced methoxymethyl 6-[(biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-carbonyl)methylsulfonyl]aminopenicillinate sulfone **14**, but in modest yield (35%). It appeared thus that the classical peptide chemistry, involving a carbodiimide-like coupling agent, was here more convenient than the pre-activation of the carboxylic partner as PFP ester.

Finally, mild hydrolysis[26] in aqueous methanol of the MOM ester **14**, quantitatively furnished the free acid **15**; the corresponding carboxylate **16** was obtained by neutralization with aqueous sodium bicarbonate (Scheme 4).

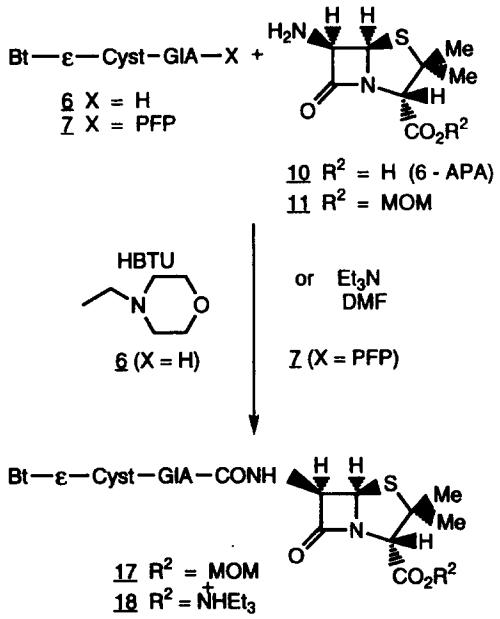
Penicillin antibiotics are N-acylated derivatives of 6-APA **10**; in the naturally-occurring penicillin F, the side chain is just a hexenoyl residue. Therefore, considering that the glutaryl moiety ending the biotinyl spacer **6** could play the role of the antibiotic side chain, we directly coupled the amino-β-lactam to the biotinyl spacer **6**, in order to obtain the activity label for penicillin binding activity selection.



Scheme 4. Coupling of the β-lactamase inhibitor to the biotinyl-spacer.

According to our previous strategy, the MOM ester **11** of 6-aminopenicillanic acid was reacted with the free acid **6** using HBTU as coupling agent (Scheme 5). Methoxymethyl 6-biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-glutaryl)aminopenicillinate **17**, isolated in 56% yield, was fully characterized by IR, ^1H and ^{13}C NMR spectroscopic data (see Experimental). Unfortunately, several attempts to deprotect the C-3 MOM ester of **17** led to extensive degradation of the β-lactam ring. The conditions examined were $MgBr_2$ in CH_2Cl_2:DMF (9:1),[16,23] and MeOH:H_2O (35:65) in the presence of anisole or *N*-ethylmorpholine. This negative result prompted us to consider the coupling of C-3 unprotected 6-APA. In this case, a selective reaction could be performed by using the activated biotinyl partner **7**. Thus, 6-APA **10** and the PFP ester **7** were mixed in DMF, in the presence of Et_3N, to furnish the target-molecule, 6-(biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-glutaryl)aminopenicillanic acid, as the triethylammonium salt **18**, in 95% yield (Scheme 5). The spectral characteristics of this compound are similar to those of the corresponding MOM ester **17** (see Experimental).

In order to show that we could select the most active enzyme from a mixture, we have prepared several mutants (T71A, T71V and V103I-Y105F) with reduced activity (between 3 and 20% of wild-type) by oligonucleotide extension mutagenesis.[30] A mixture of phages containing 2% of wild type enzyme and 24.5% of each of the three mutants described above and of S70A inactive mutant[5] was labeled with the biotinylated suicide inhibitor **16**, incubated in the presence of streptavidin coated magnetic beads and then immobilized phages were recovered by proteolysis of the factor Xa recognition site between the β-lactamase and the phage coat protein g3p as described in Ref. 5. Three rounds of selection were run. The main β-lactamase activity on phage increased from 43 sec^{-1} to 76 sec^{-1}, 215 sec^{-1} and 463 sec^{-1}. After the last selection, ≥ 75% of the phages were displaying the wild type enzyme as judged by screening on a plate with nitrocefin.



Scheme 5. Coupling of 6-APA to the biotinyl-spacer.

Conclusion

We have prepared two bifunctional activity labels allowing the selection of phage-enzymes displaying β-lactamase and D,D-carboxypeptidase related penicillin binding activities respectively. The first label, Bt-ε-Cyst-I(SO_2), was obtained in 5 steps, in 29% overall yield, from (*R*)-(+)-biotinyl-*N*-ε-aminocaproic acid. Our convergent approach implied the independent synthesis of the β-lactamase inhibitor **13** from 6-APA and chlorosulfonylacetyl chloride **8**, in 33% overall yield for the 4 steps. The second label, Bt-ε-Cyst-GlA-I(S), was linearly constructed in 6 steps from (*R*)-(+)-biotinyl-*N*-ε-aminocaproic acid and 6-APA, with an overall yield of 44%.

The biotinyl spacers we developed are either NH_2-ended (**4**) or CO_2H-ended (**6** and **7**), both including a cleavable disulfide bond provided by the cystamine building block. These new compounds will be used for the convergent construction of other activity labels, by coupling the designed enzyme inhibitors via a peptide linkage.

Finally we have shown that the labeling with suicide inhibitor can be used to extract the most active enzymes from a mixture.

Experimental

The reagents were purchased from Janssen Chimica, Aldrich or Fluka. *N*-Hydroxysuccinimide (NHS) was dried by azeotropic distillation with benzene. The other reagents were used without further purification. The solvents were dried as follows, then distilled: *N*-methylpyrrolidone, *N*,*N*-dimethylformamide (DMF) and dichloromethane over phosphorous pentoxide; ether over sodium/benzophenone; methanol over sodium/diethylsuccinate. *N*-Ethylmorpholine and triethylamine were freshly distilled over calcium hydride.

Column-chromatographies were carried out with silica gel 60, 70–230 mesh ASTM, supplied by Merck. Thin-layer chromatography was carried out on silica gel 60 plates F254 (Merck, 0.2 mm thick). Visualization was effected with ultraviolet light, iodine vapor and a spray of *p*-dimethylaminocinnamaldehyde (DMACA) which is specific for biotin derivatives.[27,28] The stock solution was obtained by dissolving DMACA (2 g) into ethanol (50 mL) and 6 N HCl (50 mL). The spray solution was prepared by dilution of the stock solution (1 mL) in ethanol (5 mL). Visualization of amino derivatives was effected, as usual, with ninhydrin.

Melting points were determined with an Electrothermal microscope and are uncorrected. Rotations (± 0.1°) were determined on a Perkin–Elmer 241 MC polarimeter. The IR spectra were taken with a Perkin–Elmer 1710 instrument (Infra Red Fourier Transformer Spectrometer) and calibrated with polystyrene (1601 cm^{-1}); only the most significant and diagnostic absorption bands are reported. The ^1H, ^{13}C and ^{19}F NMR spectra were recovered on Varian Gemini-200, Varian Gemini-300 or Bruker AM-500 spectrometers. Chemical shifts are reported in ppm (δ) downfield from internal TMS for ^1H and ^{13}C NMR, and from internal $CFCl_3$ for ^{19}F NMR. The atom numbering used for the description of the spectra is shown in the Scheme 1. The mass spectra were obtained on a Finnigan MAT TSQ-70 instrument in the FAB mode (Fast Atom Bombardment). The microanalyses were performed at the Christopher Ingold Laboratories, University College London (Dr Alan Stones).

*N-Hydroxysuccinimide ester of biotinyl-*N*-ε-aminocaproic acid (3)*

The NHS activation of biotin **1** was realized according to the method of Parameswaran;[18] the coupling of biotinyl-*N*-hydroxysuccinimide ester to ε-aminocaproic acid was made according to the method of Wilchek and Bayer.[17] Biotinyl-*N*-ε-aminocaproic acid **2** (4.10 g, 11.48 mmol) was heated, under stirring, in *N*-methylpyrrolidone (82

mL) at 90 °C, until dissolution occurred. The solution was allowed to cool off, without stirring. *N*-Hydroxysuccinimide (1.32 g, 11.48 mmol), *N*,*N*-dimethylaminopyridine (1.4 g, 11.48 mmol) and dicyclohexylcarbodiimide (2.84 g, 13.78 mmol, 1.2 equiv.) were added successively at room temperature. The mixture was stirred for two days, then filtered. The NHS ester of **2** was precipitated by addition of ether to the filtrate. The white solid obtained was dried overnight, under vacuum, in the presence of P_2O_5. Yield of **3**: 4.79 g (92%); mp 170 °C; R_f (CH_2Cl_2:MeOH:HOAc, 9:1:1) = 0.66; IR (KBr) 1820, 1790, 1733, 1696, 1627, 1568, 1540 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 1.24 (2H, H-17), 1.31 (2H, H-10), 1.35 (2H, H-16), 1.44 (1H, H-9), 1.49 (2H, H-11), 1.59 (1H, H'-9), 1.60 (2H, H-18), 2.03 (2H, H-12), 2.56 (1H, H-2), 2.65 (2H, H-19), 2.79 (4H, succinimide), 2.81 (1H, H'-2), 2.98 (2H, H-15), 3.08 (1H, H-8), 4.11 (1H, H-7), 4.28 (1H, H-3), 6.36 (1H, H-4), 6.43 (1H, H-6), 7.69 (1H, H-14); ^{13}C NMR (125 MHz, DMSO-d_6) ppm 23.92 (C-18), 25.26 (C-11), 25.42 (C-17 and $\underline{C}H_2$ succinimide), 28.0 (C-9), 28.18 (C-10), 28.59 (C-16), 30.12 (C-19), 35.18 (C-12), 38.03 (C-15), 39.59 (C-2), 55.38 (C-8), 59.15 (C-7), 60.91 (C-3), 162.67 (C-5), 168.88 (C-20), 170.19 (CO succinimide), 171.8 (C-13).

*Biotinyl-*N*-ε-aminocaproyl-*N*-cystamine (4)*

Cystamine dihydrochloride (11.25 g, 50 mmol) was dissolved in aqueous 2 M NaOH (50mL). Extraction (5 ×) with CH_2Cl_2, drying over $MgSO_4$ and concentration gave the free base (yield: 5.54 g, 73%). Cystamine (4.07 g, 26.8 mmol, 3 equiv.) was dissolved in DMF (15 mL) and cooled in an ice-bath. A solution of **3** (4.05 g, 8.9 mmol) in DMF (40 mL) was added dropwise (during 5 min). After 15 min of stirring, ether (200 mL) was added. The precipitate was filtered off, washed with ether and dried under vacuum (crude yield: 5.71 g). This material was suspended in dioxane:water 4:1 (200 mL), under vigorous stirring. Triethylamine (5.44 g, 53.8 mmol) and BOC-ON (9.72 g, 39.5 mmol) dissolved in dioxane:water (25 mL) were added successively at room temperature. After 1 h 30 min, the homogeneous mixture was extracted with ethylacetate (5 × 200 mL). The organic layers were dried over $MgSO_4$ and concentrated under vacuum. The solid residue (≈ 13 g) was purified by column chromatography on silica gel to give biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-*t*-butoxycarbonyl **5**: yield 3.25 g (61%); R_f (CH_2Cl_2:MeOH, 80:20) = 0.7; mp 123 °C; $[\alpha]^{22}_D$ = + 32.2° (MeOH, *c* 1); IR (KBr) 1704, 1640, 1545, 1367 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 1.21 (*m*, 2H, H-17), 1.35 (*m*, 2H, H-16), 1.28 (*m*, 2H, H-10), 1.35 (*s*, 9H, *t*Bu), 1.47 (*m*, 5H, H-9 + H-11+ H-18), 1.60 (*m*, 1H, H'-9), 2.03 (*t*, 4H, H-12 + H-19), 2.55 (*d*, 1H, H-2), 2.74 (*t*, 4H, H-23 + H-26), 2.81 (*dd*, 1H, H'-2), 2.98 (*m*, 2H, H-15), 3.08 (*m*, 1H, H-8), 3.18 (*m*, 2H, H-27), 3.29 (*m*, 2H, H-22), 4.11 (*m*, 1H, H-7), 4.29 (*m*, 1H, H-3), 6.33 (*s*, 1H, H-6), 6.39 (*s*, 1H, H-4), 6.95 (*t*, 1H, H-28), 7.70 (*t*, 1H, H-14), 7.92 (*t*, 1H, H-21); ^{13}C NMR (125 MHz, DMSO-d_6) ppm 24.91 (C-11), 25.26 (C-17), 26.05 (C-18), 27.98 (C-9), 28.16 (C-10), 28.16 (C $\underline{Me}_3$), 28.92 (C-16), 35.17 (C-12), 35.26 (C-19), 37.37 (C-23 + C-26), 37.54 (C-27), 37.81 (C-22), 38.24 (C-15), 39.75 (C-2), 55.35 (C-8), 59.15 (C-7), 60.99 (C-3), 77.74 ($\underline{C}Me_3$), 155.46 (C-29), 162.60 (C-5), 171.72 (C-13), 172.13 (C-20). Anal. calcd for $C_{25}H_{45}O_5N_5S_3$ (591): C, 50.74%; H, 7.66%; N, 11.83%; found: C, 50.46%; H, 7.72%; N, 11.50%.

Biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-BOC **5** (1.58 g, 2.7 mmol) was treated at room temperature with freshly distilled trifluoroacetic acid (15 mL). After 30 min, the mixture was concentrated under vacuum. The residue was triturated with ether to give biotinyl-*N*-ε-aminocaproyl-*N*-cystaminammonium trifluoroacetate **4** as a pale beige solid: yield 1.62 g (100%); IR (KBr) 3295 (br), 1700, 1650, 1552 cm^{-1}; ^{13}C NMR (125 MHz, DMSO-d_6) ppm 24.56 (C-18), 24.93 (C-11), 25.78 (C-17), 27.74 (C-9), 27.80 (C-10), 28.58 (C-16), 34.30 (C-26), 34.97 (C-12), 35.06 (C-19), 37.23 (C-23), 37.73 (C-27), 37.82 (C-22), 38.06 (C-15), 54.96 (C-8), 59.07 (C-7), 60.86 (C-3), 162.33 (C-5), 171.54 (C-13), 171.99 (C-20); ^1H NMR (500 MHz, DMSO-d_6) δ (see **5** for Bt-ε-spacer) 2.78 (*t*, 2H, H-23), 2.91 (*t*, 2H, H-26), 3.08 (*m*, 2H, H-27), 3.32 (*m*, 2H, H-22), 7.92 (*s*, *t*, 4H, H-28, H-21); mass (FAB-MNBA,+Q1MS) m/z 492 ($C_{20}H_{38}O_3N_5S_3$ + 1, 77%), 415 (10%), 340 (5%), 227 (16%); (-Q1MS) m/z 604 ($C_{22}H_{38}O_5N_5S_3F_3$–1).

*Biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-glutaryl acid (6)*

A solution of biotinyl-*N*-ε-aminocaproyl-*N*-cystaminammonium trifluoroacetate **4** (600 mg, 0.99 mmol) in DMF (8 mL) was treated with Et_3N (0.304 mL, 1.19 mmol, 2.2 equiv.) and glutaric anhydride (136 mg, 2.18 mmol, 1.2 equiv.), at room temperature, under stirring. After 30 min, the solvent was distilled under high vacuum; the residue was dissolved in water and treated with 1 N HCl to reach a pH between 1 and 2. After 15 min, the precipitate was filtered off and dried overnight, under vacuum, in the presence of P_2O_5, to furnish the biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-glutaryl acid **6** as a white powder: yield 522 mg (87%); mp 144 °C; R_f (CH_2Cl_2:MeOH:HOAC, 9:1:1) = 0.25; IR (KBr) 3309, 1712, 1636, 1551 cm^{-1}; ^{13}C NMR (125 MHz, DMSO-d_6) ppm (see **17** for Bt-ε-Cyst-spacer) 20.58 (C-31), 32.94 (C-32), 34.38 (C-30), 172.22 (C-29), 174.07 (C-33); ^1H NMR (500 MHz, DMSO-d_6) δ (see **17** for Bt-ε-Cyst-spacer) 1.68 (*m*, 2H, H-31), 2.08 (*t*, 2H, H-30), 2.18 (*t*, 2H, H-32), 11.94 (1H, H-34); mass (FAB-MNBA, +Q1MS) m/z 606 ($C_{25}H_{43}O_6N_5S_3$ + 1).

*Pentafluorophenyl biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-glutarylate (7)*

Biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-glutaryl acid **6** (250 mg, 0.41 mmol) was dissolved under stirring at 80 °C, in DMF (4 mL). The solution was allowed to cool off, without stirring. Pentafluorophenol (89 mg, 0.48 mmol, 1.2 equiv.) and dicyclohexylcarbodiimide (110 mg, 0.53 mmol, 1.3 equiv.) dissolved in DMF (1 mL), were added successively at room temperature. After 36 h of stirring, the dicyclohexylurea was filtered off and the filtrate was concentrated under high vacuum. The residue was triturated and washed with ether (5 ×), then dried under

vacuum in the presence of P_2O_5 to give the pentafluorophenyl ester **7**: yield: 303 mg (95%); mp 137–138 °C; R_f (CH_2Cl_2:MeOH, 80:20) = 0.6; $[\alpha]^{25}_D$ + 28.8° (MeOH, *c* 1); IR (KBr) 1790, 1705, 1645, 1521 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ (see **17** for Bt-ε-Cyst-spacer); 1.87 (*m*, 2H, H-31), 2.19 (*t*, 2H, H-30), 2.79 (*t*, 2H, H-32), 8.05 (1H, H-28); ^{13}C NMR (125 MHz, DMSO-d_6) ppm (see **17** for Bt-ε-Cyst spacer) 20.24 (C-31), 31.82 (C-32), 33.67 (C-30), 169.19 (C-33), 171.26 (C-29); C_6F_5 not visible; mass (FAB-MNBA, +Q1MS) m/z 772 (M + 1), 415 (S–S cleavage). Anal. calcd for $C_{31}H_{42}O_6N_5S_3F_5$ (771.8): C, 48.18%; H, 5.61%; N, 9.06%; found: C, 50.03%; H, 5.99%; N, 9.37%.

Methoxymethyl 6-aminopenicillinate (11)

The esterification was performed according to the general procedure of Manhas *et al.*[29] from 6-APA **10** (21.67 g, 0.1 mol), Et_3N (27.6 mL, 0.2 mol), and methyl acetoacetate (11.6 g, 0.1 mol) in CH_2Cl_2 (300 mL) for NH_2 protection. Reaction with methoxymethylchloride (7.6 mL, 0.1 mol) in DMF (200 mL) for 3 h at 20 °C, usual work-up and treatment with *p*-toluene sulfonic acid (22.8 g, 0.12 mol) in acetone (60 mL) gave the *p*-toluene sulfonate salt of **11**: yield 34 g (79%). The free amine was obtained by treatment of the salt with Et_3N (1.2 equiv.) in CH_2Cl_2 solution for 2 h at 20 °C, then washing with water (2 ×), drying over $MgSO_4$ and concentration under vacuum. Compound **11** was recovered as a yellowish oil (to be stored at –20 °C): IR (film) 3378, 1785, 1731 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.55 (*s*, 3H, *H-9*), 1.67 (*s*, 3H, *H-8*), 1.90 (*br s*, 2H, NH_2), 3.51 (*s*, 3H, *OMe*), 4.41 (*s*, 1H, *H-3*), 4.59 (*d*, 1H, *J* = 4.3 Hz, *H-6*), 5.31 (AB, 2H, *J* = 5.9 Hz, O-CH_2-O), 5.52 (*d*, 1H, *J* = 4.3 Hz, *H-5*) ; ^{13}C NMR (125 MHz, DMSO-d_6) ppm 26.49 (*C-9*), 30.43 (*C-8*), 57.38 (*OMe*), 62.58 (*C-6*), 63.05 (*C-2*), 69.62 (*C-5*), 69.99 (*C-3*), 91.01 (O-CH_2-O), 167.42 (*C-7*), 172.78 (*C-10*).

Pentafluorophenyl chlorosulfonylacetate (9b)

A mixture of pentafluorophenol (704 mg, 3.8 mmol) and freshly distilled sulfoacetyl dichloride **8** (677 mg, 3.8 mmol) was heated under argon atmosphere at 70 °C during two days; then HCl was removed by azeotropic distillation with benzene (2 ×). The crude pentafluorophenyl ester **9b** was not purified (brown oil): IR (film) 1796, 1392, 1181 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.68 (*s*); (acetone-d_6) 5.23 (*s*); ^{13}C NMR (50 MHz, acetone-d_6) ppm 67.6 (T, *J* = 143 Hz), 162.1 (S); ^{19}F NMR (200 MHz, acetone-d_6) ppm –152.5 ($2F_{ortho}$, *d*, J_{o-m} = 19.6 Hz), –157.4 ($1F_{para}$, *t*, J_{p-m} = 21.4 Hz), –162.7 ($2F_{meta}$, *t*, J_{m-p} = 17.5 Hz); mass (EI) m/z 324 ($C_8H_2F_5O_4S\ Cl^{35}$, 3%), 326 ($C_8H_2F_5O_4S\ Cl^{37}$, 1.5%), 289 (M – Cl, 7%), 184 (PFP, 100%), 141 (M – PFP, 12%), 143 (M – PFP, 6%).

Methoxymethyl 6-[(pentafluorophenoxycarbonyl)methylsulfonyl]aminopenicillinate (12b)

To a solution of methoxymethyl 6-aminopenicillinate **11** (320 mg, 1.23 mmol) in CH_2Cl_2 (10 mL), cooled in a dry ice-acetone bath, were added successively Et_3N (0.173 mL, 1.23 mmol) and pentafluorophenyl chlorosulfonylacetate **9b** (400 mg, 1.23 mmol) in CH_2Cl_2 (3 mL). The mixture was stirred for 30 min at –40 °C, then the cooling bath was removed and the mixture was left for 30 min at room temperature. Washing with water (3 ×), drying over $MgSO_4$ and concentration gave the crude sulfonamide **12b** as an amorphous solid: yield 443 mg (66%); R_f (CH_2Cl_2:EtOAc, 80:20) = 0.7; IR (KBr) 1791, 1747, 1359, 1164 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.55 (*s*, 3H, *H-9*), 1.63 (*s*, 3H, *H-8*), 3.55 (*s*, 3H, *OMe*), 4.55 (*d*, 1H, *J* = 16.5 Hz, *H-13*), 4.51 (*s*, 1H, *H-3*), 4.80 (*d*, 1H, *J* = 16.5 Hz, *H'-13*), 5.21 (*dd*, 1H, *J* = 4.6 and 10.7 Hz, *H-6*), 5.32 (sharp AB, 2H, O-CH_2-O), 5.67 (*d*, 1H, *J* = 4.6 Hz, *H-5*), 5.98 (*d*, 1H, *J* = 10.7 Hz, *H-11*); ^{13}C NMR (125 MHz, $CDCl_3$) ppm 26.72 (*C-9*), 32.86 (*C-8*), 56.68 (*C-13*), 58.17 (OCH_3), 61.96 (*C-6*), 64.97 (*C-2*), 67.20 (*C-5*), 70.12 (*C-3*), 91.67 (O-CH_2-O), 137.76, 140.73 and 140.9 (PFP, J_{C-F} = 259 Hz), 160.31 (*C-14*), 166.74 (*C-7*), 172.1 (*C-10*); ^{19}F NMR (300 MHz, acetone-d_6) ppm –148.7 ($2F_{ortho}$), –154.5 (F_{para}), –159.6 ($2F_{meta}$); mass (FAB-NMBA, +Q1MS) m/z 549 ($C_{18}H_{17}F_5N_2O_8S_2$ + 1).

Methoxymethyl 6-[(pentafluorophenoxycarbonyl)methylsulfonyl]aminopenicillinate S,S-dioxide (13b)

To a cold solution (–13 °C) of crude penicillinate **12b** (196 mg, 0.36 mmol) in HOAc:H_2O 4:1 (14 mL) was added dropwise (during 30 min) a solution of $KMnO_4$ (119 mg, 0.75 mmol, 2.1 equiv.) in water (3 mL). The mixture was stirred for 1 h at –10 °C, then H_2O_2 (10% in H_2O) was added dropwise until decolourization occurred. Extraction with CH_2Cl_2 (3 ×), washing with water (2 ×) and with 5% $NaHCO_3$, drying over $MgSO_4$ and concentration gave the crude sulfone **13b** as an amorphous yellow solid: yield 95 mg (46%); IR (KBr) 1811, 1757, 1364, 1327, 1164, 1118 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) δ 1.45 (*s*, 3H, *H-9*), 1.61 (*s*, 3H, *H-8*), 3.50 (*s*, 3H, *OMe*), 4.55 (*s*, 1H, *H-3*), 4.99 (AB, 2H, *H-13*), 5.34 (*d*, 1H, *J* = 4.8 Hz, *H-5*), 5.36 (*d*, 1H, *J* = 6 Hz, O-*CH*-O), 5.44 (*d*, 1H, *J* = 6 Hz, O-*CH*-O), 5.79 (*dd*, 1H, *J* = 4.6 and 10.9 Hz, *H-6*), 7.25 (*d*, 1H, *J* = 10.9 Hz, *H-11*); ^{13}C NMR (125 MHz, acetone-d_6) ppm 17.80 (*C-9*), 20.12 (*C-8*), 57.07 (*C-13*), 58.30 (OCH_3), 61.50 (*C-6*), 64.51 (*C-2*), 65.32 (*C-3*), 66.83 (*C-5*), 92.94 (O-CH_2-O), 141.95 and 138.91 (PFP), 161.09 (*C-14*), 166.99 (*C-7*), 174.11 (*C-10*); ^{19}F NMR (300 MHz, acetone-d_6) ppm –152.4 (*d*, $2F_{ortho}$, J_{o-m} = 23.7 Hz), –158.2 (*t*, $1F_{para}$, J_{p-m} = 23.7 Hz), –163.2 (*t*, $2F_{meta}$); mass (FAB-MNBA, +Q1MS) m/z 581 ($C_{18}H_{17}F_5S_2O_{10}N_2$ + 1).

Methoxymethyl 6-[(biotinyl-N-ε-aminocaproyl-N-cystamine-N'-carbonyl)methylsulfonyl]aminopenicillinate S,S-dioxide (14)

Method A. To an ice-cool solution of penicillinate sulfone **13c** (204 mg, 0.38 mmol, 1 equiv.), biotinyl-*N*-ε-aminocaproyl-*N*-cystaminammonium trifluoroacetate **4** (233 mg, 0.38 mmol, 1 equiv.) and *N*-ethylmorpholine (0.108 mL, 0.85 mmol, 2.2 equiv.) in DMF (10 mL) was added HBTU (175 mg, 0.46 mmol, 1.2 equiv.). The mixture was stirred for 2 h at room temperature. Dichloromethane (70 mL) was added and the solution was washed with water (1 × 20 mL), 10% citric acid (2 × 20

mL), 5% $NaHCO_3$ (1 × 20 mL) and water (1 × 20 mL). After drying over $MgSO_4$ and concentration, the gummy residue was triturated and washed with ether to give the crude coupling product **14** as an amorphous beige solid: yield 176 mg (52%). This material could be purified by column chromatography on silica gel (CH_2Cl_2:MeOH 80:20); mp 100–103 °C; R_f (CH_2Cl_2:MeOH, 80:20) = 0.74; $[\alpha]^{25}_D$ = + 84.4° (MeOH, *c* 1); IR (KBr) 1808 (CO β-lactam), 1754 (CO ester), 1707 (CO urea), 1646 (CO amides), 1546 (CO amides), 1355, 1325, 1162 and 1117 (SO_2, SO_2NH) cm^{-1}; Anal. calcd for $C_{32}H_{53}O_{12}N_7S_5 \cdot 2H_2O$ (924.11): C, 41.59%; H, 6.21%; N, 10.61%; found: C, 41.16%; H, 6.03%; N, 9.81%. Compound **14** was dried under vacuum in the presence of P_2O_5 and stored at –20 °C.

Method B. To an ice-cool solution of penicillinate sulfone **13b** (168 mg, 0.29 mmol, 1 equiv.) and biotinyl-*N*-ε-aminocaproyl-*N*-cystaminammonium trifluoroacetate **4** (175 mg, 0.3 mmol, 1 equiv.) in DMF (2 mL) was added *N*-ethylmorpholine (0.044 mL, 0.36 mmol, 1.2 equiv.). The mixture was stirred at room temperature for 24 h. Dichloromethane (10 mL) was added and the solution was washed with 10% citric acid, 5% $NaHCO_3$ and water. After drying over $MgSO_4$ and concentration, the residue was triturated and washed with ether (3 ×), then dried under vacuum. Yield of **14**: 90 mg (35%); ^1H NMR (500 MHz, DMSO-d_6) δ (see **17** for Bt-ε-spacer) 1.36 (*s*, 3H, *H-9*), 1.50 (*s*, 3H, *H-8*), 2.73–2.83 (*t*, 4H, H-23 + H-26), 3.35–3.42 (*m*, 4H, H-22 + H-27), 3.43 (*s*, 3H, *OMe*), 4.16 (AB, 2H, *H-13*), 4.60 (*s*, 1H, *H-3*), 5.28 (*d*, 1H, *O-CH-O*), 5.36 (*d*, 1H, *O-CH-O*), 5.42 (*d*, 1H, *H-5*), 5.57 (*dd*, 1H, *H-6*), 7.94 (*t*, 1H, H-21), 7.98 (*d*, 1H, *H-11*), 8.41 (*t*, 1H, H-28); ^{13}C NMR (125 MHz, DMSO-d_6) ppm (see **17** for Bt-ε-spacer) 17.14 (*C-9*), 19.26 (*C-8*), 36.63 (C-26), 37.19 (C-23), 37.82 (C-22), 38.16 (C-27), 57.54 (*OMe*), 57.88 (*C-13*), 59.83 (*C-6*), 62.88 (*C-3*), 64.08 (*C-2*), 65.76 (*C-5*), 91.80 (O-*CH*$_2$-O), 161.93 (C-14), 166.22 (*C-7*), 173.49 (*C-10*); mass (FAB-MNBA, –Q1MS) m/z 887 ($C_{32}H_{53}O_{12}N_7S_5$ – 1).

*6-[(Biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-carbonyl)methylsulfonyl]aminopenicillanic acid S,S-dioxide (15)*

A solution of methoxymethyl ester **14** (19 mg, 0.02 mmol) in MeOH (2 mL) and H_2O (3.6 mL) was stirred overnight at room temperature. Evaporation of MeOH and lyophilization gave the acid **15**: yield 17 mg (100%); R_f (CH_2Cl_2:MeOH, 80:20) ≈ 0; IR (KBr) 3399 (br), 1804, 1720–1640 (br), 1547, 1440, 1325, 1206, 1162, 1117 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ (see **17** for Bt-ε-spacer) 1.35 (3H, *H-9*), 1.46 (*s*, 3H, *H-8*), 2.76–2.78 (*t*, 4H, H-23 + H-26), 3.31 (*m*, 2H, H-22), 3.38 (*m*, 2H, H-27), 4.16 (AB, 2H, *H-13*), 4.36 (*s*, 1H, *H-3*), 5.35 (*d*, 1H, *J* = 4 Hz, *H-5*), 5.52 (*dd*, 1H, *J* = 4 and 10 Hz, *H-6*), 7.95 (*d* + *t*, 2H, *H-11* + H-21), 8.40 (*t*, 1H, H-28); ^{13}C NMR (125 MHz, DMSO-d_6) ppm (see **17** for Bt-ε-spacer) 17.21 (*C-9*), 19.37 (*C-8*), 36.63 (C-26), 37.20 (C-23), 37.83 (C-22), 38.17 (C-27), 57.90 (*C-13*), 59.75 (*C-6*), 63.14 (*C-3*), 64.01 (*C-2*), 65.72 (*C-5*), 161.94 (*C-14*), 167.88 (*C-7*), 173.55 (*C-10*); mass (FAB-MNBA,–Q1MS) m/z 842 ($C_{30}H_{49}O_{11}N_7S_5$ – 1), 798 (M – CO_2), 413, 414 (S–S cleavage). The sodium salt **16** was obtained by dissolution of the acid **15** into aqueous $NaHCO_3$ (1 equiv.) and lyophilization; IR (KBr) 3300 (br), 1796, 1690, 1645 (br), 1551, 1463, 1386, 1319, 1160, 1114 cm^{-1}; mass (FAB-MNBA, –Q1MS) m/z 864 ($C_{30}H_{48}O_{11}N_7S_5Na$ – 1), 842 (M – Na).

*Methoxymethyl 6-(biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-glutaryl)aminopenicillinate (17)*

A mixture of biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-glutaryl acid **6** (440 mg, 0.726 mmol), 1 equiv.) and *N*-ethylmorpholine (0.185 mL, 1.45 mmol, 2 equiv.) in DMF (45 mL) was heated at 50 °C until dissolution occurred. Methoxymethyl 6-aminopenicillinate **11** (189 mg, 0.726 mmol, 1 equiv.) was added and the solution was cooled in an ice-bath. HBTU (330 mg, 0.87 mmol, 1.2 equiv.) was added; after 10 min at 0 °C, the mixture was stirred for 1 h 30 min at room temperature. Dilution with CH_2Cl_2 (200 mL), washing with H_2O (1 × 70 mL), 10% citric acid (2 × 60 mL), 5% $NaHCO_3$ (1 × 70 mL) and H_2O (2 × 60 mL), drying over $MgSO_4$ and concentration gave the crude coupling product **17**. This was triturated and washed with ether, then dried under vacuum and stored at –20 °C: yield 345 mg (beige amorphous solid, 56%). Dissolution in CH_2Cl_2:MeOH (80:20) and rapid filtration over silica gel gave the analytical sample: mp 105 °C; $[\alpha]^{25}_D$ + 108.3° (MeOH, *c* 1); R_f (CH_2Cl_2:MeOH, 80:20) = 0.6; IR (KBr) 3300 (br), 1786 (CO β-lactam), 1750 (CO ester), 1701 (CO urea), 1680 (CO amide), 1645 (CO amides), 1460 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 1.21 (*m*, 2H, H-17), 1.28 (*m*, 2H, H-10), 1.35 (*m*, 2H, H-16), 1.44 (*s* + *m*, 4H, *H-8* + H-9), 1.47 (*m*, 4H, H-11 + H-18), 1.57 (*m*, 1H, H'-9), 1.59 (*s*, 3H, *H-9*), 1.68 (*m*, 2H, H-31), 2.03 (*t*, 4H, H-12 + H-19), 2.06 (*t*, 2H, H-32), 2.18 (*t*, 2H, H-30), 2.56 (*d*, 1H, H-2), 2.74 (*t*, 4H, H-23 + H-26), 2.81 (*dd*, 1H, H'-2), 2.98 (*m*, 2H, H-15), 3.08 (*m*, 1H, H-8), 3.29 (*m*, 4H, H-22 + H-27), 3.41 (*s*, 3H, *OMe*), 4.11 (*m*, 1H, H-7), 4.28 (*m*, 1H, H-3), 4.37 (*s*, 1H, *H-3*), 5.27 (*d*, 1H, O-*CH*-O), 5.32 (*d*, 1H, O-*CH*-O), 5.45–5.52 (AB, 2H, *H-5* + *H-6*), 6.36 (*s*, 1H, H-4), 6.43 (*s*, 1H, H-6), 7.71 (*t*, 1H, H-14), 7.95–8.00 (*t*, 2H, H-21 + H-28), 8.69 (*d*, 1H, *H-11*); ^{13}C NMR (125 MHz, DMSO-d_6) ppm 21.28 (C-31), 24.90 (C-18), 25.25 (C-11), 26.04 (C-17), 26.41 (*C-8*), 27.97 (C-9), 28.14 (C-10), 28.90 (C-16), 30.31 (*C-9*), 33.92 (C-32), 34.60 (C-30), 35.16 (C-12), 35.24 (C-19), 37.24 (C-23), 37.31 (C-26), 37.84 (C-22), 37.87 (C-27), 38.24 (C-15), 39.59 (C-2), 55.34 (C-8), 57.41 (*OMe*), 58.68 (*C-6*), 59.14 (C-7), 60.98 (C-3), 63.84 (*C-2*), 67.53 (*C-5*), 69.93 (*C-3*), 91.14 (O-*CH*$_2$-O), 162.62 (C-5), 167.07 (*C-7*), 171.71 (C-13), 171.75 (C-33), 172.07 (C-29), 172.15 (C-20), 173.58 (*C-10*); mass (FAB-MNBA, +Q1MS) m/z 849, 588, 415. Anal. calcd for $C_{35}H_{57}N_7O_9S_4 \cdot H_2O$ (866.11): C, 48.53%; H, 6.86%; N, 11.32%; found: C, 48.43%; H, 6.69%; N, 10.79%.

*Triethylammonium 6-(biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*'-glutaryl)aminopenicillinate (18)*

To a suspension of 6-APA **10** (28 mg, 0.129 mmol, 1 equiv.) in DMF (2.5 mL) were added successively Et_3N

(0.036 mL, 0.259 mmol, 2 equiv.) and pentafluorophenyl biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N'*-glutarylate **7** (100 mg, 0.129 mmol, 1 equiv.) dissolved in DMF (1 mL). The mixture was stirred overnight at room temperature. The solvent was removed under high vacuum; the residue was triturated and washed with ether (5 ×), then dried under vacuum in the presence of P_2O_5, to give the coupling product **18** as an amorphous beige solid: yield 113 mg (96.5%, this product was stored at –20 °C); R_f (CH_2Cl_2:MeOH, 80:20) ≈ 0; IR (KBr) 3299 (br), 1780 (CO β-lactam), 1704 (CO urea), 1646 (br, CO amides), 1547 cm^{-1}; mass (FAB-MNBA, –Q1MS) m/z 802 ($C_{33}H_{52}N_7O_8S_4$); ^1H NMR (500 MHz, DMSO-d_6) δ (see **17** for Bt-ε-Cyst GlA-spacer) 1.57 (*s*, 3H, *H-8*), 1.44 (*s*, 3H, *H-9*), 4.14 (*s*, 1H, *H-3*), 5.42 (AB, 2H, J = 4.1 Hz and 10 Hz, *H-5* + *H-6*), 8.62 (*d*, 1H, *H-11*), 1.14 (*t*, 9H, N-CH_2-CH_3), 3.02 (*q*, 6H, N-CH_2-CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) ppm (see **17** for Bt-ε-Cyst-GlA-spacer) 26.78 (*C-9*), 30.57 (*C-8*), 58.34 (*C-6*), 63.72 (*C-2*), 67.30 (*C-5*), 69.89 (*C-3*), 169.04 (*C-7*), 173.47 (*C-10*), 8.68 (N-CH_2-CH_3), 45.43 (N-CH_2-CH_3).

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