Abstract—The use of methanethiosulfonates as thiol-specific modifying reagents in the strategy of combined site-directed mutagenesis and chemical modification allows virtually unlimited opportunities for creating new protein surface environments. As a consequence of our interest in electrostatic manipulation as a means of tailoring enzyme activity and specificity, we have adopted this approach for the controlled incorporation of multiple negative charges at single sites in the representative serine protease, subtilisin \textit{Bacillus lentus} (SBL). A series of mono-, di- and triacidic acid methanethiosulfonates were synthesized and used to modify cysteine mutants of SBL at positions 62 in the S2 site, 156 and 166 in the S1 site and 217 in the S1\textsubscript{0} site. Kinetic parameters for these chemically modified mutant (CMM) enzymes were determined at pH 8.6 under conditions which ensured complete ionization of the unnatural amino acid side-chains introduced. The presence of up to three negative charges in the S1, S1\textsubscript{0} and S2 subsites of SBL resulted in up to 11-fold lowered activity, possibly due to interference with oxyanion stabilization of the transition state of the hydrolytic reactions catalyzed. Each unit increase in negative charge resulted in a raising of \( K_M \) and a reduction of \( k_{cat} \). However, no upper limit was observed for increases in \( K_M \), whereas decreases in \( k_{cat} \) reached a limiting value. Comparison with sterically similar but uncharged CMMs revealed that electrostatic effects of negative charges at positions 62, 156 and 217 are detrimental, but are beneficial at position 166. These results indicate that the ground-state binding of SBL to the standard substrate, Suc-AAPF-pNA, to SBL is reduced, but without drastic attenuation of catalytic efficiency, and show that SBL tolerates high levels of charge at single sites. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

The introduction of charge into wild-type (WT) enzymes, through random\textsuperscript{1–3} or site-directed\textsuperscript{4–16} mutagenesis or chemical modification,\textsuperscript{17,18} can be used to broaden substrate specificity or to enhance catalytic activity. The successful tailoring of specificity towards charged substrates has confirmed the validity of exploiting the electrostatic attraction between complementary ions as a viable strategy for improving binding. For example, the introduction of negative charge into the active sites of subtilisin BPN',\textsuperscript{4} aspartate aminotransferase\textsuperscript{6} and \( \text{L-} \)lactate dehydrogenase\textsuperscript{11} has expanded their structural specificities towards substrates with positively charged side chains.

Introductions of charge by protein engineering have typically been limited to naturally occurring amino acids such as negatively charged aspartate\textsuperscript{4,6,9,11–16} or positively charged arginine\textsuperscript{4,7,9} or lysine.\textsuperscript{4,5,9,12} Chemical modification allows a greater variety of charged groups to be introduced, but the reactions used for their introduction are often non-specific in nature.\textsuperscript{17} For example, the reaction of cyclic anhydrides with trypsin caused indiscriminate modification of lysine residues, the effects of which could only be interpreted in general terms.\textsuperscript{17} Furthermore, although ingenious molecular biological techniques have been developed for the introduction of non-natural charged amino acids into proteins,\textsuperscript{20–22} they are not yet suitable for large-scale synthesis or routine application. In addition, the use of charged amino acids in such techniques can result in poor levels of incorporation.\textsuperscript{20,21}

The use of site-directed mutagenesis combined with chemical modification\textsuperscript{23–28} of single sites offers a potential solution to these problems. In this technique cysteine is introduced at preselected positions and its
thiol residue is then reacted with methanethiosulfonate (MTS) reagents (Scheme 1). Methanethiosulfonate reagents react specifically and quantitatively with thiols. Recently, using the serine protease subtilisin *Bacillus lentus* (SBL) as our model, we have used this technique to improve enzyme activity and alter specificity. SBL is a near-ideal enzyme for evaluating the validity of this strategy since its does not contain a natural cysteine. In the current work we describe the use of this method to introduce locally high charge density into SBL through the incorporation of single residues bearing multiple charges.

Using the X-ray structure of SBL as our guide, four sites were chosen for mutation because of their seminal positions in the active site. Two of these, N62 (subtilisin BPN' numbering) and L217, occupy positions that are equidistant from S221 of the catalytic triad, in the S235 and S1p pockets, respectively. The other two sites, S156 and S166, are located at the base of the S1 pocket and their side chains are directed towards SBL’s surface and catalytic triad, respectively. The MTS reagents 1a–e were selected to modify these positions.

Results and Discussion

Synthesis of carboxyalkyl methanethiosulfonates 1b–e

Previous work has demonstrated that, of the methods available, direct nucleophilic displacement of a primary alkyl bromide by methanethiosulfonate ion provides the most efficient method for the preparation of alkyl methanethiosulfonates. This general method was therefore adopted as the basis for the preparation of all of 1b–e. The aliphatic monocarboxylate MTS 1b was prepared from 5-bromopentanoic acid and NaSSO2CH3 in 80% yield.

The aromatic dicarboxylate MTS 1c was prepared from toluene-3,5-dicarboxylic acid (2) via a precursor benzylc bromide as shown in Scheme 2. Treatment of 3 with NaSSO2CH3 gave the protected aromatic MTS 4 in 60% yield. Hydrolysis of 4 with TFA gave 1c as a white solid (91% yield, 44% overall yield from 2).

The aliphatic di- and tricarboxylates 1d,e were prepared from Meldrum’s acid (5a) using 1,2-dibromoethane to introduce a brominated linker group as shown in Scheme 3. The low pKa of 5a allowed the use of mildly basic conditions compatible with this choice of linker. For the sake of simplicity, we chose methyl Meldrum’s acid (5b) as a starting material for 1d in which one alkylation site is blocked as a methyl group. The synthesis of 1e utilized this position to introduce a third carboxylic moiety.

Alkylation of 5b with 1,2-dibromoethane afforded bromide 6b in 71% yield. Treatment of 6b with NaSSO2CH3 in DMF at 50°C led to the displacement of the remaining bromide and resulted in the formation of protected dicarboxylate MTS 7b. Hydrolysis of 7b using acidic ion exchange resin allowed the successful formation of the aliphatic diacidic MTS 1d (79% yield, 37% overall yield from 5b).

The synthesis of the triacidic reagent 1e required the construction of a protected tricarboxylate 5c before
elaboration. Alkylation of Meldrum’s acid (5a) with tert-butyl bromoacetate allowed the formation of 5c with moderate selectivity in 59% yield. Elaboration of tricarboxylate 5c was carried out using 1,2-dibromoethane and then NaSSO₂CH₃ in an analogous manner to that used for the synthesis of 1d and gave protected tricarboxylate MTS 7c in 57% yield over 2 steps. Complete deprotection of 7c using CF₃COOD in D₂O was followed by ¹H NMR, and resulted in the formation of target 1e (70% yield, 23% overall yield from 5a).

Preparation of chemically modified mutants (CMMs)
MTS reagents 1a–e were used to modify the chosen SBL cysteine mutants, N62C, S156C, S166C and L217C under conditions described previously.31–33 These reactions proceeded rapidly and quantitatively, as judged by the monitoring of changes in specific activity and by titration of residual free thiols with Ellman’s reagent,46 respectively. The structure of the charged CMMs was confirmed by ES–MS. Non-reducing native PAGE was used to determine the purity of all the enzymes, which appeared as single bands. Consistent with the introduction of negative charge, each of the CMMs showed retarded mobility in the direction of the cathode relative to WT. The active enzyme concentration of the resulting CMM solutions was determined by active site titration with α-toluenesulfonyl fluoride (PMSF) using a fluoride ion-sensitive electrode.47

Kinetic effects of site specific modification
The effects of modification upon SBL were assessed by the determination of $k_{cat}$ and $K_M$ for the hydrolysis of succinyl-AAPF-p-nitroanilide (Suc-AAPF-pNA). Our usual assay pH of 8.6 ensured complete ionization of the unnatural amino acid side-chains introduced. The kinetic parameters of the 20 CMMs generated are compared with those of WT and unmodified mutants in Table 1 and Figure 2.

### Table 1. Kinetic parameters for modified enzymes

<table>
<thead>
<tr>
<th>Entry</th>
<th>Enzyme Pocket</th>
<th>R</th>
<th>Level of charge</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SBL-WT</td>
<td>—</td>
<td>—</td>
<td>153 ± 4</td>
<td>0.73 ± 0.05</td>
<td>209 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>N62C S₂</td>
<td>H</td>
<td>0</td>
<td>174 ± 9</td>
<td>1.90 ± 0.20</td>
<td>92 ± 11</td>
</tr>
<tr>
<td>3</td>
<td>a</td>
<td>1</td>
<td>119 ± 4</td>
<td>0.93 ± 0.07</td>
<td>128 ± 11</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>b</td>
<td>1</td>
<td>106 ± 2</td>
<td>1.01 ± 0.05</td>
<td>105 ± 6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>c</td>
<td>2</td>
<td>113 ± 7</td>
<td>1.00 ± 0.10</td>
<td>113 ± 13</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>d</td>
<td>2</td>
<td>90 ± 4</td>
<td>1.47 ± 0.14</td>
<td>61 ± 6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>e</td>
<td>3</td>
<td>129 ± 3</td>
<td>1.46 ± 0.06</td>
<td>88 ± 4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>L217C S₁’</td>
<td>H</td>
<td>0</td>
<td>41 ± 1</td>
<td>0.80 ± 0.04</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>9</td>
<td>a</td>
<td>1</td>
<td>89 ± 6</td>
<td>1.80 ± 0.20</td>
<td>50 ± 6</td>
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<td>10</td>
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<td>1</td>
<td>54 ± 1</td>
<td>1.03 ± 0.06</td>
<td>52 ± 3</td>
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</tr>
<tr>
<td>11</td>
<td>c</td>
<td>2</td>
<td>69 ± 2</td>
<td>0.81 ± 0.06</td>
<td>85 ± 7</td>
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</tr>
<tr>
<td>12</td>
<td>d</td>
<td>2</td>
<td>63 ± 2</td>
<td>1.65 ± 0.11</td>
<td>38 ± 3</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>e</td>
<td>3</td>
<td>55 ± 2</td>
<td>1.48 ± 0.08</td>
<td>37 ± 3</td>
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</tr>
<tr>
<td>14</td>
<td>S156C S₁</td>
<td>H</td>
<td>0</td>
<td>125 ± 4</td>
<td>0.85 ± 0.06</td>
<td>147 ± 11</td>
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<tr>
<td>15</td>
<td>a</td>
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<td>87 ± 2</td>
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<tr>
<td>16</td>
<td>b</td>
<td>1</td>
<td>76 ± 1</td>
<td>1.08 ± 0.04</td>
<td>70 ± 3</td>
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</tr>
<tr>
<td>17</td>
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<td>61 ± 1</td>
<td>1.39 ± 0.10</td>
<td>44 ± 3</td>
<td></td>
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<tr>
<td>18</td>
<td>d</td>
<td>2</td>
<td>53 ± 1</td>
<td>1.67 ± 0.06</td>
<td>32 ± 1</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>e</td>
<td>3</td>
<td>74 ± 2</td>
<td>1.87 ± 0.08</td>
<td>39 ± 2</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>S166C H</td>
<td>0</td>
<td>42 ± 1</td>
<td>0.50 ± 0.05</td>
<td>84 ± 9</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>a</td>
<td>1</td>
<td>25 ± 1</td>
<td>1.34 ± 0.08</td>
<td>19 ± 1</td>
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</tr>
<tr>
<td>22</td>
<td>b</td>
<td>1</td>
<td>48 ± 1b</td>
<td>1.52 ± 0.06</td>
<td>31 ± 1b</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>c</td>
<td>2</td>
<td>47 ± 3</td>
<td>1.60 ± 0.20</td>
<td>29 ± 4</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>d</td>
<td>2</td>
<td>67 ± 2</td>
<td>2.26 ± 0.10</td>
<td>30 ± 2</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>e</td>
<td>3</td>
<td>76 ± 2</td>
<td>2.46 ± 0.11</td>
<td>31 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

a Michaelis–Menten constants were measured at 25 °C according to the initial rates method in 0.1 M Tris–HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, Suc-AAPF-pNA as the substrate.
b Based on total protein concentration.48
Position 62 in the S2 pocket is the most tolerant of mutation and modification (Fig. 2(a)) and mutation to cysteine reduces $k_{\text{cat}}/K_M$ by only a factor of 2 (Table 1, Entry 2). Upon modification, activity is partially restored and values of $k_{\text{cat}}/K_M$ for N62C-a,b,c are raised approximately 1.5-fold relative to N62C (Table 1, entries 3–5). Modifications with aliphatic di- and tri-carboxylate MTS reagents d,e elicit further drops in $k_{\text{cat}}/K_M$, with N62C-d being 3.5-fold lower than WT. However, despite the increased charge, this $k_{\text{cat}}/K_M$ drop is less marked for N62C-e.

The deleterious effect of negative charges in the S2 pocket upon $k_{\text{cat}}$ is apparent in the 1.3-fold decrease observed for N62C-a (Table 1, entry 3) relative to WT. However, as the level of negative charge increases, $k_{\text{cat}}$ values do not decrease further to any significant extent.

The effects of mutation at position 217 in the S1’ pocket (Fig. 2(b)) are intrinsically more dramatic than at all three other sites since the value of $k_{\text{cat}}/K_M$ for L217C is 4-fold lower than WT. The introduction of a single negative charge only affects $k_{\text{cat}}/K_M$ a little and leads to near-identical $k_{\text{cat}}/K_M$ values for L217C-a,b (Table 1, Entries 9,10). As negative charge increases further, two opposite trends are observed, with the $k_{\text{cat}}/K_M$ value for aromatic L217C-e being raised 1.6-fold relative to L217C-a,b, while those for aliphatic L217C-d,e are reduced by 1.4-fold.

These slight $k_{\text{cat}}/K_M$ changes seen at position 217 are the result of larger, but counteracting changes in each of $k_{\text{cat}}$ and $K_M$. For example, while L217C-a has the highest value of $k_{\text{cat}}$, it also has the highest $K_M$ value (both 2.2-fold higher than L217C). As at position 62, when the level of negative charge increases, from L217C-a to L217C-e, $k_{\text{cat}}$ values decrease only slightly and remain 1.3- to 1.7-fold higher than L217C (Table 1, entries 10–13). $K_M$ values increase unevenly to 2-fold higher than WT. Interestingly, the underlying cause of the out-of-line $k_{\text{cat}}/K_M$ of L217C-e is an unusually low $K_M$ (0.81 mM, entry 11), which may be a consequence of complementary aromatic interactions between the substrate and the phenyl ring of side chain e.

The effects of mutation and modification at positions 156 and 166 in the S1 pocket are shown in Figure 2(c) and (d). Mutation at position 156 to cysteine causes a 1.4-fold drop in $k_{\text{cat}}/K_M$ (S156C, Table 1, entry 14). From S156C-a to S156C-d $k_{\text{cat}}/K_M$ decreases monotonically to a value that is 6-fold lower than WT. The additional negative charge present in S156C-e partially restores this value, to only 5.4-fold lower than WT.

Mutation and modification at position 166 leads to the least active negatively charged CMMs, with $k_{\text{cat}}/K_M$ 6 to 11-fold lower than WT. This partly reflects the intrinsically lower $k_{\text{cat}}/K_M$ value of the unmodified mutant S166C, which is already 2.5-fold lower than WT. However, the presence of the sulfonatoethyl side chain in S166C-a causes a dramatic drop to a value that is 11-fold lower than WT. $k_{\text{cat}}/K_M$ is increased 1.5-fold for S166C-b and remains steady as the level of negative charge increases from S166C-c to S166C-e.

The $k_{\text{cat}}$ values for S156C and S166C CMMs are similar to those found for L217C CMMs, typically 2 to
2.5-fold lower than WT. As at positions 62 and 217, the
detrimental effect of a single negative charge on $k_{\text{cat}}$ is
not amplified by the introduction of additional negative
charges. In fact, $k_{\text{cat}}$ values for S166C CMMs increase
steadily from 6-fold lower than WT for S166C-a (Table
1, entry 21) to 2-fold lower than WT for S166C-e (Table
1, entry 25).

The $K_M$ values for both S156C and S166C CMMs
increase steadily with increasing negative charge and are
largest for S166C-e ($K_M$ 2.46 mM, 3.5-fold higher than
WT, Table 1, entry 25). Consistent with its surface-
exposed nature these effects are less pronounced at
position 156, with $K_M$ increasing to only 2.5-fold higher
than WT for S156C-e (Table 1, entry 19).

Kinetic effects of negative charge

To separate the contribution of electrostatics from steric
effects, a comparison of these charged CMMs with
those containing sterically similar uncharged side
chains\(^{49}\) was made. For example, N62C-a was com-
pared with N62C-S-ethyl. N62C-b, d, e were compared
with N62C-S-n-pentyl and N62C-c was compared with
N62C-S-benzyl. Figure 3 illustrates the results of intro-
ducing charge to these near-isosteric systems. This pro-
vides an estimate of the effect of negative charge upon
the kinetics of SBL when corrected for underlying steric
and hydrophobic effects.

Two differing trends emerge from Figure 3. At positions
62, 217 and 156, the electrostatic contribution of each of
side chains a-e is detrimental to $k_{\text{cat}}/K_M$. The reductions
caued are similar for each side chain, vary little from
site to site and increase with the level of negative charge
introduced. These reduced $k_{\text{cat}}/K_M$ values resulting from
the introduction of negative charge are consistent with
earlier findings.\(^ {12,31\text{--}33}\) Such effects may be attributed, in
part, to destabilization of the tetrahedral oxyanion
intermediate that is formed in the rate limiting step of
catalysis.\(^ {50}\)

In contrast, the introduction of negative charge at posi-
tion 166 partially restores some of the activity lost
through the introduction of near-isosteric uncharged
side chains. Therefore, the drastically lowered $k_{\text{cat}}/K_M$
of CMMs S166C-a-e relative to WT are, in fact, a result
of steric or hydrophobic effects. Mutation analysis of
subtilisin BPN’ has shown that $k_{\text{cat}}/K_M$ decreases dra-
matically when the optimal binding volume of the S1-
pocket is exceeded.\(^ {51}\) The effect of introducing even
small groups at position 166 of SBL\(^ {52}\) is to fill the S1-
pocket and this dramatically decreases $k_{\text{cat}}/K_M$. For
example, uncharged CMM S166C-S-ethyl has a $k_{\text{cat}}/K_M$
13.5-fold lower than WT.\(^ {49}\) Molecular mechanics ana-
lysis of S166C CMMs has shown that charged side
chains introduced at position 166 may orientate them-
sefls towards external solvent.\(^ {33}\) This serves to reduce
the volume of the S1 pocket that is occupied by the side
chain. The existence of such an orientation for S166C-
a-e, which is lacking in uncharged CMM counterparts,
might, in part, explain the beneficial effects of introdu-
cing charge. As a result, charged CMM S166C-S-
EtSO\(_3^+\), side chain a, has a $k_{\text{cat}}/K_M$ only 11-fold lower
than WT.

Conclusions

In summary, we have devised short and efficient syn-
thetich routes to three novel multiply charged methane-
thiosulfonates, 1c, d and e. Such compounds, as well
as being of interest in our approach to the controlled
tailoring of enzyme activity, may prove useful in the
study of ion channels. The use of MTS reagents in
techniques such as the substituted-cysteine accessibility
method (SCAM)\(^ {53\text{--}55}\) has allowed aspects of membrane
ion channel topology and conformation to be deter-
mined. In particular the use of charged MTS reagents
has given an invaluable insight into ion specificity\(^ {56}\) and
mechanism of action.\(^ {57\text{--}61}\)

Using our established methodology, we selectively
modified the cysteine thials of SBL mutants, N62C,
S156C, S166C, and L217C, with these reagents. With-
out exception, mutation and modification at all four
sites led to reduced catalytic efficiency in the hydrolysis
of Suc-AAPF-pNA. However these reductions do not
exceed 11-fold relative to WT and the lowest $k_{\text{cat}}$ values
determined were only 6-fold reduced. This reduced effi-
ciency is manifested largely through decreased binding
interactions, i.e. decreased $K_M$ values, that increase with
the level of charge introduced. In contrast, $k_{\text{cat}}$ values
responding S156C CMMs increases with increasing negative charge.

The hydrolysis of different substrates, including those
containing basic residues, and pH-activity profiles, are
being investigated. The pK\(_a\) effects and specificity con-
sequences will be presented in due course.

![Figure 3](image-url)

Figure 3. The effects of introducing negative charge to CMMs: In ($k_{\text{cat}}/K_M$), with suc-AAPF-pNA as the substrate, of the negatively
charged N62C, L217C and S156C CMMs decreases relative to that of
near-isosteric uncharged CMMs as the level of negative charge
increases (from side chain a to e). In contrast, this value for the cor-
responding S166C CMMs increases with increasing negative charge.
Experimental

Mutants of subtilisin *Bacillus licheniformis* (SBL) were generated, and WT and mutant enzymes purified as described previously.\(^{32,33}\) NaSSO\(_2\)CH\(_3\) (mp 269–269.5°C (dec.) (lit.,\(^{29}\) mp 272–273.5°C) and toluene-3,5-dicarboxylic acid (2)\(^{62}\) (mp 294.5–296 °C (water) (lit.,\(^{62}\) mp 287–288°C) were prepared according to literature methods. DMF was distilled under N\(_2\) from CaH\(_2\) and stored over molecular sieves under N\(_2\) before use. CCl\(_4\) was fractionally distilled before use. Sulfonatoethyl methane-thiosulfonate (1a) was purchased from Toronto Research Chemicals (2 Brisbane Rd., Toronto, ON, Canada). All other chemicals were used as received from Sigma-Aldrich or Baker. All flash chromatography was performed using silica gel (Whatman, 60 A, 230–400 mesh). Melting points were determined using an Electrothermal IA9000 series digital melting point apparatus and are uncorrected. IR spectra were recorded and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:50) to give a colorless oil which solidified upon standing to give di-tert-butyl toluene-3,5-dicarboxylate (4.8 g, 84%) as a white solid; mp 86.5–87.5°C (hexane); IR (film) 1712 cm\(^{-1}\) (C=O), 1606, 1476 cm\(^{-1}\) (Ar C=C); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.60 (s, 18H, C(CH\(_3\))\(_3\)), 2.43 (s, 3H, CH\(_3\)), 7.95 (br s, 2H, H-2, H-6), 8.38 (br s, 1H, H-4); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 21.4 (CH\(_3\)), 28.2 (C(CH\(_3\))\(_3\)), 81.4 (C(CH\(_3\))\(_3\)), 127.7, 132.1, 133.7, 138.1 (Ar), 165.2 (COO).

NBS (0.521 g, 2.93 mmol) and 1,1’-azobis(cyclohexanecarbonitrile) (30 mg, 0.12 mmol) were added to solution of this diester (0.712 g, 2.44 mmol) in CCl\(_4\) (10 mL) and heated under reflux under N\(_2\). After 3 h a second portion of initiator (30 mg, 0.12 mmol) was added. After a further 3 h the reaction solution was cooled and filtered. The filtrate was washed with sat. NaHCO\(_3\) (aq), dried (MgSO\(_4\)), and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:50) to afford crude 3,5-di((tert-butoxy-carbonyl)benzyl bromide (3) (0.872 g, 96%). A solution of 3 (0.872 g, 2.35 mmol) and NaSSO\(_2\)CH\(_3\) (0.327 g, 2.44 mmol) in DMF (1 mL) was heated at 50°C under N\(_2\). After 1 h the reaction solution was cooled, diluted with water (5 mL) and extracted with ether (15 mL \times 3). The combined extracts were washed with brine, dried (MgSO\(_4\)), and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:8) to give 3,5-di((tert-butoxy-carbonyl)benzyl methanethiosulfonate (4) (0.570 g, 60%) as a colorless oil; IR (film) 1717 cm\(^{-1}\) (C=O), 1604, 1477, 1456 cm\(^{-1}\) (Ar C=C), 1328, 1135 cm\(^{-1}\) (S=SO\(_2\)); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.59 (s, 18H, C(CH\(_3\))\(_3\)), 3.07 (s, 3H, SO\(_2\)CH\(_3\)), 4.43 (s, 2H, CH\(_2\)), 8.13 (s, 2H, H-2, H-6), 8.48 (s, 1H, H-4); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 28.2 (C(CH\(_3\))\(_3\)), 40.0 (CH\(_2\)), 51.3 (SO\(_2\)CH\(_3\)), 82.1 (C(CH\(_3\))\(_3\)), 130.2, 133.2, 133.5, 135.7 (Ar), 164.3 (COO).

A solution of 4 (0.941 g, 2.30 mmol) in CF\(_3\)COOH: CH\(_2\)Cl\(_2\) (1: v/v, 10 mL) was stirred at room temperature for 3 h, during which time a white precipitate was formed. The solvents were removed and the residue triturated with CH\(_2\)Cl\(_2\) (5 mL). The resulting mixture was filtered, and the residue washed with CH\(_2\)Cl\(_2\) and dried under vacuum to give 1b (1.167 g, 80%) as a white solid; mp 61–62.5°C (lit.,\(^{42}\) mp 69–71°C); IR (KBr) 1703 cm\(^{-1}\) (C=O), 1311, 1125 cm\(^{-1}\) (S=SO\(_2\)); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.70–2.00 (m, 4H, H-2, H-3), 2.43 (t, \(J=1.9\) Hz, 2H, H-4), 3.20 (t, \(J=6.8\) Hz, 2H, H-1), 3.34 (s, 3H, SSO\(_2\)CH\(_3\)), 8.82 (br s, 1H, COOH); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 23.4, 28.9, 33.1, 35.9 (CH\(_2\))\(_3\), 50.7 (SSO\(_2\)CH\(_3\)), 178.7 (COOH); MS \textit{m/z} (EI\(^{+}\)) 213 (M+H\(^{+}\), 2), 195 (M+H\(^{+}\)-H\(_2\)O, 11), 133 (50), 115 (M\(^+\)-CH\(_3\)SO\(_2\)H, 100%); HRMS \textit{m/z} (EI\(^+\)) Found 213.0251 (M+H\(^{+}\)); C\(_{6}\)H\(_{13}\)O\(_{4}\)S\(_{2}\) requires 213.0255.

3,5-Dicarboxybenzyl methanethiosulfonate (1c). 1,1’-Carbonyldiimidarozole (6.67 g, 0.0441 mol) was added to a solution of toluene-3,5-dicarboxylic acid (2) (3.364 g, 0.0187 mol) in DMF (30 mL) and the resulting mixture stirred at 40°C under N\(_2\). After 1.5 h DBU (6.15 mL, 0.041 mol) and -BuOH (7.7 mL, 0.0822 mol) were added. After 24 h the solution was cooled, ether (150 mL) added and the mixture acidified (HCl (aq), 1.5 M). The etheral layer was separated and the aqueous layer further extracted (ether, 150 mL). The organic fractions were combined, washed with water and 10% K\(_2\)CO\(_3\) (aq), dried (MgSO\(_4\)), and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:50) to afford a colorless oil which solidified upon standing to give di-tert-butyl toluene-3,5-dicarboxylate (4.58 g, 84%) as a white solid; mp 86.5–87.5°C (hexane); IR (film) 1717 cm\(^{-1}\) (C=O), 1606, 1476 cm\(^{-1}\) (Ar C=C); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.60 (s, 18H, C(CH\(_3\))\(_3\)), 2.43 (s, 3H, CH\(_3\)), 7.95 (br s, 2H, H-2, H-6), 8.38 (br s, 1H, H-4); \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 21.4 (CH\(_3\)), 28.2 (C(CH\(_3\))\(_3\)), 81.4 (C(CH\(_3\))\(_3\)), 127.7, 132.1, 133.7, 138.1 (Ar), 165.2 (COO).

NBS (0.521 g, 2.93 mmol) and 1,1’-azobis(cyclohexanecarbonitrile) (30 mg, 0.12 mmol) were added to solution of this diester (0.712 g, 2.44 mmol) in CCl\(_4\) (10 mL) and heated under reflux under N\(_2\). After 3 h a second portion of initiator (30 mg, 0.12 mmol) was added. After a further 3 h the reaction solution was cooled and filtered. The filtrate was washed with sat. NaHCO\(_3\) (aq), dried (MgSO\(_4\)), and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:8) to give 3,5-di(tert-butoxy-carbonyl)benzyl thioanisylate (1d). Anhydrous K\(_2\)CO\(_3\) (1.67 g, 12.0 mmol) was added to a solution of methyl Meldrum’s acid (5b) (1 g, 6.33 mmol) in
DMF (33 mL) under N₂ and stirred vigorously. After 1 h the supernatant liquid was added dropwise to a solution of 1,2-dibromoethane (1.9 mL, 22.2 mmol) in DMF (11 mL) under N₂. After 89 h TLC (EtOAc:hexane, 1:3) indicated conversion of starting material (R₀ 0.3) to a major product (R₀ 0.5). The reaction mixture was added to water (100 mL) and extracted with ether (100 mL × 3). The organic fractions were combined, dried (MgSO₄), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 3:17 to 1:3) to give 5-(2'-bromoethyl)-2,2,5-trimethyl-1,3-dioxocyclohexa-4,6-dione (1e) (1.18 g, 71%) as a white solid; mp 84–85°C (ether:hexane); IR (KBr) 1738, 1784 cm⁻¹ (C=O); ¹H NMR (CDCl₃) δ 1.66 (s, 3H, C(CH₃)2), 1.76, 1.78 (s × 2, 3H × 2, C(CH₃)3), 2.61 (t, J = 8 Hz, 2H, H-1'), 3.32 (t, J = 8 Hz, 2H, H-2'); ¹³C NMR (CDCl₃) δ 25.2, 29.3, 29.9, 32.0, 31.9, 49.2 (C(CH₃)2, C(CH₃)2, C-1, C-2), 49.2 (C-5'), 51.2 (SSO₂CH₃), 106.1 (C(CH₃)2), 169.5 (C₄, C-6); MS m/z (EI+): 249, 251 (M⁺-CH₂SO₂CH₃), 206, 208 (M⁺-OC(CH₃)2, C(CH₃)2), 162, 164 (M⁺-C(O)OC(O)CH₃), 42, 69 (M⁺-C(CH₃)2CH₂Br, 100%).

NaSSO₂CH₃ (376 mg, 5.80 mmol) was added to a solution of 6b (1.18 g, 4.46 mmol) in DMF (40 mL) under N₂ and the resulting solution warmed to 50°C. After 29 h the reaction solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 3:7 to give 2-(2',2',5'-trimethyl-1,3-dioxocyclohexa-4,6-dionyl)ethyl methanethiosulfonate (7b) (1.10 g, 83%) as a cloudy oil; IR (film) 1737, 1771, 1300, 1133 cm⁻¹; ¹HNMR (CDCl₃) δ 0.84, 2.61, 2.62, 3.07, 3.08 (C(CH₃)3), 4.91, 4.92 (H₂COOBu), 107.8 (C(CH₃)2), 169.3 (C₄, C-6); MS m/z (EI+): 281 (M⁺-CH₃, 1), 269 (2), 239 (M⁺-H₂COOBu, 3), 159 (100), 141 (56), 113 (96), 103 (23), 87 (78), 69 (M⁺-C(O)OC(O)CH₃), 254.9997 (M⁻[H⁺]), 100), 200 (8), 159 (25) 143 (32%).

Anhydrous K₂CO₃ (300 mg, 2.17 mmol) was added to a solution of 5e (400 mg, 1.55 mmol) in DMF (10 mL) under N₂ and stirred vigorously. After 1 h the supernatant liquid was added dropwise to a solution of 1,2-dibromoethane (0.7 mL, 8.06 mmol) in DMF (3 mL) under N₂ at 50°C. After 70 h TLC (EtOAc:hexane, 1:9) indicated the conversion of starting material (R₀ 0.1) to a major product (R₀ 0.3). The reaction mixture was cooled, added to distilled water (50 mL) and extracted with ether (50 mL × 3). The organic fractions were combined, dried (MgSO₄), filtered and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:9) to give 5-(2'-bromoethyl)-5-(tert-butoxy carbonyl)-2,2,5-trimethyl-1,3-dioxocyclohexa-4,6-dione (6e) (372 mg, 66%) as a white solid; mp 120–123°C (ether:hexane); IR (KBr) 1773, 1731 cm⁻¹ (C=O); ¹H NMR (CDCl₃) δ 1.40 (s, 9H, C(CH₃)3), 1.80 (s, 6H, C(CH₃)2), 3.09 (d, J = 4 Hz, 2H, CH₂COOBu), 3.70 (t, J = 4 Hz, 1H, H-5'); ¹³C NMR (CDCl₃) δ 18.7, 28.8, 33.0, 43.4 (CH₂COOBu), C(CH₃)2, 28.5 (C(CH₃)2), 82.8 (C(CH₃)2), 105.6 (C(CH₃)2), 165.6, 169.6 (C-4, C-6, COOBu); MS m/z (CI-): 257 (M⁺-[H⁺]), 254, 200 (8), 159 (25) 143 (32%).

NaSSO₂CH₃ (143 mg, 1.07 mmol) was added to a solution of 6c (301 mg, 0.82 mmol) in DMF (20 mL) under N₂ and the resulting solution warmed to 50°C. After 29 h the reaction solution was cooled and the solvent removed. The residue was purified by flash chromatography (EtOAc:hexane, 1:3) and crystallized from ether to give 2-(5'-tert-butoxy carbonyl)-2,2'-dimethyl-1,3-dioxocyclohexa-4,6-dionyl)ethyl methanethiosulfonate (7e) (280 mg, 86%) as a colorless crystalline solid; mp 103–105°C (ether:hexane); IR (KBr) 1772, 1738,
1717 cm$^{-1}$ (C=O) 1314, 1129 cm$^{-1}$ (S=SO$_2$); $^1$H NMR CDC$_3$ $\delta$ 1.141 (s, 9H, C(CH$_3$)$_3$), 1.83, 1.93 (s x 2, 3H x 2, C(CH$_3$)$_2$), 2.33–2.41 (m, 2H, H-2), 3.10–3.20 (m, 2H, H-4), 3.12–3.20 (m, 2H, H-2), 3.45 (s, 3H, SSO$_2$CH$_3$); $^{13}$C NMR (CDCl$_3$) $\delta$ 28.0 (C(CH$_3$)$_3$), 28.9, 29.2, 30.7, 37.9, 40.2 (C$_2$COOBU$^-$, C(CH$_3$)$_2$), C-1, C-2, 50.0 (C-3), 50.6 (SSO$_2$CH$_3$), 82.8 (C(CH$_3$)$_3$), 107.3 (C(CH$_3$)$_3$), 167.2, 169.7 (C$^4$, C$^6$, C$^0$COOBU$^-$); MS m/z (CI$^-$): 395 ([M-H]$^-$ 1), 381 (M$^-$CH$_3$), 281 (M$^-$CH$_2$COOBU$^-$ 5), 257 (M$^-$CH$_2$SSO$_2$CH$_3$), 100, 215 (45), 158 (37%).

A solution of 7c (138 mg, 0.35 mmol) in CF$_3$COOD:D$_2$O (7:3, 2 mL) was heated to 50°C. After 32 h, $^1$H NMR spectroscopy showed the conversion of starting material to a single product. The solution was cooled and the solvent removed. The residue was purified by flash chromatography (butan-1-ol:AcOH:water, 4:1) and ion exchange chromatography (Amberlyst A21, 30% v/v CF$_3$COOH (aq) as eluent) to give 1e (73 mg, 70%) as an amorphous solid; IR (KBr) 1706 cm$^{-1}$ (C=O) 1310, 1127 cm$^{-1}$ (S=SO$_2$); $^1$H NMR (D$_2$O) $\delta$ 2.25–2.34 (m, 2H, H-2), 3.01 (s, 2H, H-4), 3.12–3.20 (m, 2H, H-2), 3.45 (s, 3H, SSO$_2$CH$_3$); $^{13}$C NMR (D$_2$O) $\delta$ 34.3, 37.7, 40.2 (C-1, C-2), 50.0 (C-3), 50.6 (SSO$_2$CH$_3$), 82.8 (C(CH$_3$)$_3$), 107.3 (C(CH$_3$)$_3$), 167.2, 169.7 (C$^4$, C$^6$, C$^0$COOBU$^-$); MS m/z (CI$^-$): 395 ([M-H]$^-$ 1), 381 (M$^-$CH$_3$), 281 (M$^-$CH$_2$COOBU$^-$ 5), 257 (M$^-$CH$_2$SSO$_2$CH$_3$), 100, 215 (45), 158 (37%).

Site-specific chemical modification. To approximately 25 mg of each of the SBL mutants in CHES buffer (2.5 mL; 70 mM CHES, 5 mM MES, 2 mM CaCl$_2$, pH 9.5) at 37°C, 37% (v/v) CF$_3$COOD:D$_2$O (5:95, 2 mL) was heated to 50°C. After 32 h, $^1$H NMR spectroscopy showed the conversion of starting material to a single product. The solution was cooled and the solvent removed. The residue was purified by flash chromatography (butan-1-ol:AcOH:water, 4:1) and ion exchange chromatography (Amberlyst A21, 30% v/v CF$_3$COOH (aq) as eluent) to give 1e (73 mg, 70%) as an amorphous solid; IR (KBr) 1706 cm$^{-1}$ (C=O) 1310, 1127 cm$^{-1}$ (S=SO$_2$); $^1$H NMR (D$_2$O) $\delta$ 2.25–2.34 (m, 2H, H-2), 3.01 (s, 2H, H-4), 3.12–3.20 (m, 2H, H-2), 3.45 (s, 3H, SSO$_2$CH$_3$); $^{13}$C NMR (D$_2$O) $\delta$ 34.3, 37.7, 41.1 (C-1, C-2, C-4), 52.9 (SSO$_2$CH$_3$), 58.0 (C-3), 171.7 (COOH, CH$_2$COOH); MS m/z (FAB$^-$): 299 ([MH]$^-$), 221 (21), 183 (40), 111 (49), 91 (100%). Anal. caled for C$_9$H$_{18}$O$_5$S: C, 32.00; H, 4.03%; found: C, 31.84; H 3.91%;

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References and Notes

of the high propensity of the anion of 5a to form cyclic products with \( \text{Zn}^{2+} \) dihalides see Ridvan L; Závada J. Tetrahedron 1997, 53, 14793.

45. Direct alkylation of 5a with 1,2-dibromoethane led only to the formation of a spirocyclopropane derivative, the product of an intramolecular cyclization, in low yield. For a recent analysis of the high propensity of the anion of 5a to form cyclic products with \( \text{Zn}^{2+} \) dihalides see Ridvan L; Závada J. Tetrahedron 1997, 53, 14793.


48. Certain inconsistencies between active enzyme concentration as determined by active site titration with PMSF, and total protein concentration have been reported for negatively charged mutants of SBL, (Murray C.J., personal communication). These are characterized by sluggish fluoride ion concentration bursts and high rates of background PMSF hydrolysis. Active enzyme concentration values for S166C-b were low and gave rise to an anomalous value for \( k_{\text{cat}} \) (270 ± 5 s\(^{-1}\)). Consequently the value shown was calculated using total protein concentration as determined by absorbance at 280 nm \((E_{280} = 23,000 \text{ M}^{-1} \text{ cm}^{-1})\). The \( K_M \) value determined (1.52 ± 0.06 M) is not concentration dependent.

49. The following kinetic parameters for the previously prepared \(^{31,32}\) near isosteric CMMs were determined under the conditions described in the experimental section: N62C-S-ethyl \( k_{\text{cat}} \) 104 ± 2 s\(^{-1}\), \( K_M \) 0.55 ± 0.04 mM, \( k_{\text{cat}}/K_M \) 189 ± 14 s\(^{-1}\) M\(^{-1}\); N62C-S-benzyl \( k_{\text{cat}} \) 129 ± 3 s\(^{-1}\), \( K_M \) 0.34 ± 0.03 mM, \( k_{\text{cat}}/K_M \) 379 ± 37 s\(^{-1}\) M\(^{-1}\); N62C-S-\( N\)pentyl \( k_{\text{cat}} \) 184 ± 5 s\(^{-1}\), \( K_M \) 0.75 ± 0.05 mM, \( k_{\text{cat}}/K_M \) 245 ± 18 s\(^{-1}\) M\(^{-1}\); L217C-S-\( N\)pentyl \( k_{\text{cat}} \) 87 ± 3 s\(^{-1}\), \( K_M \) 0.52 ± 0.05 mM, \( k_{\text{cat}}/K_M \) 167 ± 17 s\(^{-1}\) M\(^{-1}\); S166C-S-benzyl \( k_{\text{cat}} \) 72 ± 2 s\(^{-1}\), \( K_M \) 0.59 ± 0.05 mM, \( k_{\text{cat}}/K_M \) 122 ± 11 s\(^{-1}\) M\(^{-1}\); S166C-S-ethyl \( k_{\text{cat}} \) 11.8 ± 0.5 s\(^{-1}\), \( K_M \) 0.76 ± 0.08 mM, \( k_{\text{cat}}/K_M \) 15.5 ± 1.8 s\(^{-1}\) M\(^{-1}\); S166C-S-benzyl \( k_{\text{cat}} \) 23.1 ± 0.5 s\(^{-1}\), \( K_M \) 1.17 ± 0.06 mM, \( k_{\text{cat}}/K_M \) 19.7 ± 1.1 s\(^{-1}\) M\(^{-1}\).


52. This space is more limited in SBL than in subtilisin BPN‘ as the peptide backbone that makes up the wall of the S1 pocket contains four less amino acid residues.


