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Covalent Modification of Subtilisin *Bacillus lentus* Cysteine Mutants with Enantiomerically Pure Chiral Auxiliaries Causes Remarkable Changes in Activity

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Abstract—Methanethiosulfonate reagents may be used to introduce virtually unlimited structural modifications in enzymes via reaction with the thiol group of cysteine. The covalent coupling of enantiomerically pure (*R*) and (*S*) chiral auxiliary methanethiosulfonate ligands to cysteine mutants of subtilisin *Bacillus lentus* induces spectacular changes in catalytic activity between diastereomeric enzymes. Amidase and esterase kinetic assays using a low substrate approximation were used to establish k_{cat}/K_M values for the chemically modified mutants, and up to 3-fold differences in activity were found between diastereomeric enzymes. Changing the length of the carbon chain linking the phenyl or benzyl oxazolidinone ligand to the mutant N62C by a methylene unit reverses which diastereomeric enzyme is more active. Similarly, changing from a phenyl to benzyl oxazolidinone ligand at S166C reverses which diastereomeric enzyme is more active. Chiral modifications at S166C and L217C give CMMs having both high esterase k_{cat}/K_M's and high esterase to amidase ratios with large differences between diastereomeric enzymes.

Introduction

Many methods for improving the activity and enantioselectivity of hydrolases have been investigated. They include extreme temperatures,[1] solvent engineering,[2] structural variation of the substrate,[3] imprinting,[4] lyoprotectants,[5] chemical modification,[6] site-directed[7] and random[8] mutagenesis. However, the chemical modification of mutant enzymes has been underused as a method for generating new hydrolases with novel properties.[9] We have applied this strategy of site-directed mutagenesis combined with chemical modification to enhance the activity and alter the specificity of subtilisin *Bacillus lentus* (SBL).[10] Using this technique, cysteine is introduced at well defined locations near important pockets around the active site, and the thiol is reacted specifically with a methanethiosulfonate (MTS) reagent. This methodology offers almost unlimited possibilities for structural variation. In addition, wild-type (WT) SBL contains no natural cysteine, so the structural modification of the cysteine mutant occurs solely at the targeted site.

In this paper, we endeavor to explore changes in enzyme catalysis induced by the covalent attachment of enantiomerically pure MTS ligands derived from chiral auxiliaries to cysteine mutants of SBL (Scheme 1). We selected mandelic acid and several oxazolidinones constructed from glycine, valine, phenylglycine, phenylalanine and *cis*-1-amino-indanol. We covalently linked the homochiral MTS ligands to cysteine mutants of SBL to create sets of diastereomeric chemically modified mutants (CMMs) allowing the observation of enzyme activity changes due solely to differences in the chiral environment at one site. This methodology acts as a very fine and precise probe of enzymatic catalysis, since any differences between diastereomeric enzymes are solely attributable to the changed absolute configuration of the ligand.

Enantiomerically pure MTS ligands, **1a–i**, were synthesized and used to chemically modify the N62C, S156C, S166C and L217C mutants of SBL. These residues were targeted on the basis of SBL's X-ray crystal structure.[11] N62C is in the S_2 pocket near His-64.[12] S156C and S166C are at the bottom of the S_1 pocket. However, S156C is surface exposed and S166C is buried pointing into the pocket. L217C is found in S_1' which is where the leaving group is bound. A kinetic assay of amidase and esterase activity was conducted on these new diastereomeric

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CMMs in order to investigate their properties and to probe any changes in selectivity.

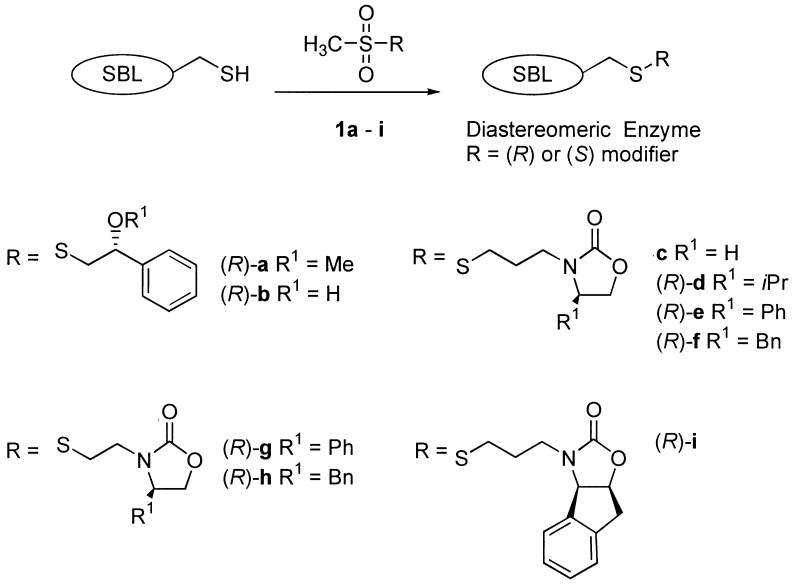
Results

Synthesis of MTS reagents 1a–i

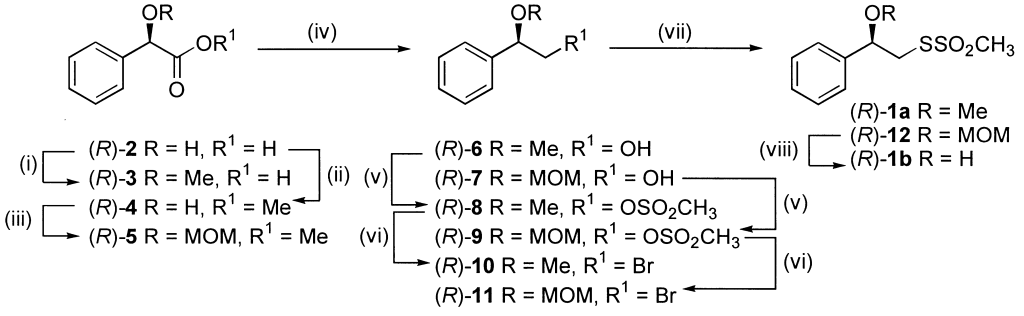
For the synthesis of the mandelate based MTS ligands, (*R*)-mandelic acid, (*R*)-**2**, was *O*-methylated with Me_2SO_4[13] in $NaOH/H_2O$ to give (*R*)-**3** in 37% yield (Scheme 2). The acid, (*R*)-**3**, was reduced in 72% yield with borane in THF to alcohol, (*R*)-**6**, which was converted quantitatively to mesylate, (*R*)-**8**, in CH_2Cl_2. The mesylate was converted to bromide, (*R*)-**10** (73%), by the action of LiBr in refluxing acetone, and methanethiosulfonate, (*R*)-**1a**, was formed in 84% yield from bromide, (*R*)-**10**, using $NaSSO_2CH_3$ in DMF. The methanethiosulfonate (*S*)-**1a** was made in an analogous fashion from (*S*)-mandelic acid.

A similar approach allowed the synthesis of (*R*)-**1b** (Scheme 2). (*R*)-Mandelic acid, (*R*)-**2**, was esterified to give (*R*)-**4** which was protected as its methoxymethyloxy ether, (*R*)-**5**, in excellent yield (90% for two steps). The ester, (*R*)-**5**, was reduced with $LiBH_4$ to the alcohol, (*R*)-**7** (98%), which was converted to the mesylate, (*R*)-**9**, and then to the bromide, (*R*)-**11** (80% for two steps), using the same conditions as for the methyl ether analogue. This bromide was reacted with $NaSSO_2CH_3$ in DMF for 2 days to give (*R*)-**12** in 61% yield. The alcohol was deprotected by the action of TFA/H_2O to give the MTS reagent, (*R*)-**1b**, in 82% yield. The methanethiosulfonate (*S*)-**1b** was made in an analogous fashion from (*S*)-mandelic acid.

The synthesis of oxazolidinone-based methanethiosulfonate ligands is shown in Scheme 3. Oxazolidinones have been widely used as chiral auxiliaries in asymmetric synthesis, and the degree of asymmetric induction can be excellent in chemical transformations ranging from alkylations to aldol reactions to Diels–Alder additions.[14] The commercially available oxazolidinones, **13**-(*R*)-**16**, were alkylated with 1,3-dibromopropane or 1,2-dibromoethane in DMSO/KOH[15] to give the bromides, **17**-(*R*)-**22**, which



Scheme 1. The corresponding author (*S*) MTS ligands follow the same code scheme [i.e. (*S*)-**a,** (*S*)-**b,** (*S*)-**d,** (*S*)-**e**, (*S*)-**f**, (*S*)-**g**, (*S*)-**h**, (*S*)-**i**].



Scheme 2. Reagents. (i) Me_2SO_4, NaOH, H_2O, 37%; (ii) MeOH, H^+; (iii) MOM-Cl, CH_2Cl_2, Et_3N (90%, 2 steps); (iv) For (*R*)-**3**: BH_3, THF, 82%; For (*R*)-**5**: $LiBH_4$, THF, 97%; (v) $MeSO_2Cl_2$, Et_3N; Dor (*R*)-**8**: 100%; (vi) LiBr, acetone; For (*R*)-**10**: 84%; For (*R*)-**11**: 78% 2 steps; (vii) $NaSSO_2CH_3$, DMF; For (*R*)-**12**: 61%; (vii) TFA, H_2O, 82%.

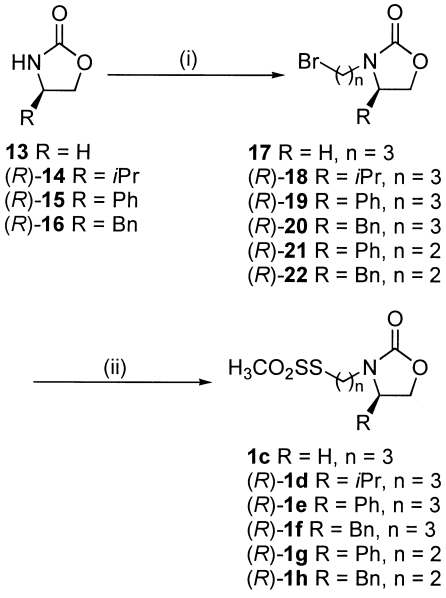
were converted to the methanethiosulfonates, **1c**-(*R*)-**1h**, in 38–61% yield over two steps. The MTS reagents (*S*)-**1d**-(*S*)-**1h** were made in an identical manner from the (*S*) oxazolidinones.

The (1*R*,2*S*) oxazolidinone, (*R*)-**24**, of *cis*-1*R*-amino-2*S*-indanol, (*R*)-**23**, was prepared in quantitative yield by the reaction of (*R*)-**23**, triphosgene and Et_3N in CH_2Cl_2 (Scheme 4).[16] (*R*)-**24** was then alkylated with 1,3-dibromopropane to make bromide, (*R*)-**25**, which was reacted with $NaSSO_2CH_3$ to give (*R*)-**1i** (49% yield for two steps). MTS reagent (*S*)-**1i** was synthesized from *cis*-1*S*-amino-2*R*-indanol in the same manner.

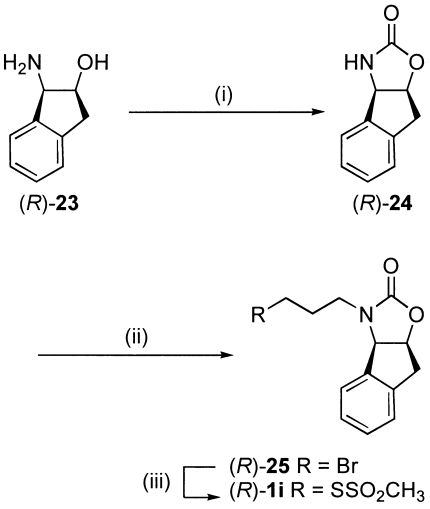
Enzyme kinetic assay

Subtilisin mutants were obtained from Genencor Inc.[17] and modified with the homochiral MTS reagents. Characterization of the new CMMs was done by PMSF titration[18] of their active sites, Ellman's titration[19] of residual thiol (≤2% in all cases), ES-MS after FPLC purification (mol. wt.±6 mass units in all cases), and by nondenaturing gradient gels, which all showed one band.

Amidase and esterase kinetic assays were conducted on these new diastereomeric CMMs. Both assays were run using a low substrate concentration in order to obtain a specificity constant (k_{cat}/K_M) that gave us an idea of the performance of the CMMs and allowed us to compare diastereomeric enzymes. (At low substrate concentration, $(k_{cat}/K_M) = v_{initial}/[Enzyme][Substrate]$). The results are presented in Table 1.



Scheme 3. Reagents: (i) KOH, DMSO, $Br(CH_2)_nBr$; (ii) $NASSo_2CH_3$, DMF.



Scheme 4. Reagents: (i) Triphosgene, CH_2Cl_2, Et_3N, 100%; (ii) KOH, DMSO, $Br(CH_2)_3Br$; (ii) $NaSSO_2CH_3$, DMF.

Discussion

Chiral auxiliaries are employed in asymmetric organic synthesis to block one diastereotopic face of a molecule

Table 1. Kinetic assay[a] of SBL CMMs

	Amidase assay k_{cat}/K_M ($mM^{-1}\,s^{-1}$)		Esterase assay k_{cat}/K_M ($mM^{-1}\,s^{-1}$)	
Enzyme	(*R*)	(*S*)	(*R*)	(*S*)
WT[b]	209±15		3560±540	
N62C[b]	92±7		4380±655	
N62C-**a**	218±9	226±11	5156±131	5483±106
N62C-**b**	187±10	220±9	3571±73	3054±171
N62C-**c**	181±6		9185±407	
N62C-**d**	333±13	284±5	5440±78	4098 ±151
N62C-**e**	458±13	308±7	13870±920	6564±157
N62C-**f**	245±3	150±1	4995±87	3261±163
N62C-**g**	185±4	244±7	3635±58	4120±159
N62C-**h**	262±5	335±7	6149±202	7591±209
N62C-**i**	165±3	228±6	4675±143	3279±135
S166C[b]	84±4		350±41	
S166C-**a**	72±2	26±1	1677±16	1246±48
S166C-**b**	48±2	15±1	1061±18	929±27
S166C-**c**	75±1		4898±196	
S166C-**d**	75±1	76±1	4215±157	4475±196
S166C-**e**	101±3	64±2	4076±111	3964±90
S166C-**f**	22±1	52±1	1495±134	3277±134
S166C-**g**	104±2	37±1	4281±96	4069±165
S166C-**h**	35±1	80±2	2150±107	5446±211
S166C-**i**	20±1	47±1	1488±54	4556±170
L217C[b]	51±4		5540±798	
L217C-**a**	204±5	144±4	10140±230	8075±144
L217C-**b**	175±3	227±6	9147±167	8714±324
L217C-**c**	85±1		5917±200	
L217C-**d**	105±3	104±2	8315±171	9296±665
L217C-**e**	120±4	184±3	8015±413	6696±255
L217C-**f**	73±2	79±2	6435±169	5128±163
L217C-**i**	118±4	171±7	7914±272	7321±330
S156C[b]	147±8		—[c]	
S156C-**a**	102±2	98±1	2468±45	1928±59
S156C-**b**	85±3	90±2	2284±81	2528±68
S156C-**e**	88±2	92±4	1796±63	2179±38

[a]The amidase assay was done at 0.05 and 0.1 mM *N*-Suc-AAPF-pNA in 0.1 M Tris at pH 8.6, and the esterase assay was conducted at 0.015 and 0.03 mM *N*-Suc-AAPF-SBn in 0.1 M Tris at pH 8.6. Assay errors are the mean standard errors from sets of six replicates.

[b]k_{cat}/K_M obtained by full kinetic run of 8 substrate concentrations and calculation of independent k_{cat} and K_M values. Errors were obtained from the curve-fitting errors in k_{cat} and K_M.

[c]Determination of esterase k_{cat}/K_M for S156C was impossible due to rapid reaction between the mutant and Ellman's reagent.

thus forcing the reaction to the other face which results in the formation of solely one diastereomer. Combining this negative, blocking methodology with the positive, accelerating effect of enzymes seemed a natural mix. In fact, the covalent coupling of enantiomerically pure (*R*) and (*S*) chiral auxiliary MTS ligands to SBL cysteine mutants has caused remarkable changes in enzyme activity. We can attribute these changes uniquely to the difference in spatial orientation at the ligand stereocentre when comparing diastereomeric enzymes. The extraordinary differences in catalytic activity between diastereomeric enzymes can be compared in Figures 1a–c.

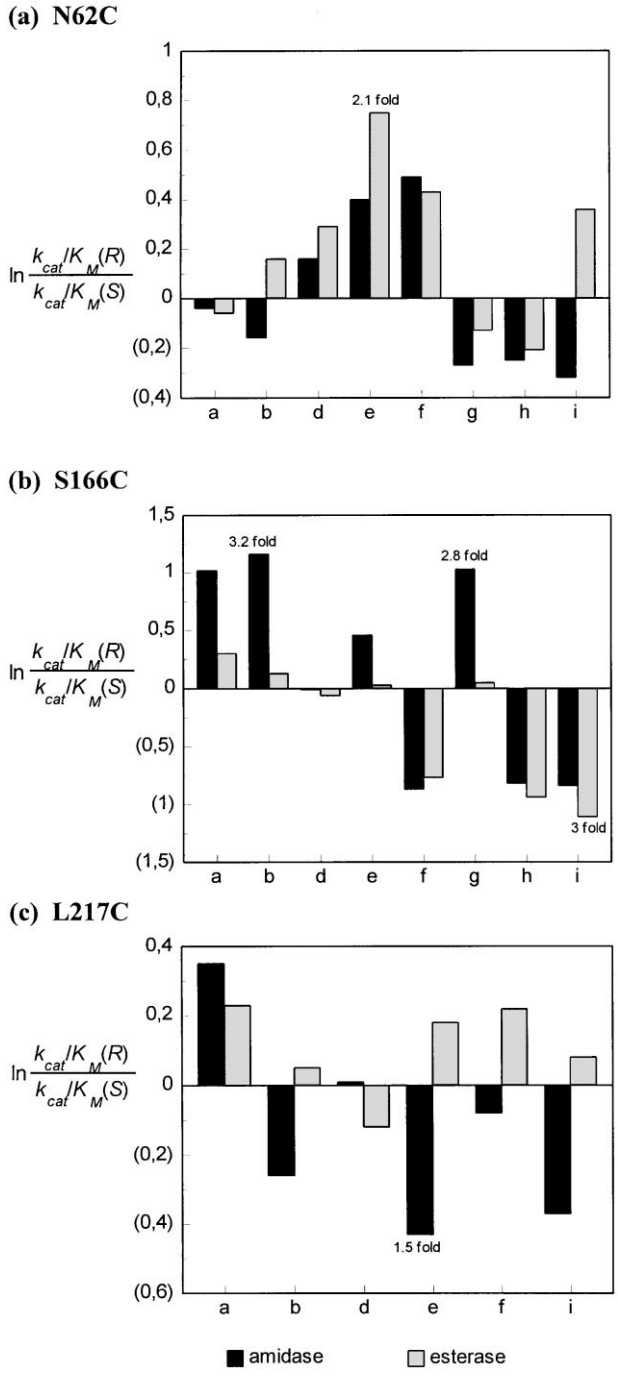


Figure 1. Comparison of specificity constants for N62C, S166C and L217C CMMs.

N62C

Of the N62C CMMs, the N62C-**e** set of diastereomeric CMMs is remarkable in displaying both high catalytic activity and a large difference between diastereomers. N62C-(*R*)-**e** is both an excellent amidase (2.2-fold better than WT) and an excellent esterase (3.9-fold better than WT). In addition, the (*S*)-diastereomer is a good amidase ($308\,mM^{-1}\,s^{-1}$) and esterase ($6564\,mM^{-1}\,s^{-1}$), but not as good as the (*R*)-diastereomer. Thus, there is a large difference between the two diastereomeric CMMs with respect to esterase performance ((*R*) is 2.1-fold better than (*S*)) and a moderate difference in amidase activity. At the same time, the achirally modified mutant (N62C-**c**) is only as good an amidase ($181\,mM^{-1}\,s^{-1}$) as WT and a poorer esterase ($9185\,mM^{-1}\,s^{-1}$) than N62C-(*R*)-**e**. These observations indicate that not only does the addition of a phenyl group at the 4 position of the oxazolidinone ring increase enzyme activity, but that the addition must be (*R*)-phenyl. Thus, the (*R*)-**e** modification at N62C is affecting the enzyme in a unique manner. Individual k_{cat} and K_M values were determined for the three enzymes, N62C-**c** and the N62C-**e** set, and these results are presented in Table 2 along with WT values for comparison. It is obvious that the kinetic assay using the low substrate approximation slightly underestimates the k_{cat}/K_M values, but the ratios of catalytic activity between diastereomeric enzymes remain approximately the same.

Modification of N62C with (*R*)-**1e**, (*S*)-**1e** and **1c** decreases K_M indicating better binding of the substrate, and in the case of amidase activity, it is this K_M effect that is the source of the increased k_{cat}/K_M, since these N62C CMMs have similar k_{cat} values to the WT. However, the changes in esterase activity for these enzymes are more complex. N62C-(*R*)-**e** and N62C-**c** show significantly higher k_{cat} and lower K_M values than WT, giving overall 5.4-fold and 3.7-fold better esterase activity than WT, respectively. The N62C-(*S*)-**e** CMM does not display these characteristics. While it does bind the substrate very well and achieve half its maximum turnover rate at low substrate concentration ($K_M = 0.15\,mM$), its k_{cat} ($1106\,s^{-1}$) is much lower than WT. Therefore, it appears that a 4*R*-phenyl substitution on the oxazolidinone improves overall catalytic performance by increasing k_{cat} and lowering K_M.

In an attempt to improve on these results, the ethyl linked phenyl and benzyl oxazolidinone N62C CMMs were prepared (N62C-**g** and N62C-**h**). Surprisingly, there was a reversal of which modification made the best enzyme. In the case of the propyl linked CMMs (N62C-**e** and N62C-**f**), the (*R*) modification was the best amidase and esterase for both phenyl and benzyl groups. However, the (*S*) modification was the best when these same groups were ethyl linked. This brings to mind the flipping of substrate preference for transesterification reactions catalyzed by WT from (*S*) to (*R*) and back to (*S*) for secondary alcohols, β-branched primary alcohols and γ-branched primary alcohols respectively.[20] However, in the present situation, the substrate does not change. Rather, the ability of the enzyme to convert substrate to product is altered depending upon the stereocentre of the covalently

Table 2. Kinetic parameters of WT and selected SBL CMMs[a]

Enzyme	Amidase				Esterase			
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)	k_{cat}/K_M ($mM^{-1}\,s^{-1}$) assay	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)	k_{cat}/K_M ($mM^{-1}\,s^{-1}$) assay
WT	153±4	0.73±0.05	209±15	—	1940±180	0.54±0.07	3560±540	—
N62C-(*R*)-**e**	163±2	0.26±0.01	627±26	458±13	2894±117	0.15±0.02	19293±2895	13868±920
N62C-(*S*)-**e**	164±2	0.41±0.02	400±20	308±7	1106±45	0.15±0.02	7373±1098	6564±157
N62C-**c**	193±3	0.63±0.03	307±16	181±6	3447±66	0.26±0.01	13258±710	9185±407

[a]Notation as in Table 1.

linked ligand as well as the number of bonds present in the link between the enzyme backbone and the stereocentre.

S166C

Modifications at S166C produced many sets of diastereomeric CMMs with large differences in activity. Primarily, the **1a**, **1b**, **1f**, **1g**, **1h** and **1i** modifications produced CMMs with greater than 2-fold variations in amidase between diastereomeric CMMs. The largest difference of any set of CMMs was achieved with S166C-**b** which has a $[k_{cat}/K_M\ (R)]/[k_{cat}/K_M\ (S)]$ ratio of 3.2. Notably, the modifications with the phenyl and benzyl oxazolidinones at S166C reverse which diastereomeric CMM has greater catalytic activity in a way similar to the same modifications at N62C. However, at S166C the reversal is caused by the addition of a methylene unit directly to the stereocentre of the oxazolidinone ligand. The (*R*)-phenyl oxazolidinone modifications ((*R*)-**e** and (*R*)-**g**) produce S166C CMMs that are better than the (*S*) analogues, but the (*S*)-benzyl oxazolidinones ((*S*)-**f** and (*S*)-**h**) give significantly better S166C CMMs than the (*R*).

Though none of these CMMs showed significantly greater than WT activity, S166C-(*S*)-**g** and S166C-(S)-**i** are good esterases (4069 $mM^{-1}\,s^{-1}$ and 4556 $mM^{-1}\,s^{-1}$, respectively) and have high esterase/amidase ratios of 110 and 97 making them good candidates to be superior peptide ligation catalysts (Fig. 2a). S166C-(*S*)-**a** and S166C-(*S*)-**b** have relatively high esterase/amidase ratios (48 and 62) compared to S166C (4) and WT (23), but these two CMMs are very poor esterases. Interestingly, for chiral modifications at S166C, the (*S*)-ligand consistently gives a CMM with a higher esterase to amidase ratio than the (*R*)-ligand, except in the case of the **1f** where the two diastereomeric enzymes have similar ratios.

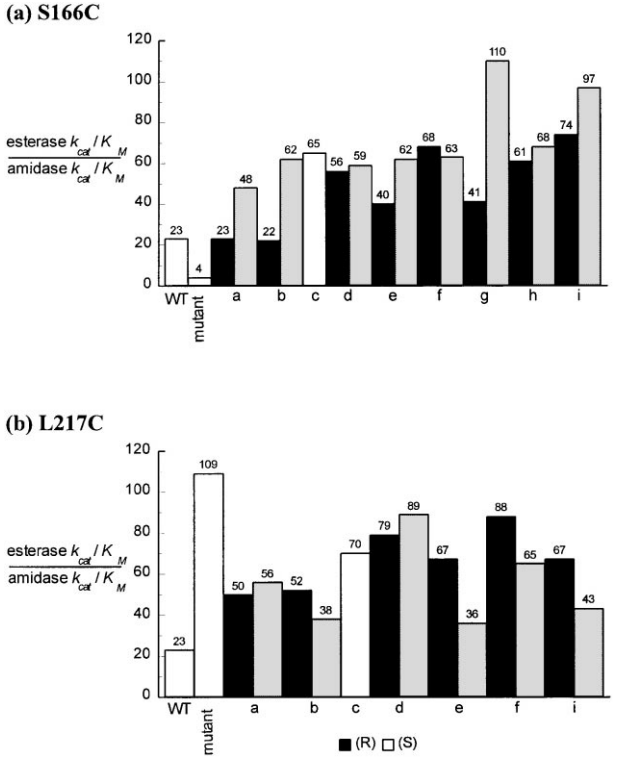


Figure 2. Changes in esterase to amidase activity ratios for S166C and L217C CMMs.

Table 3. Electro-spray mass spectral Data for CMMs[a]

Enzyme	Calculated mass		Found mass	
	(*R*)	(*S*)	(*R*)	(*S*)
N62C-**a**	26853	26853	26855	26854
N62C-**b**	26839	26839	26841	26838
N62C-**c**	26846		26850	
N62C-**d**	26888	26888	26889	26889
N62C-**e**	26922	26922	26921	26921
N62C-**f**	26936	26936	26939	26939
N62C-**g**	26908	26908	26910	26907
N62C-**h**	26922	26922	26924	26924
N62C-**i**	26934	26934	26937	26936
S166C-**a**	26880	26880	26881	26886
S166C-**b**	26866	26866	26862	26872
S166C-**c**	26873		26877	
S166C-**d**	26915	26915	26915	26916
S166C-**e**	26949	26949	26950	26951
S166C-**f**	26963	26963	26964	26963
S166C-**g**	26935	26935	26937	26934
S166C-**h**	26949	26949	26951	26949
S166C-**i**	26961	26961	26964	26964
L217C-**a**	26854	26854	26850	26850
L217C-**b**	26840	26840	26842	26840
L217C-**c**	26847		26847	
L217C-**d**	26889	26889	26892	26892
L217C-**e**	26923	26923	26922	26923
L217C-**f**	26937	26937	26938	26940
L217C-**i**	26935	26935	26937	26937
S156C-**a**	26880	26880	26883	26883
S156C-**b**	26866	26866	26866	26868
S156C-**e**	26949	26949	26949	26949

[a]Mol. wt.±6 mass units in all cases.

L217C

While no large differences were observed between diastereomeric CMMs, the chiral modifications at L217C produced many CMMs that could be used as peptide ligation catalysts due to their high esterase/amidase ratio (Fig. 2b). L217C-(*S*)-**d** has a very high esterase k_{cat}/K_M ($9296\ mM^{-1}\ s^{-1}$) and a low amidase value ($104\ mM^{-1}\ s^{-1}$) giving it a relatively high esterase/amidase ratio of 89. L217C-(*R*)-**f** has a similar ratio of 88 and a good esterase k_{cat}/K_M ($6435\ mM^{-1}\ s^{-1}$). While it is true that the unmodified L217C mutant has the highest ratio in the group (109), this is mitigated by its lower esterase k_{cat}/K_M ($5540\ mM^{-1}\ s^{-1}$). Encouragingly, all of these CMMs should catalyze the formation of peptide bonds from an ester acyl donor and amine nucleophile very efficiently.

S156C

Modification of S156C by **1a**, **1b** and **1e** revealed no enzymes with either high activity or large difference between diastereomers. This is not surprising, because the S156C side chain is surface exposed, so it is probable that the ligand modifiers either point out of, or are not closely associated with the S_1 pocket. For this reason, the kinds of subtle variations expected due to spatial orientation were not found at S156C. As a result, no further modifications were made of this mutant.

Conclusion

It has been found that the modification of cysteine mutants of SBL with enantiomerically pure MTS ligands effects considerable changes in enzyme activity. Amidase and esterase kinetic assays using a low substrate approximation, found up to 3-fold differences in activity between diastereomeric enzymes. N62C-(*R*)-**e** was particularly remarkable. Its amidase k_{cat}/K_M was 1.56-fold better than its diastereomer, N62C-(*S*)-**e**, and 3-fold better than WT. Also, the esterase k_{cat}/K_M of N62C-(*R*)-**e** was 2.6-fold better than its diastereomer and 5.4-fold better than WT. Changing the length of the carbon chain linking the phenyl or benzyl oxazolidinone ligand to N62C by a methylene unit reverses which diastereomeric enzyme is more active. In a similar fashion, changing from a phenyl to benzyl oxazolidinone ligand at S166C reverses which diastereomeric enzyme is more active. Work is in progress investigating the peptide ligation and transesterification capabilities of the CMMs discussed in this paper. In addition, the attachment of enantiomerically pure ligands containing charged groups to SBL mutants is being pursued.

Experimental

The N62C, L217C, S166C, and S156C mutants of subtilisin *Bacillus lentus* were prepared and purified by the general method.[17] Spectrophotometric measurements were made on a Perkin–Elmer Lamda 2 spectrophotometer. Melting points were determined using an Electrothermal IA9000 series Digital Melting Point Apparatus, and are uncorrected. Optical rotation data were obtained using a Perkin–Elmer 243B polarimeter. Compounds were identified by their ^1H (200 MHz) and ^{13}C (50.3 MHz) NMR spectra, run using a Varian Gemini NMR spectrometer, their IR spectra using a Perkin–Elmer FTIR Spectrum 1000 instrument, and HRMS data were acquired using a Micromass 70-250S (double focussing) mass spectrometer for EI spectra and a Micromass ZAB-SE for FAB spectra. Enantiomeric excesses of methanethiosulfonates ((*R*)-**1a**, (*S*)-**1a**, (*R*)-**1b** and (*S*)-**1b**) were determined by HPLC on a Chiralcel OJ column using a hexane: isopropanol eluent system. Enantiomeric excesses (ee) of bromides ((*R*)-**18**, (*S*)-**18**, (*R*)-**19**, (*S*)-**19**, (*R*)-**20**, (*S*)-**20**, (*R*)-**21**, (*S*)-**21**, (*R*)-**22**, (*S*)-**22**, (*R*)-**25** and (*S*)-**25**) were determined by HPLC on a Chiralcel OD column using the same eluent system. The standard substrate *N*-Succinyl AlaAlaProPhe-paranitroanilide (*N*-Suc-SSPF-pNA) and *N*-Succinyl AlaAlaProPhe-thiobenzyl ester (*N*-Suc-SSPF-SBn) were purchased from BACHEM California.

Preparation of methanethiosulfonate reagents

(*R*)-2-Methoxy-2-phenyl-ethylmethanethiosulfonate ((*R*)-1a). (*R*)-Mandelic acid (4.678 g, 30.75 mmol) was dissolved in 6 M NaOH (50 mL, 300 mmol) and dimethyl sulfate (14.6 mL, 154 mmol) was added over 1 h so that the temperature stayed at 50 °C. After another 1 h of stirring, H_2O (50 mL) was added, and the solution was acidified to pH 1 with 12 M HCl. The mixture was saturated with NaCl, extracted with EtOAc (3×100 mL), and the extracts dried with Na_2SO_4. After filtration and evaporation under reduced pressure, the solid was pulverized, refluxed in hexanes (100 mL) for 15 min and hot filtered. The insoluble (*R*)-mandelic acid (2.71 g, 58%) was recovered, and the hexanes evaporated under reduced pressure to give (*R*)-2-methoxy-mandelic acid, (*R*)-**3** (1.91 g, 37%) which was used directly in the next step.

(*R*)-**3** (1.91 g, 11.46 mmol), was placed under Ar and dry THF (15 mL) was added. The resulting solution was cooled to 0 °C and 1 M BH_3·THF (17.2 mL, 17.2 mmol) was added over 1 min. The ice bath was removed, and the reaction was allowed to warm to 20 °C. After stirring overnight, the reaction mixture was poured into a stirred mixture of EtOAc (200 mL)/saturated aqueous $NaHCO_3$ (100 mL). The aqueous layer was saturated with NaCl and extracted with EtOAc (3×150 mL). The combined EtOAc fractions were dried with $MgSO_4$, filtered and evaporated under reduced pressure. Flash chromatography was conducted using a step gradient (25% EtOAc/75% hexanes to 33% EtOAc/67% hexanes) to give (*R*)-2-methoxy-2-phenyl-1-ethanol, (*R*)-**6** (1.26 g, 72%), as a colorless oil. $[\alpha]_D^{25}$ −114.6° (*c* 1.27, EtOH) [lit.[21] $[\alpha]_D^{25}$ −117.3° (*c* 1.006, EtOH)]; IR, ^1H NMR and ^{13}C NMR data were identical to the literature.[21]

(*R*)-**6** (1.25 g, 8.213 mmol) and Et_3N (2.29 mL, 16.43 mmol) were dissolved in CH_2Cl_2 (20 mL) under Ar and cooled to 0 °C. MsCl (0.95 mL, 12.27 mmol) was added over 1 min, and stirred for 10 min. The ice bath was removed, and the solution was stirred overnight.

The reaction was then poured into EtOAc (200 mL)/saturated aqueous $NaHCO_3$ (100 mL), and then stirred and saturated with NaCl. The aqueous layer was extracted with EtOAc (3×150 mL), and the combined organic fractions were dried with $MgSO_4$. After filtration and evaporation under reduced pressure, the crude product was purified by flash chromatography using 50% EtOAc/50% hexanes to give (*R*)-2-methoxy-2-phenyl-1-ethylmethanesulfonate, (*R*)-**8**, quantitatively (1.88 g) as a colorless oil. $[\alpha]_D^{25}$ −97.4° (*c* 1.36, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.30–7.40 (5H, m), 4.47–4.52 (1H, m), 4.20–4.36 (2H, m), 3.30 (3H, s), 2.99 (3H, s); ^{13}C NMR ($CDCl_3$) δ 136.6, 128.8, 126.9, 81.5, 72.7, 57.0, 37.6.

(*R*)-**8** (1.88 g, 8.160 mmol) and LiBr (3.54 g, 40.76 mmol) were refluxed in freshly distilled acetone (20 mL) for 20 h under a $CaCl_2$ drying tube. After cooling and evaporation to dryness under reduced pressure, hexanes (30 mL) were added and the mixture filtered. The filtrate was evaporated under reduced pressure, and flash chromatography of the crude product was done using a step gradient (hexanes to 5% EtOAc/95% hexanes) to give (*R*)-2-methoxy-2-phenyl-1-ethyl bromide, (*R*)-**10**, (1.284 g, 73%), as a colorless oil. $[\alpha]_D^{25}$ −71.6 (*c* 1.26, MeOH) [lit.[22] for the (*S*) enantiomer $[\alpha]_D^{25}$ +73° (MeOH)]; ^1H NMR ($CDCl_3$) δ 7.31–7.40 (5H, m), 4.36–4.42 (1H, m), 3.45–3.60 (2H, m), 3.32 (3H, s); ^{13}C NMR ($CDCl_3$) δ 139.0, 128.6, 128.5, 126.7, 83.4, 57.2, 36.2; HRMS (EI) *m*/*z*: calcd for $C_9H_{11}OBr$, 213.9993; found, 213.9988.

(*R*)-**10** (1.28 g, 5.951 mmol) and sodium methanethiosulfonate (1.04 g, 7.752 mmol) were dissolved in dry DMF (10 mL) under Ar and heated to 70 °C. After stirring for 24 h, the DMF was evaporated under reduced pressure. The crude product was dissolved in EtOAc, filtered, and the filtrate was evaporated under reduced pressure. flash chromatography using a step gradient (5% EtOAc/95% hexanes to 33% EtOAc/67% hexanes) gave the title compound, (*R*)-**1a** (1.235 g, 84%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ −90.4° (*c* 0.94, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.31–7.39 (5H, m), 4.42–4.48 (1H, m), 3.41–3.46 (2H, m), 3.27 (3H, s), 3.24 (3H, s); ^{13}C NMR ($CDCl_3$) δ 139.0, 128.7, 128.5, 126.6, 82.3, 56.9, 50.3, 43.4; HRMS (FAB+) *m*/*z*: calcd for $C_{10}H_{14}O_3S_2$ + H, 247.0463; found, 247.0470.

(*S*)-2-Methoxy-2-phenyl-ethylmethanethiosulfonate ((*S*)-1a). (*S*)-**3** was prepared in the same manner as the (*R*)-**3**. From (*S*)-mandelic acid (4.00 g, 26.29 mmol) was obtained (*S*)-**1** (1.301 g, 30%). (*S*)-**6** was prepared in the same manner as the (*R*)-**6**. From (*S*)-**3** (1.20 g, 7.221 mmol) was obtained (*S*)-**6** (0.903 g, 82%). Its IR, ^1H NMR and ^{13}C NMR data were identical to (*R*)-**6**. $[\alpha]_D^{25}$ +115.0° (*c* 1.26, EtOH).

(*S*)-**8** was prepared in the same manner as the (*R*)-**8**. From (*S*)-**6** (0.883 g, 5.802 mmol) was obtained (*S*)-**8** (1.33 g, 100%). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**8**. $[\alpha]_D^{25}$ +95.0° (*c* 1.70, $CHCl_3$).

(*S*)-**10** was prepared in the same manner as (*R*)-**10**. From (*S*)-**8** (1.33 g, 5.773 mmol) was obtained (*S*)-**10** (1.02 g, 81%). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**10**. $[\alpha]_D^{25}$ +72.4° (*c* 1.15, MeOH).

(*S*)-**1a** was prepared in the same manner as (*R*)-**1a**. From (*S*)-**10** (1.00 g, 4.649 mmol) was obtained (*S*)-**1a** (0.961 g, 84%, ee≥98%). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**1a**. $[\alpha]_D^{25}$ +93.8° (*c* 1.002); HRMS (FAB+) *m*/*z*: calcd for $C_{10}H_{14}O_3S_2$ + H, 247.0463; found, 247.0474.

(*R*)-2-Hydroxy-2-phenyl-ethylmethanethiosulfonate ((*R*)-1b). (*R*)-Mandelic acid (2.568 g, 16.87 mmol) and 2,2-dimethoxypropane (5.1 mL, 41.48 mmol) were dissolved in MeOH (100 mL) and 12 M HCl (100 mL) was added. The resulting solution was stirred for 20 h under a $CaCl_2$ tube and evaporated to dryness under reduced pressure. EtOAc (100 mL) and saturated aqueous $NaHCO_3$ (100 mL) were added, and the aqueous phase was extracted with EtOAc (3×100 mL). The organic fractions were dried with $MgSO_4$, and evaporated under reduced pressure to give (*R*)-methyl mandelate, (*R*)-**4**, quantitatively (2.78 g) as a white solid which was of sufficient purity for the next step.

(*R*)-**4** (1.695 g, 10.20 mmol) and Huenig's base (6.22 mL, 35.70 mmol) were dissolved in dry CH_2Cl_2 (25 mL) at 0 °C under Ar. MOM-Cl (2.32 mL, 30.55 mmol) was dripped into the solution over 1 min, and the reaction was stirred at 20 °C for 16 h. The solution was poured into a mixture of EtOAc (200 mL)/ice/3 M HCl (100 mL) and stirred for 5 min. The aqueous layer was extracted with EtOAc (3×150 mL), and the combined organic fractions were dried with $MgSO_4$. Flash chromatography was performed using a step gradient (10% EtOAc/90% hexanes to 25% EtOAc/75% hexanes) to give (*R*)-2-methyloxymethoxy methyl mandelate, (*R*)-**5** (1.935 g, 90%), as a colorless oil. $[\alpha]_D^{25}$ −133.5° (*c* 1.41, $CHCl_3$); [lit.[23] for the (*S*) enantiomer $[\alpha]_D^{25}$ +5.9° (*c* 1.11, $CHCl_3$); IR, ^1H NMR and ^{13}C NMR data were identical to the literature.[23]

(*R*)-**5** (1.924 g, 9.152 mmol) was dissolved in dry THF (50 mL) at 0 °C under Ar, and $LiBH_4$ (0.498 g, 22.87 mmol) was added. The reaction was stirred for 16 h at 20 °C, and then poured into a stirred mixture of EtOAc (200 mL)/saturated aqueous $NaHCO_3$ (150 mL). After the reaction had subsided, the aqueous layer was extracted with EtOAc (3×200 mL), and the combined organic fractions were dried with $MgSO_4$. The crude product was purified by flash chromatography using a step gradient (25% EtOAc/75% hexanes to 33% EtOAc/67% hexanes) to give (*R*)-2-methyloxymethoxy-2-phenyl-1-ethanol, (*R*)-**7** (1.63 g, 98%), as a colorless oil. $[\alpha]_D^{25}$ −189.9° (*c* 1.72, $CHCl_3$); [lit.[24] for the (*S*) enantiomer $[\alpha]_D^{20}$ +196° (*c* 2.67, $CHCl_3$)]; IR, ^1H NMR and ^{13}C NMR data were identical to the literature.

(*R*)-2-Methyloxymethoxy-2-phenyl-1-ethylmethanesulfonate, (*R*)-**9**, was prepared in the same manner as (*R*)-**8**. (*R*)-**7** (1.530 g, 8.396 mmol) was converted quantitatively to (*R*)-**9** (2.175 g). $[\alpha]_D^{25}$ −141.6° (*c* 1.10, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.35 (5H, s), 4.89–4.95 (1H, m), 4.56–4.65 (2H, AB q), 4.25–4.40 (2H, m), 3.36 (3H, s), 2.95 (3H, s); ^{13}C NMR ($CDCl_3$) δ 136.6, 128.7, 127.1, 94.4, 75.5, 72.3, 55.6, 37.4.

(*R*)-2-Methyloxymethoxy-2-phenyl-1-ethyl bromide, (*R*)-**11**, was prepared in the same manner as (*R*)-**10**. (*R*)-**9**

(2.035 g, 7.817 mmol) was converted to (*R*)-**11** (1.536 g, 80%). $[\alpha]_D^{25}$ −130.9° (*c* 1.29, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.35 (5H, s), 4.82–4.88 (1H, m), 4.57–4.66 (2H, AB q), 3.49–3.65 (2H, m), 3.43 (3H, s); ^{13}C NMR ($CDCl_3$) δ 139.0, 128.6, 128.5, 126.9, 94.5, 77.7, 55.8, 36.2; HRMS (EI) *m*/*z*: calcd for $C_{10}H_{13}O_2Br$, 244.0099; found, 244.0091.

(*R*)-2-Methyloxymethoxy-2-phenyl-1-ethylmethanethiosulfonate, (*R*)-**12**, was prepared in the same manner as (*R*)-**1a**. (*R*)-**10** (1.458 g, 5.948 mmol) was converted to (*R*)-**12** (1.005 g, 61%). $[\alpha]_D^{25}$ −149.6° (*c* 2.23, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.36 (5H, s), 4.88–4.94 (1H, m), 4.56 (2H, s), 3.48–3.51 (2H, m), 3.40 (3H, s), 3.23 (3H, s); ^{13}C NMR ($CDCl_3$) δ 139.0, 128.7, 128.6, 126.9, 94.3, 76.3, 55.9, 50.5, 43.4; HRMS (FAB+) *m*/*z*: calcd for $C_{11}H_{16}O_4S_2+H$, 277.0569; found, 277.0600.

(*R*)-**12** (0.864 g, 3.126 mmol) was suspended in H_2O (10 mL) and trifluoroacetic acid (10 mL) was added at 0 °C. The solution was stirred at 20 °C for 40 h, and the volatiles were evaporated under reduced pressure to near dryness. H_2O (20 mL) was added, and the suspension was evaporated to dryness. Finally, toluene (50 mL) was added, and the solution was evaporated to dryness. The crude product was purified by flash chromatography using a step gradient (25% EtOAc/75% hexanes to 33% EtOAc/67% hexanes) to give the title compound, (*R*)-**1b** (0.689 g, 95%, ee≥98%), as white crystals. An analytical sample was recrystallized from ether/hexanes. Mp 48.5–49.5 °C; $[\alpha]_D^{25}$ −63.1° (*c* 0.89, $CHCl_3$); IR (neat) 3470 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.38 (5H, s), 5.00–5.06 (1H, m), 3.44–3.49 (2H, m), 3.26 (3H, s), 2.60 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 141.5, 128.7, 128.5, 125.9, 73.0, 50.5, 44.8; HRMS (FAB+) *m*/*z*: calcd for $C_9H_{12}O_3S_2+H$, 233.0307; found, 233.0326.

(*S*)-2-Hydroxy-2-phenyl-ethylmethanethiosulfonate ((*S*)-1b). (*S*)-**4** was prepared in the same manner as (*R*)-**4**. From (*S*)-mandelic acid (3.176 g, 20.87 mmol) was obtained crude (*S*)-**4** (3.45 g, quantitative) which was used directly in the next step.

(*S*)-**5** was prepared in the same manner as (*R*)-**5**. From (*S*)-**4** (3.45 g, 20.76 mmol) was obtained (*S*)-**5** (3.014 g, 69%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**5**. $[\alpha]_D^{25}$ +131.6° (*c* 1.74, $CHCl_3$).

(*S*)-**7**, was prepared in the same manner as (*R*)-**7**. From (*S*)-**5** (2.995 g, 14.25 mmol) was obtained (*S*)-**7** (2.565 g, 99%) Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**7**. $[\alpha]_D^{25}$ +193.2° (*c* 1.30, $CHCl_3$).

(*S*)-**9** was prepared in the same manner as (*R*)-**9**. From (*S*)-**7** (2.467 g, 13.54 mmol) was obtained (*S*)-**9** (3.486 g, 99%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**9**. $[\alpha]_D^{25}$ +135.5° (*c* 1.40, $CHCl_3$).

(*S*)-**11**, was prepared in the same manner as (*R*)-**11**. From (*S*)-**9** (3.486 g, 13.39 mmol) was obtained (*S*)-**11** (2.822 g, 86%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**11**. $[\alpha]_D^{25}$ +125.8° (*c* 1.21, MeOH).

(*S*)-**12** was prepared in the same manner as (*R*)-**12**. From (*S*)-**11** (0.863 g, 3.521 mmol) was obtained (*S*)-**12** (0.541 g, 56%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**12**. $[\alpha]_D^{25}$ +153.4° (*c* 2.43, $CHCl_3$).

The title compound, (*S*)-**1b**, was prepared in the same manner as (*R*)-**1b**. From (*S*)-**12** (0.526 g, 1.903 mmol) was obtained (*S*)-**1b** (0.419 g, 95%, ee≥98%), as white crystals, which were recrystallized from ether/hexanes. Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**1b**. Mp 47.0–48.0 °C; $[\alpha]_D^{25}$ +63.3° (*c* 1.676, $CHCl_3$); HRMS (FAB+) *m*/*z*: calcd for $C_9H_{12}O_3S_2+H$, 233.0307; found, 233.0311.

***N*-(3′-Methanethiosulfonatopropyl)-2-oxazolidinone (1c).** To a cooled solution (15–20 °C) of 1,3-dibromopropane (6.4 mL, 63.05 mmol) in dry DMSO (5 mL) was added powdered KOH (0.920 g, 16.40 mmol). 2-Oxazolidinone (1,100 g, 12.63 mmol) was added in small amounts over 5 min, and the reaction was stirred for 4 h at 20 °C. The mixture was diluted with ether (100 mL) and H_2O (20 mL), and the aqueous phase was extracted with ether (3×50 mL). After drying with $MgSO_4$, the crude product was purified by flash chromatography using a step gradient (25% EtOAc/75% hexanes to 50% EtOAc/50% hexanes) to give *N*-(3′-bromopropyl)-2-oxazolidinone, **17** (1.48 g, 56%). IR (neat) 1747 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.30 (2H, t, *J* = 7.2 Hz), 3.57 (2H, t, *J* = 8.2 Hz), 3.33–3.43 (4H, q), 2.03–2.17 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.4, 61.7, 45.0, 43.0, 30.4, 29.9; HRMS (FAB+) *m*/*z*: calcd for $C_6H_{10}NO_2Br$, 207.9972; found, 207.9957.

The title compound, **1c**, was prepared in the same manner as (*R*)-**1a**. **17** (1.316 g, 6.325 mmol) was converted to **1c** (1.013 g, 67%). It was recrystallized from EtOAc/ether. Mp 36–37.5 °C; IR (neat) 1748 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.32 (2H, t, *J* = 7.4 Hz), 3.56 (2H, t, *J* = 8.4 Hz), 3.35 (2H, t, *J* = 6.7 Hz), 3.31 (3H, s), 3.14 (2H, t, *J* = 7.0 Hz), 1.96–2.10 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.5, 61.7, 50.4, 44.6, 42.9, 33.2, 27.6; HRMS (FAB+) *m*/*z*: calcd for $C_7H_{13}NO_4S_2+H$, 240.0364; found, 240.0365.

***N*-(3′-Methanethiosulfonatopropyl)-(*R*)-4-isopropyl-2-oxazolidinone ((*R*)-1d).** *N*-(3′-Bromopropyl)-(*R*)-4-isopropyl-2-oxazolidinone, (*R*)-**18**, was prepared in the same manner as **17**. From (*R*)-4-isopropyl-2-oxazolidinone (0.518 g, 4.011 mmol) was obtained (*R*)-**18** (0.626 g, 62%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ −2.7° (*c* 1.87, $CHCl_3$); IR (neat) 1748 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.20 (1H, t, *J* = 8.8 Hz), 4.04 (1H, dd, *J* = 9.0, 5.3), 3.69–3.77 (1H, m), 3.47–3.58 (1H, m), 3.40 (2H, t, *J* = 6.5 Hz), 3.06–3.20 (1H, m), 2.25–1.99 (3H, m), 0.86 (6H, t, *J* = 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ 158.3, 62.7, 59.6, 40.6, 30.2, 27.7, 17.5, 14.2; HRMS (FAB+) *m*/*z*: calcd for $C_9H_{16}NO_2Br$, 250.0441; found, 250.0419.

The title compound, (*R*)-**1d** was prepared in the same manner as (*R*)-**1a**. (*R*)-**18** (0.530 g, 2.119 mmol) was converted to (*R*)-**1d** (0.492 g, 83%). $[\alpha]_D^{25}$ −22.3° (*c* 1.37, $CHCl_3$); IR (neat) 1744 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.25 (1H, t, *J* = 9.0 Hz), 4.07 (1H, dd, *J* = 9.0, 5.4 Hz), 3.73–3.81 (1H, m), 3.50–3.65 (1H, m), 3.33 (3H, s), 3.07–3.21 (3H, m), 1.98–2.13 (3H, m), 0.90 (3H, d, *J* = 7.0 Hz), 0.86 (3H, d, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ 158.6,

62.9, 59.2, 50.5, 40.5, 33.5, 27.9, 27.6, 17.6, 14.2; HRMS (FAB+) m/z: calcd for $C_{10}H_{19}NO_4S_2+H$, 282. 0834; found, 282.0842.

***N*-(3′-Methanethiosulfonatopropyl)-(*S*)-4-isopropyl-2-oxazolidinone ((*S*)-1d).** (*S*)-**18** was prepared in the same manner as (*R*)-**18**. From (*S*)-4-isopropyl-2-oxazolidinone (0.504 g, 3.902 mmol) was obtained (*S*)-**18** (0.558 g, 57%, ee≥98%)). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**18**. $[\alpha]_D^{25}$ +3.4° (*c* 3.42, $CHCl_3$).

The title compound, (*S*)-**1d**, was prepared in the same manner as (*R*)-**1d**. From (*S*)-**18** (0.493 g, 1.971 mmol) was obtained (*S*)-**1d** (0.435 g, 78%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**1d**. $[\alpha]_D^{25}$ +23.2° (2.27, $CHCl_3$); HRMS (EI) m/z: calcd for $C_{10}H_{19}NO_4S_2+H$, 282. 0834; found, 282.0833.

***N*-(3′-Methanethiosulfonatopropyl)-(*R*)-4-phenyl-2-oxazolidinone ((*R*)-1e).** *N*-(3′-Bromopropyl)-(*R*)-4-phenyl-2-oxazolidinone, (*R*)-**19**, was prepared in the same manner as **17**. From (*R*)-4-phenyl-2-oxazolidinone (0.322 g, 1.970 mmol) was obtained (*R*)-**19** (0.370 g, 66%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ −35.8° (*c* 3.10, $CHCl_3$); IR (neat) 1748 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.26–7.45 (5H, m), 4.79 (1H, dd, J = 8.8, 6.3 Hz), 4.63 (1H, dd, J = 8.6, 8.6 Hz), 4.15 (1H, dd, J = 8.6, 6.4 Hz), 3.30–3.54 (3H, m), 2.89–3.03 (1H, m), 1.90–2.12 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.2, 137.7, 129.3, 129.2, 126.9, 69.8, 60.3, 41.1, 30.2, 29.9; HRMS (EI) m/z: calcd for $C_{12}H_{14}NO_2Br$, 283.0208; found, 283.0197.

The title compound, (*R*)-**1e**, was prepared in the same manner as (*R*)-**1a**. (*R*)-**19** (0.346 g, 1.218 mmol) was converted to (*R*)-**1e** (0.344 g, 89%). $[\alpha]_D^{25}$ −70.5° (*c* 0.84, $CHCl_3$); IR (neat) 1746 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.26–7.43 (5H, m), 4.81 (1H, dd, J = 8.8, 6.6 Hz), 4.65 (1H, dd, J = 8.6, 8.6 Hz), 4.16 (1H, dd, J = 8.6, 6.6 Hz), 3.40–3.55 (1H, m), 3.29 (3H, s), 2.90–3.15 (3H, m), 1.82–1.97 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.4, 137.5, 129.4, 129.3, 127.1, 69.9, 60.0, 50.6, 41.0, 33.4, 27.5; HRMS (FAB+) m/z: calcd for $C_{13}H_{17}NO_4S_2+H$, 316.0678; found, 316.0678.

***N*-(3′-Methanethiosulfonatopropyl)-(*S*)-4-phenyl-2-oxazolidinone ((*S*) - 1e).** (*S*)-**19** was prepared in the same manner as (*R*)-**19**. From (*S*)-4-phenyl-2-oxazolidinone (0.964 g, 5.911 mmol) was obtained (*S*)-**19** (0.955 g, 57%, ee≥98%)). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**19**. $[\alpha]_D^{25}$ +33.3° (*c* 2.50, $CHCl_3$). The title compound, (*S*)-**1e**, was prepared in the same manner as (*R*)-**1e**. From (*S*)-**19** (0.870 g, 3.062 mmol) was obtained (*S*)-**1e** (0.814 g, 84%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**1e**. $[\alpha]_D^{25}$ +68.8° (*c* 1.21, $CHCl_3$); HRMS (EI) m/z: calcd for $C_{13}H_{17}NO_4S_2+H$, 316.0678; found, 316.0683.

***N*-(3′-Methanethiosulfonatopropyl)-(*R*)-4-benzyl-2-oxazolidinone ((*R*)-1f).** *N*-(3′-Bromopropyl)-(*R*)-4-benzyl-2-oxazolidinone, (*R*)-**20**, was prepared in the same manner as **17**. From (*R*)-4-benzyl-2-oxazolidinone (0.499 g, 2.816 mmol) was obtained (*R*)-**20** (0.454 g, 54%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ −14.3° (*c* 2.06, $CHCl_3$); IR (neat) 1751 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.14–7.36 (5H, m), 3.96–4.21 (3H, m), 3.10–3.65 (5H, m), 2.61–2.72 (1H, m), 2.04–2.27 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.0, 135.2, 128.9, 128.8, 127.1, 66.7, 56.6, 40.8, 38.5, 30.5, 30.2; HRMS (FAB+) m/z: calcd for $C_{13}H_{16}NO_2Br$, 298.0441; found, 298.0416.

The title compound, (*R*)-**1f**, was prepared in the same manner as (*R*)-**1a**. (*R*)-**20** (0.364 g, 1.221 mmol) was converted to (*R*)-**1f** (0.362 g, 90%). $[\alpha]_D^{25}$ −31.7° (*c* 1.33, $CHCl_3$); IR (neat) 1745 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.14–7.34 (5H, m), 3.98–4.21 (3H, m), 3.48–3.61 (1H, m), 3.32 (3H, s), 3.04–3.30 (4H, m), 2.61–2.73 (1H, m), 1.98–2.11 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.2, 135.2, 128.9, 128.8, 127.1, 66.7, 56.1, 50.4, 40.7, 38.4, 33.3, 27.8; HRMS (FAB+) m/z: calcd for $C_{14}H_{19}NO_4S_2+H$, 330.0834; found, 330.0834.

***N*-(3′-Methanethiosulfonatopropyl)-(*S*)-4-benzyl-2-oxazolidinone ((*S*)-1f).** (*S*)-**20** was prepared in the same manner as (*R*)-**20**. From (*S*)-4-benzyl-2-oxazolidinone (0.504 g, 2.844 mmol) was obtained (*S*)-**20** (0.558 g, 66%, ee≥98%)). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**20**. $[\alpha]_D^{25}$ +14.1° (*c* 2.50, $CHCl_3$). The title compound, (*S*)-**1f**, was prepared in the same manner as (*R*)-**1f**. From (*S*)-**20** (0.449 g, 1.506 mmol) was obtained (*S*)-**1f** (0.458 g, 92%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**1f**. $[\alpha]_D^{25}$ +29.9° (*c* 1.19, $CHCl_3$); HRMS (EI) m/z: calcd for $C_{14}H_{19}NO_4S_2+H$, 330.0834; found, 330.0844.

***N*-(2′-Methanethiosulfonatoethyl)-(*R*)-4-phenyl-2-oxazolidinone ((*R*)-1g).** *N*-(3′-Bromoethyl)-(*R*)-4-phenyl-2-oxazolidinone, (*R*)-**21**, was prepared in the same manner as **17**, except 10 equiv of 1,2-dibromoethane and 3 equiv of KOH were used. From (*R*)-4-phenyl-2-oxazolidinone (0.261 g, 1.599 mmol) was obtained (*R*)-**21** (0.387 g, 90%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ −54.1° (*c* 1.80, $CHCl_3$); IR (neat) 1749 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.26–7.46 (5H, m), 4.98 (1H, dd, J = 8.8, 6.6 Hz), 4.67 (1H, dd, J = 8.8, 8.8 Hz), 4.16 (1H, dd, J = 8.8, 6.6 Hz), 3.75–3.87 (1H, m), 3.42–3.53 (1H, m), 3.12–3.36 (2H, m); ^{13}C NMR ($CDCl_3$) δ 158.0, 137.4, 129.4, 129.3, 127.0, 70.0, 60.4, 43.8, 28.6; HRMS (EI) m/z: calcd for $C_{11}H_{12}NO_2Br$, 269.0051; found, 269.0055.

The title compound, (*R*)-**1g**, was prepared in the same manner as (*R*)-**1a**. (*R*)-**21** (0.392 g, 1.462 mmol) was converted to (*R*)-**1g** (0.320 g, 73%). $[\alpha]_D^{25}$ −28.8° (*c* 1.32, $CHCl_3$); IR (neat) 1749 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.29–7.43 (5H, m), 4.88 (1H, dd, J = 8.9, 6.6 Hz), 4.67 (1H, dd, J = 8.8, 8.8 Hz), 4.18 (1H, dd, J = 8.8, 6.5 Hz), 3.59–3.76 (1H, m), 3.28 (3H, s), 3.10–3.26 (3H, m); ^{13}C NMR ($CDCl_3$) δ 158.1, 137.3, 129.4, 129.3, 127.1, 69.9, 60.3, 50.7, 41.8, 33.6; HRMS (EI) m/z: calcd for $C_{12}H_{15}NO_4S_2+H$, 302.0521; found, 302.0529.

***N*-(2′-Methanethiosulfonatoethyl)-(*S*)-4-phenyl-2-oxazolidinone ((*S*)-1g).** (*S*)-**21** was prepared in the same manner as (*R*)-**21**. From (*S*)-4-phenyl-2-oxazolidinone (0.381 g, 2.335 mmol) was obtained (*S*)-**21** (0.564 g, 89%, ee≥98%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**21**. $[\alpha]_D^{25}$ +54.6° (*c* 1.85, $CHCl_3$). The title compound, (*S*)-**1g**, was prepared in the same manner as (*R*)-**1g**. From (*S*)-**21** (0.532 g, 1.969 mmol) was obtained (*S*)-**1g** (0.450 g, 76%). Its ^{1}H NMR and ^{13}C NMR data were identical to (*R*)-**1g**. $[\alpha]_D^{25}$ +27.8° (1.30, $CHCl_3$); HRMS

(EI) m/z: calcd for $C_{12}H_{15}NO_4S_2+H$, 302.0521; found, 302.0534.

***N*-(2′-Methanethiosulfonatoethyl)-(*R*)-4-benzyl-2-oxazolidinone ((*R*)-1h).** *N*-(3′-Bromoethyl)-(*R*)-4-benzyl-2-oxazolidinone, (*R*)-**22**, was prepared in the same manner as **17**, except 10 equiv of 1,2-dibromoethane and 3 equiv of KOH were used. From (*R*)-4-benzyl-2-oxazolidinone (0.386 g, 2.178 mmol) was obtained (*R*)-**22** (0.372 g, 60%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ −16.7° (*c* 1.35, $CHCl_3$); IR (neat) 1748 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.12–7.40 (5H, m), 3.81–4.30 (4H, m), 3.38–3.63 (3H, m), 3.11–3.20 (1H, m), 2.66–2.76 (1H, m); ^{13}C NMR ($CDCl_3$) δ 157.8, 135.2, 129.0, 127.3, 67.1, 56.9, 44.1, 38.7, 29.1; HRMS (EI) m/z: calcd for $C_{12}H_{14}NO_2Br$, 284.0286; found, 284.0281.

The title compound, (*R*)-**1h**, was prepared in the same manner as (*R*)-**1a**. (*R*)-**22** (0.334 g, 1.175 mmol) was converted to (*R*)-**1h** (0.363 g, 98%). $[\alpha]_D^{25}$ +4.5 (*c* 1.10, $CHCl_3$); IR (neat) 1748 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.15–7.39 (5H, m), 4.02–4.29 (3H, m), 3.72–3.89 (1H, m), 3.14–3.58 (4H, m), 3.38 (3H, s), 2.65–2.75 (1H, m); ^{13}C NMR ($CDCl_3$) δ 158.1, 135.2, 129.0, 127.3, 67.2, 57.0, 50.7, 42.0, 38.7, 33.9; HRMS (EI) m/z: calcd for $C_{13}H_{17}NO_4S_2+H$, 316.0677; found, 316.0683.

***N*-(2′-Methanethiosulfonatoethyl)-(*S*)-4-benzyl-2-oxazolidinone ((*S*)-1h).** (*S*)-**22** was prepared in the same manner as (*R*)-**22**. From (*S*)-4-benzyl-2-oxazolidinone (0.371 g, 2.094 mmol) was obtained (*S*)-**22** (0.375 g, 63%, ee≥98%). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**22**. $[\alpha]_D^{25}$ +15.6° (*c* 1.55, $CHCl_3$). The title compound, (*S*)-**1h**, was prepared in the same manner as (*R*)-**1h**. From (*S*)-**22** (0.328 g, 1.154 mmol) was obtained (*S*)-**1h** (0.245 g, 67%). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**1h**. $[\alpha]_D^{25}$ −5.8° (*c* 1.20, $CHCl_3$); HRMS (EI) m/z: calcd for $C_{13}H_{17}NO_4S_2+H$, 316.0677; found, 316.0664.

***N*-(3′-Methanethiosulfonatopropyl)-(3a*R*-*cis*)-3,3a,8,8a-tetrahydro-2*H*-indeno[1,2-*d*]-oxazol-2-one ((*R*)-1i).** (1*R*, 2*S*)-*cis*-1-Amino-2-indanol (0.980 g, 6.569 mmol) was placed in a round-bottomed flask and a dry Ar atmosphere was established. Dry CH_2Cl_2 (50 mL) and Et_3N (1.9 mL, 13.63 mmol) were added, and the resulting solution was cooled to −60 °C. On addition of triphosgene (0.64 g, 2.157 mmol), the cooling bath was removed, and the reaction was allowed to warm to 20 °C over 1 h. The reaction was then poured into CH_2Cl_2 (100 mL) and H_2O (50 mL) and the aqueous phase was extracted with CH_2Cl_2 (3×100 mL). After drying with $MgSO_4$, the organic layer was evaporated under reduced pressure to give (3a*R*-*cis*)-3,3a,8,8a-tetrahydro-2*H*-indeno[1,2-*d*]-oxazol-2-one, (*R*)-**24** (1.15 g, quantitative) as white crystals, which were of sufficient purity for the next step in the reaction sequence. An analytical sample was recrystallized from CH_2Cl_2/hexanes. Mp 205.5–206.5 °C; [lit.[25] for enantiomer mp 205 °C]; $[\alpha]_D^{25}$ +107.7° (*c* 1.25, $CHCl_3$); [lit.[25] for enantiomer $[\alpha]_D^{25}$ −79.4° (*c* 1.4, $CHCl_3$)]. IR (KBr) 3255, 1752, 1707 cm^{-1}; ^1H NMR (acetone-d_6) δ 7.24–7.43(4H, m), 5.39 (1H, t, J=7.5 Hz), 5.21 (1H, d, J=7.0 Hz), 3.42 (1H, dd, J=17.7, 6.2 Hz), 3.20 (1H, d, J=17.9 Hz), 2.90 (1H, br s); ^{13}C NMR (acetone-d_6) δ 159.1, 142.5, 141.0, 129.7, 128.3, 126.2, 125.8, 80.8, 61.7, 39.3; HRMS (FAB+) m/z: calcd for $C_{10}H_9NO_2+H$, 176.0771; found, 176.0681.

N-(3′-Bromopropyl)-(3a*R*-*cis*)-3,3a,8,8a-tetrahydro-2*H*-indeno[1,2-*d*]-oxazol-2-one, (*R*)-**25**, was prepared in the same manner as **17**. From (*R*)-**24** (1.007 g, 5.748 mmol) was obtained (*R*)-**25** (1.11 g, 65%, ee≥98%), as a colorless oil. $[\alpha]_D^{25}$ +31.3° (*c* 1.61, $CHCl_3$); IR (neat) 1748 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.24–7.45 (4H, m), 5.31 (1H, dt, J=7.4, 3.1 Hz), 5.14 (1H, d, J=7.7 Hz), 3.23–3.70 (6H, m), 2.12–2.34 (2H, m); ^{13}C NMR ($CDCl_3$) δ 157.1, 140.5, 138.0, 129.8, 127.4, 125.8, 125.1, 77.1, 64.1, 41.0, 39.3, 30.4, 30.1; HRMS (FAB+) m/z: calcd for $C_{13}H_{14}NO_2Br$, 296.0285; found, 296.0254.

The title compound, (*R*)-**1i**, was prepared in the same manner as (*R*)-**1a**. (*R*)-**25** (0.925 g, 3.123 mmol) was converted to (*R*)-**1i** (0.882 g, 86%). It was recrystallized from EtOAc/hexanes. Mp 94.0–95.0 °C; $[\alpha]_D^{25}$ +17.7° (*c* 1.28, $CHCl_3$); IR (KBr) 1729 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.26–7.38 (4H, m), 5.32 (1H, dt, J=7.4, 3.0 Hz), 5.14 (1H, d, J=7.6 Hz), 3.36–3.69 (4H, m), 3.32 (3H, s), 3.14–3.22 (2H, m), 2.10–2.23 (2H, m); ^{13}C NMR ($CDCl_3$) δ 157.2, 140.6, 137.9, 129.7, 127.4, 125.8, 125.0, 77.2, 63.7, 50.4, 40.9, 39.2, 33.4, 27.5; HRMS (FAB+) m/z: calcd for $C_{14}H_{17}NO_4S_2+H$, 328.0677; found, 328.0683.

***N*-(3′-Methanethiosulfonatopropyl)-(3a*S*-*cis*)-3,3a,8,8a-tetrahydro-2*H*-indeno[1,2-*d*]-oxazol-2-one ((*S*)-1i).** (*S*)-**24** was prepared in the same manner as (*R*)-**24**. From (1*S*, 2*R*)-*cis*-1-amino-2-indanol (1.09 g, 7.306 mmol) was obtained (*S*)-**24** (1.27 g, quantitative). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**24**. Mp 205.0–207.0 °C; $[\alpha]_D^{25}$ −109.7° (*c* 1.30, $CHCl_3$).

(*S*)-**25** was prepared in the same manner as (*R*)-**25**. From (*S*)-**24** (1.023 g, 5.839 mmol) was obtained (*S*)-**25** (0.940 g, 54%, ee≥98%). Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**25**. $[\alpha]_D^{25}$ −30.5° (*c* 1.82, $CHCl_3$).

The title compound, (*S*)-**1i**, was prepared in the same manner as (*R*)-**1i**. From (*S*)-**25** (0.840 g, 2.836 mmol) was obtained (*S*)-**1i** (0.838 g, 90%). It was recrystallized from EtOAc/hexanes. Its ^1H NMR and ^{13}C NMR data were identical to (*R*)-**1i**. Mp 94.0–95.0 °C; $[\alpha]_D^{25}$ −18.7° (*c* 1.38, $CHCl_3$); HRMS (EI) m/z: calcd for $C_{14}H_{17}NO_4S_2+H$, 328.0677; found, 328.0694.

Site-specific chemical modification

To 1.25 mL of a SBL mutant stored in MES buffer (10 mM MES, 1 mM $CaCl_2$, pH 5.8) was added 0.75 mL CHES buffer (70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) at 20 °C and one of the methanethiosulfonate reagents (100 mL of a 0.5 M solution in CH_3CN) in a PEG (10,000) coated polypropylene test tube, and the mixture agitated in an end-over-end rotator. After 30 min, all modification reactions were negative to the Ellman's test indicating the absence of free thiol. In order to ensure complete reaction, a further 100 mL of methanethiosulfonate solution was added and the reaction was continued for another 30 min. The reaction solution was purified on a disposable desalting column (Pharmacia

Biotech PD-10, Sephadex G-25 M) pre-equilibrated with MES buffer (5 mM MES, 2 mM $CaCl_2$, pH 6.5). The CMM was eluted with MES-buffer (5.0 mL), dialyzed (MWCO 12-14,000) against MES buffer (10 mM MES, 1 mM $CaCl_2$, pH 5.8) then flash frozen and stored at −20 °C. Modified enzymes were analyzed by non-denaturing gradient (8–25%) gels at pH 4.2, run towards the cathode on the Pharmacia Phast-System™,[26] and appeared as one single band. Each of the CMMs was analyzed in parallel with its parent cysteine mutant and the WT enzyme.

Enzyme characterization

Prior to ES-MS analysis, CMMs were purified by FPLC (BioRad, Biologic System) on a Source 15 RPC matrix (17-0727-20 from Pharmacia) with 5% acetonitrile, 0.01% TFA as the running buffer and eluted with 80% acetonitrile, 0.01% TFA in a one step gradient. Electrospray mass spectra were recorded on a PE SCIEX API III Biomolecular Mass Analyzer (Table 3).

The free thiol content of N62C, L217C, S166C, S156C and their CMMs, was determined spectrophotometrically by titration with Ellman's reagent ($\epsilon_{412} = 13{,}600$ $M^{-1}\,cm^{-1}$) in phosphate buffer 0.25 M, pH 8.0.

The active enzyme concentration was determined as previously described[18] by monitoring fluoride release upon enzyme reaction with a-toluenesulfonyl fluoride (Aldrich Chemical Co. Inc.) as measured by a fluoride ion sensitive electrode (Orion Research 96-09). The active enzyme concentration determined in this way was used to calculate kinetic parameters for each CMM.

Kinetic measurements

Specificity constants determined using the low substrate approximation were measured at 0.05 and 0.1 mM *N*-Suc-AAPF-*p*NA at 25 °C in 0.1 M Tris containing 0.005% Tween 80 and 1% DMSO at pH 8.6 for amidase activity ($\epsilon_{410} = 8800\ M^{-1}\,cm^{-1}$), and at 0.015 and 0.03 mM *N*-Suc-AAPF-SBn at 25 °C in 0.1 M Tris containing 0.005% Tween 80 and 1% 37.5 mM DTNB in DMSO at pH 8.6 for esterase activity ($\epsilon_{412} = 13{,}600\ M^{-1}\,cm^{-1}$). A general run consisted of equilibrating six plastic cuvettes containing 980 mL of 0.1 M Tris, 0.005% Tween 80 at pH 8.6 to 25 °C. The substrate (10 mL) in DMSO was added and the cuvette was shaken twice before returning it to the machine for zeroing. Immediately, the enzyme (10 mL) in 20 mM MES, 1 mM $CaCl_2$ at pH 5.8 was added and the cuvette was returned to the machine with an eight second delay. The initial rate data was recorded and used to calculate k_{cat}/K_M. Esterase data was adjusted to account for background hydrolysis of the substrate.

Michaelis–Menten constants were measured at 25 °C by curve fitting (GraFit® 3.03) of the initial rate data determined at eight concentrations (0.05–3.0 mM) of the *N*-Suc-AAPF-pNA substrate for amidase activity and eight concentrations (0.015–2.0 mM) of the *N*-Suc-AAPF-SBn substrate for esterase activity.

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