VOL. 56 NO. 2, FEB. 2003 THE JOURNAL OF ANTIBIOTICS pp. 160 — 168

N(2)-Substituted D,L-Cycloserine Derivatives: Synthesis and Evaluation as Alanine Racemase Inhibitors

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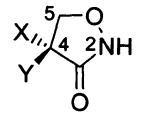
(Received for publication August 19, 2002)

A select series of *N*(2)-substituted D,L-cycloserine derivatives were prepared and evaluated for inhibitory activity against purified alanine racemases (*alr* gene product) from *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, as well as in a growth inhibition assay. *N*(2)-Modification led to loss of enzymatic inhibitory activity in most cases consistent with a recent proposal for cycloserine function.

D-Cycloserine (**1**) is a bioactive microbial product from *Streptomyces* (*S. garyphalus* sive *orchidaceus*)[1,2] that is clinically used as a second-line drug for the treatment of tuberculosis.[3] The primary macromolecular target for **1** is alanine racemase, an essential prokaryotic enzyme that is necessary for the conversion of L- to D-alanine, which is required for cell wall synthesis.[4]

Structure-activity relationship (SAR) studies have documented that most **1** structural changes gave diminished *in vitro* cell-based antimicrobial activities compared with **1**.[1,2] These investigations provided information concerning the antibiotic properties of cycloserine analogues, but they did not determine whether these derivatives interacted with alanine racemase since most cell growth studies do not monitor substrate cellular uptake, efflux and metabolism. In this study, we take advantage of new methodologies for protein expression[3,4] to examine the importance of the *N*(2)-structural site in cycloserine for bioactivity. This position has been largely overlooked in earlier investigations.[1,2] The biochemical properties of eighteen *N*(2)-modified cycloserine derivatives were tested against alanine racemases from *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, and then evaluated in growth assays using *E. coli* and *S. aureus*. We found that *N*(2)-modification in D,L-cycloserine led to loss of enzymatic inhibitory activity and that the loss of drug function was not enzyme specific. These findings are explained in context of a recently proposed mechanisms for inactivation of PLP-dependent enzymes by cycloserine.[7,8]



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a X=NH_2, Y=H (D-cycloserine)
b X=H, Y=NH_2 (L-cycloserine)

Results and Discussion

Selection of Compounds

The parent compound in our study is D,L-cycloserine (**1**). We modified only the *N*(2) ring position in **1**. The eighteen compounds (**2**~**19**) selected for evaluation are listed in

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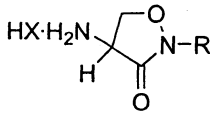
Table 1. Chemical, biochemical, and biological properties of *N*(2)-substituted D,L-cycloserine derivatives.



Cpd No.	R	% Inhibition[a]			K_i[b] [mM]			MIC[c] [mM]	
		MTb[d]	EC[e]	SA[f]	MTb[d]	EC[e]	SA[f]	EC[e]	SA[f]
1	H	93	93	94	0.0086	0.0138	0.0073	0.3	0.2
2	CH_3[g]	16	17	12	ND[h]	ND	ND	>2.5	>2.5
3	CH_2CH_3[i]	35	10	20	ND	ND	ND	>2.5	>2.5
4	$(CH_2)_2F$[g]	15	8	5	ND	ND	ND	>2.5	>2.5
5	CH_2OCH_3[g]	48	4	36	1.59	19.5	3.00	3.0	2.5
6	CH_2SCH_3[g]	2	5	3	ND	ND	ND	>2.5	>2.5
7	$(CH_2)_2OCH_3$[i]	10	8	12	ND	ND	ND	>2.5	>2.5
8	$(CH_2)_2OBn$[g]	3	5	5	ND	ND	ND	>2.5	>2.5
9	$(CH_2)_2OH$[g]	40	7	21	1.56	11.93	5.02	>2.5	>2.5
10	$(CH_2)_3OH$[g]	15	6	18	ND	ND	ND	>2.5	>2.5
11	$CH_2CO_2CH_3$[i]	16	13	22	ND	ND	ND	>2.5	>2.5
12	CH_2CO_2H	19	7	19	ND	ND	ND	>2.5	>2.5
13	$CH(CH_3)CO_2CH_3$[g]	6	7	14	ND	ND	ND	>2.5	>2.5
14	$CH(CH_3)CO_2CH_3$[g]	6	7	14	ND	ND	ND	>2.5	>2.5
15	CH_2CONH_2[g]	15	10	8	ND	ND	ND	>2.5	>2.5
16	$CH_2CONHOCH_3$[g]	46	77	43	0.36	0.47	1.16	>2.5	>2.5
17	$CH_2CON(CH_3)OH$[g]	24	11	11	ND	ND	ND	>2.5	>2.5
18	CH_2Ph[g]	17	7	7	ND	ND	ND	>2.5	>2.5
19	$CH_2Ph(p\text{-}F)$[g]	40	5	8	ND	ND	ND	>2.5	>2.5

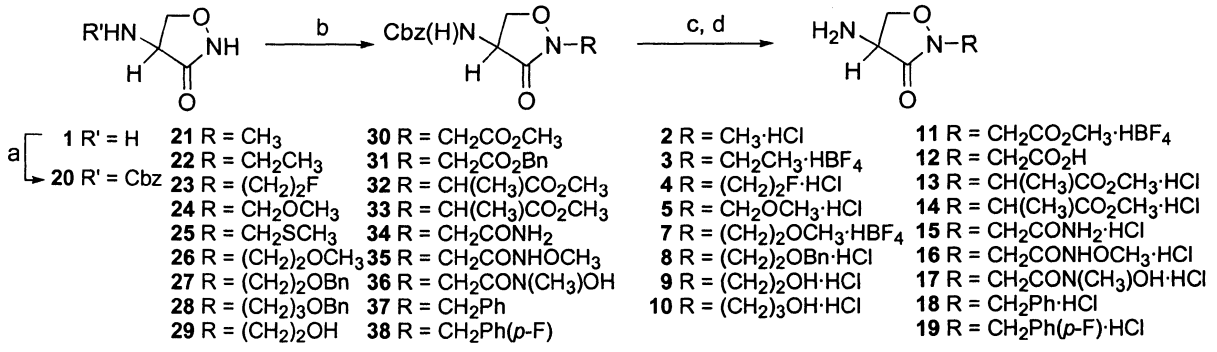
[a]% Inhibition of alanine racemase activity in the D→ L direction determined from duplicate measurements. [b]K_i = kinetic inhibition constant. [c]MIC = minimal inhibitory concentration of the tested compound determined from duplicate tests. [d]MTb = *M. tuberculosis*. [e]EC = *E. coli* Alr. [f]SA = *S. aureus* Alr. [g]Isolated as the HCl salt. [h]ND = not determined. [i]Isolated as the HBF_4 salt.

Table 1. We varied the size and electronic properties of the *N*(2) amino substituent. Included in our list were *N*(2) amino moieties that can enter into hydrogen bonding interactions with amino acid residues that comprise the alanine racemase PLP binding pocket. In all cases, the compounds were prepared as the *N*(2)-modified cycloserine racemate. This choice permitted our use of synthetic methods for **2**~**19** known to racemize **1**.

Chemistry

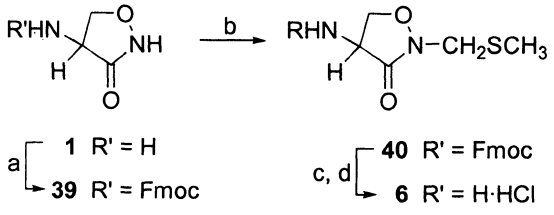
Two synthetic strategies (Schemes 1 and 2) were used to prepare *N*(2)-substituted D,L-cycloserines **2**~**19** from D,L-cycloserine[9] (**1**). First, D,L-cycloserine (**1**) was converted to the Cbz-protected derivative **20**[10] in 44% yield with Cbz-Cl under Schotten-Bauman conditions. *N*(2)-Substitution of **20** proceeded smoothly with the requisite alkyl halide and base (*tert*-BuOK) in DMF[11] to give **21**~**38** (37~91% yield). Under these conditions, we observed no evidence of the isomeric *O*-substituted imidate. Catalytic hydrogenation (10% Pd-C, H_2, 1 atm) of **21**~**24** and **26**~**38** followed by acidification (HCl, HBF_4) of the reaction mixture provided the desired *N*(2)-substituted D,L-cycloserine salts **2**~**5** and **7**~**19**. Of these compounds, only D-**18** has been previously reported.[10] The cycloserine product was isolated as the salt to prevent dimerization of the substituted D,L-cycloserine free base to the corresponding 2,5-bis-substituted-3,6-diketopiperazine.[12] Our use of catalytic conditions to remove the Cbz-protecting group in **21**~**24** and **26**~**38** complemented an existing HBr/HOAc deprotection

Scheme 1. Synthesis of *N*(2)-substituted D,L-cycloserine derivatives through Cbz-**20**.



(a) Cbz-Cl, 1 N NaOH, 0°C to rt, 44%; (b) R-X, *tert*-BuOK, DMF, 55~60°C; (c) 10% Pd-C, H_2, MeOH, rt; (d) 1 M HCl or 54 wt.% HBF_4, Et_2O, 0~5°C.

Scheme 2. Synthesis of *N*(2)-substituted D,L-cycloserine derivatives through Fmoc-**39**.



(a) Fmoc-Cl, 1 N NaOH, 61%; (b) *tert*-BuOK, DME, $ClCH_2SCH_3$, rt, 38%; (c) Et_2NH, THF, 0~5°C; (d) 1 M HCl, Et_2O, 0~5°C (67% from **40**).

procedure.[10] Efforts to extend this method to **25** were unsuccessful and led to the recovery of starting material. Accordingly, we replaced the Cbz-protecting group with 9-fluorenylmethoxycarbonyl (Fmoc) moiety (Scheme 2). Installation of the Fmoc unit in **39**[13] with Fmoc-Cl and base proceeded in 61% yield. Treatment of **39** with *tert*-BuOK and $ClCH_2SCH_3$ in DME gave **40** (38% yield). Deprotection of the Fmoc group with Et_2NH followed by acidification (HCl) gave the desired product **6** (67% yield from **40**).

Structural characterization of products **2**~**19** and Cbz-(**21**~**38**) and Fmoc-(**40**, **41**) intermediates was accomplished using IR, ^1H NMR, ^{13}C NMR, and MS (low- and high-resolution) spectroscopy (Tables 2 and 3). In the case of **32** and **33**, the diastereomeric mixture obtained after *N*(2) alkylation was separated by PTLC and then the Cbz-group was removed (10% Pd-C, H_2) and the product converted to the racemic **13** and **14** hydrochloride salts. We did not identify the stereochemical identity of the *N*(2) substituent in **13** and **14**.

Biochemical and Biological Properties

The inhibitory activities[14] of D,L-cycloserine derivatives **2**~**19** were evaluated against purified alanine racemases, products of the *alr* gene, from *E. coli*,[5] *S. aureus*, and *M. tuberculosis*[5] (Table 1). Three enzymes were evaluated since alanine racemases display approximately 50% amino acid identity.[15] In our experiments, D,L-**1** served as the control. We also determined the antibiotic activity of the D,L-cycloserine analogues against *E. coli* and *S. aureus* by establishing their minimal inhibitory concentration (MIC) in a microtiter plate growth assay (Table 1).

All of the modifications at the *N*(2) site resulted in reduced activity in both the enzyme and growth assay. These results underscore the importance of unsubstituted *N*(2)-cycloserine modification on drug function. There were three compounds **5**, **9** and **16**, however, with *N*(2) substitutions that retained significant, albeit reduced, activity in both assays. For these compounds we determined their kinetic inhibition constant, K_i, for the three alanine racemases (Table 1). We found that the K_i ranged from 0.36~11.93 mM, which was 34~864-fold higher than that observed for **1**. Compounds **5**, **9** and **16** contained an ether, hydroxyl, and hydroxamate containing side chain, respectively. These side chains could serve as sites for

Table 2. Physicochemical data of 2-substituted-*N*-Cbz-DL-cycloserine derivatives **21**~**38**.

Compd	Yield (%) mp (°C)[a]	Formula	HR-MS (+CI)[b]	^{1}H NMR[c] (300 MHz, CD_3OD) δ	^{13}C NMR[d] (75 MHz, CD_3OD) δ	IR[e] (cm^{-1})
21	81 107-110	$C_{12}H_{14}N_2O_4$	251.10351 $[M+1]^+$ (251.10318)	3.16 (3H, s), 4.06 (1H, dd, 8.7, 9.6), 4.54 (1H, t, 8.7), 4.73 (1H, br dd, 8.7, 9.6), 5.10 (2H, s), 7.29-7.36 (5H, m)	32.1, 54.8, 68.0, 71.9, 129.0, 129.2[f], 129.5[f], 138.0, 158.4, 168.2	3259 1718 1693
22	88 116-118	$C_{13}H_{16}N_2O_4$	265.11889 $[M+1]^+$ (265.11883)	1.21 (3H, t, 7.2), 3.52-3.65 (2H, m), 4.04 (1H, dd, 8.4, 9.6), 4.55 (1H, t, 8.4), 4.73 (1H, br dd, 8.4, 9.6), 5.10 (2H, s), 7.27-7.35 (5H, m)	12.3, 41.2, 55.1, 68.0, 72.0, 129.0, 129.1[f], 129.5[f], 138.0, 158.4, 167.6	3261 1720 1691
23	71 100-102	$C_{13}H_{15}FN_2O_4$	283.11004 $[M+1]^+$ (283.10941)	3.81 (1H, br t, 3.9), 3.90 (1H, br t, 3.9), 4.08 (1H, br dd, 8.7, 9.6), 4.49 (1H, br t, 3.9), 4.59 (1H, t, 8.7), 4.65 (1H, br t, 3.9), 4.78 (1H, br dd, 8.7, 9.6), 5.11 (2H, s), 7.31-7.35 (5H, m)	47.0 (d, 21.0), 54.8, 68.0, 72.0, 80.1 (d, 169.7), 129.0, 129.2[f], 129.5[f], 138.0, 152.1, 169.3	3307 1713
24	50 75-77	$C_{13}H_{16}N_2O_5$	280.10571 $[M]^+$ (280.10592)	3.37 (3H, s), 4.11 (1H, dd, 8.4, 9.3), 4.57 (1H, t, 8.4), 4.78 (1H, br dd, 8.4, 9.3), 4.83 (1H, d, 12.0), 4.88 (1H, d, 12.0), 5.10 (2H, s), 7.27-7.35 (5H, m)	54.7, 57.5, 68.0, 71.9, 76.5, 129.0, 129.2[f], 129.5[f], 138.0, 158.4, 169.8	3307 1715 1702
25	37 73-75	$C_{13}H_{16}N_2O_4S$	297.08988 $[M+1]^+$ (297.09090)	2.23 (3H, s), 4.11 (1H, dd, 8.1, 9.3), 4.60 (1H, t, 8.1), 4.65 (2H, s), 4.76 (1H, br dd, 8.1, 9.3), 5.10 (2H, s), 7.28-7.35 (5H, m)	15.9, 49.6, 54.7, 68.0, 72.0, 129.0, 129.2[f], 129.5[f], 138.0, 158.3, 168.6	3299 1709 1687
26	66 56-58	$C_{14}H_{18}N_2O_5$	295.12987 $[M+1]^+$ (295.12940)	3.32 (3H, s), 3.51-3.75 (4H, m), 4.03 (1H, dd, 8.4, 9.3), 4.54 (1H, t, 8.4), 4.76 (1H, br dd, 8.4, 9.3), 5.10 (2H, s), 7.28-7.35 (5H, m)	45.9, 54.8, 58.8, 68.0, 68.7, 72.0, 129.0, 129.1[f], 129.5[f], 138.0, 158.4, 168.9	3265 1723 1685
27	79 73-76	$C_{20}H_{22}N_2O_5$	371.15983 $[M+1]^+$ (371.16070)	3.60-3.82 (4H, m), 4.00 (1H, br dd, 8.4, 9.3), 4.49 (2H, s), 4.50 (1H, t, 8.4), 4.72 (1H, br dd, 8.4, 9.3), 5.09 (2H, s), 7.25-7.34 (10H, m)	46.2, 54.9, 66.3, 68.0, 71.9, 73.7, 128.8, 128.9[f], 129.0, 129.1[f], 129.4[f], 129.5[f], 138.0, 139.4, 158.3, 168.9	3309 1718 1701
28	75 75-77	$C_{21}H_{24}N_2O_5$	385.17694 $[M+1]^+$ (385.17635)	1.90 (2H, br quin, 6.0), 3.52 (2H, t, 6.0), 3.62-3.72 (2H, m), 4.03 (1H, br dd, 8.7, 9.9), 4.47 (2H, s), 4.50 (1H, t, 8.7), 4.70 (1H, br dd, 8.7, 9.9), 5.10 (2H, s), 7.29-7.36 (10H, m)	28.1, 43.5, 55.0, 67.9, 68.3, 71.9, 74.1, 128.7, 128.9[f], 129.0, 129.1[f], 129.4[f], 129.5[f], 138.0, 139.7, 158.3, 167.8	3254 1725 1683
29	91 g	$C_{13}H_{16}N_2O_5$	281.11385 $[M+1]^+$ (281.11375)	3.67-3.72 (4H, m), 4.07 (1H, br dd, 8.7, 9.6), 4.56 (1H, t, 8.7), 4.78 (1H, br dd, 8.7, 9.6), 5.11 (2H, s), 7.29-7.36 (5H, m)	48.9, 55.0, 58.6, 68.0, 72.1, 129.0, 129.2[f], 129.5[f], 138.0, 158.4, 168.8	3303 1717 1695
30	89 71-73	$C_{14}H_{16}N_2O_6$	309.10858 $[M+1]^+$ (309.10866)	3.74 (3H, s), 4.10 (1H, br dd, 8.4, 9.0), 4.30 (1H, d, 19.0), 4.42 (1H, d, 19.0), 4.56 (1H, t, 8.4), 4.79 (1H, br dd, 8.4, 9.0), 5.10 (2H, s), 7.30-7.34 (5H, m)	47.8, 53.2, 54.5, 68.0, 72.0, 129.0, 129.2[f], 129.5[f], 138.0, 158.4, 168.8, 171.2	3334 1759 1727
31	79 100-102	$C_{20}H_{20}N_2O_6$	385.13976 $[M+1]^+$ (385.13996)	4.08 (1H, br dd, 8.4, 9.6), 4.34 (1H, d, 18.0), 4.48 (1H, d, 18.0), 4.55 (1H, t, 8.4), 4.79 (1H, br dd, 8.4, 9.6), 5.10 (2H, s), 5.19 (2H, s), 7.31-7.34 (10H, m)	48.1, 54.5, 68.0, 68.6, 72.0, 129.0, 129.1[f], 129.2, 129.4[f], 129.5[f], 129.7[f], 136.8, 138.0, 158.4, 168.2, 171.4	3321 1749 1702
32	26 h	$C_{15}H_{18}N_2O_6$	h	1.49 (3H, d, 7.5), 3.74 (3H, s), 4.10 (1H, br dd, 8.4, 9.3), 4.58 (1H, t, 8.4), 4.82 (1H, br dd, 8.4, 9.3), 5.11 (2H, s), 7.28-7.35 (5H, m)	14.3, 53.3, 54.7, 55.3, 68.0, 72.7, 129.0, 129.2[f], 129.5[f], 138.0, 158.5, 169.9, 171.2	3311 1746 1703
33	23 99-101	$C_{15}H_{18}N_2O_6$	323.12475 $[M+1]^+$ (323.12431)	1.50 (3H, d, 7.2), 3.72 (3H, s), 4.03 (1H, br dd, 8.4, 9.6), 4.55 (1H, t, 8.4), 4.74 (1H, br dd, 8.4, 9.6), 4.88 (1H, q, 7.2), 5.10 (2H, s), 7.29-7.35 (5H, m)	13.8, 53.3, 54.9, 55.2, 68.0, 71.5, 129.0, 129.2[f], 129.5[f], 138.0, 158.4, 170.9, 171.4	h
34	75 120-122	$C_{13}H_{15}N_3O_5$	294.10948 $[M+1]^+$ (294.10900)	4.14 (1H, dd, 8.7, 9.3), 4.17 (1H, d, 17.4), 4.27 (1H, d, 17.4), 4.57 (1H, t, 8.7), 4.79 (1H, br dd, 8.7, 9.3), 5.10 (2H, s), 7.28-7.35 (5H, m)	48.9, 54.5, 68.0, 72.1, 129.0, 129.2[f], 129.5[f], 137.9, 158.4, 170.2, 170.8	3404 3316 1694
35	85 123-126	$C_{14}H_{17}N_3O_6$	324.11963 $[M+1]^+$ (324.11956)	3.70 (3H, s), 4.12 (1H, br dd, 8.7, 9.6), 4.18 (2H, s), 4.57 (1H, t, 8.7), 4.79 (1H, br dd, 8.7, 9.6), 5.11 (2H, s), 7.28-7.37 (5H, m)	47.5, 54.5, 64.6, 68.1, 72.1, 129.0, 129.2[f], 129.6[f], 138.0, 158.4, 165.1, 170.8	3307 1727 1681
36	66 132-125	$C_{14}H_{17}N_3O_6$	324.11854 $[M+1]^+$ (324.11956)	3.20 (3H, s), 4.11 (1H, br dd, 8.4, 9.6), 4.53 (2H, s), 4.56 (1H, t, 8.4), 4.87 (1H, br dd, 8.4, 9.6), 5.11 (2H, s), 7.28-7.37 (5H, m)	36.6, 48.0, 54.7, 68.0, 72.2, 129.0, 129.2[f], 129.5[f], 138.0, 158.5, 167.6, 171.8	3312 1727 1690
37[i]	74 109-111	$C_{18}H_{18}N_2O_4$	327.13391 $[M+1]^+$ (327.13448)	4.02 (1H, dd, 8.4, 9.6), 4.50 (1H, t, 8.4), 4.65 (1H, d, 15.6), 4.76 (1H, d, 15.6), 4.77 (1H, br dd, 8.4, 9.6), 5.10 (2H, s), 7.28-7.37 (10H, m)	49.8, 54.8, 68.0, 71.9, 129.0, 129.1, 129.2[f], 129.5[f], 129.6[f], 129.7[f], 136.3, 138.0, 158.4, 168.1	3278 1709 1679
38	77 105-107	$C_{18}H_{17}FN_2O_4$	345.12454 $[M+1]^+$ (345.12506)	4.03 (1H, dd, 8.7, 9.0), 4.50 (1H, t, 8.7), 4.62 (1H, d, 15.9), 4.75 (1H, d, 15.9), 4.76 (1H, br dd, 8.7, 9.0), 5.10 (2H, s), 7.05 (2H, t, 8.7), 7.26-7.36 (7H, m)	49.1, 54.8, 68.0, 71.9, 116.4 (d, 22.2)[f], 129.0, 129.2[f], 129.5[f], 131.1 (d, 8.0)[f], 131.4 (d, 3.5), 138.0, 158.4, 163.9 (d, 243.1), 168.2	3304 1711 1679

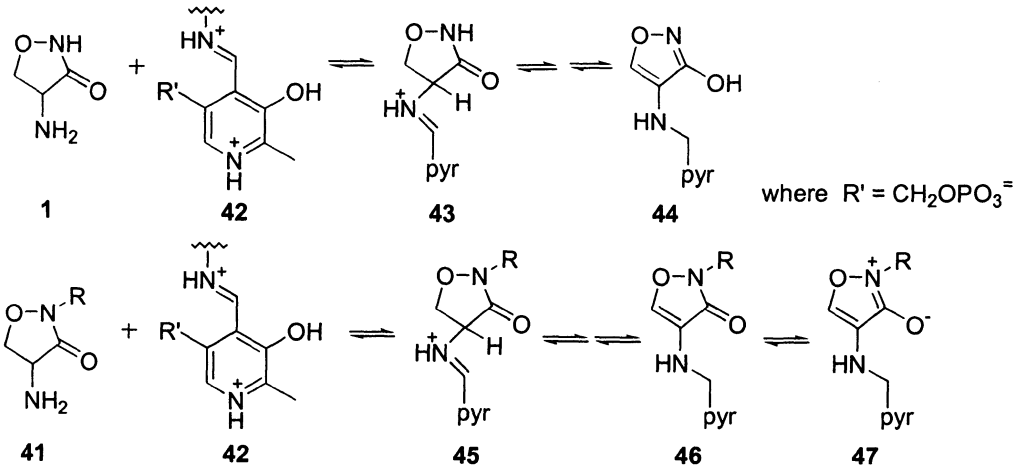
[a]Melting points are uncorrected. [b]The value in parentheses is the calculated value. [c]The number in each entry is the chemical shift value (δ) observed in ppm relative to Me_4Si, followed by the multiplicity of the signal and the coupling constant(s) in Hz. [d]The number in the entry is the chemical shift value (δ) observed in ppm relative to the solvent peak, followed in select cases by the multiplicity and the coupling constant in Hz. [e]IR were taken either neat or KBr pellets. [f]Peak corresponded to two signals. [g]Compound existed as an oily soild. [h]Data not available. [i]Reference 10.

Table 3. Physicochemical data of 2-substituted-DL-cycloserine derivatives **2**~**5**, **7**~**9**, **11**~**19**.

Compd	Yield (%) mp (°C)[a]	Formula	HR-MS (+CI)[b]	^{1}H NMR[c] (300 MHz, CD_3OD) δ	^{13}C NMR[d] (75 MHz, CD_3OD) δ	IR[e] (cm^{-1})
2	70 164-168	$C_4H_8N_2O_2$ ·HCl	117.06613 [(M-HCl) + 1]$^+$ (117.06640)	3.21 (3H, s), 4.24 (1H, br t, 8.9), 4.47 (1H, br dd, 8.4, 8.9), 4.70 (1H, br dd, 8.4, 8.9)	32.1, 52.6, 70.2, 164.4	3404 1685
3	67 162-164	$C_5H_{10}N_2O_2$ ·HBF_4	131.08241 [(M-HBF_4) + 1]$^+$ (131.08205)	1.24 (3H, t, 7.2), 3.60-3.69 (2H, m), 4.20 (1H, br t, 9.0), 4.48 (1H, br dd, 8.7, 9.0), 4.71 (1H, br dd, 8.7, 9.0)	12.3, 41.4, 52.8, 70.4, 163.7	3420 1699
4	74 118-121	$C_5H_9FN_2O_2$ ·HCl	149.07237 [(M-HCl) + 1]$^+$ (149.072 63)	3.87 (1H, dt, 4.5, 4.8), 3.95 (1H, dt, 4.5, 4.8), 4.25 (1H, br t, 9.3), 4.52 (1H, t, 4.5), 4.54 (1H, br dd, 8.7, 9.3), 4.68 (1H, t, 4.5), 4.76 (1H, br dd, 8.7, 9.3)	47.2 (d, 20.5), 52.5, 70.5, 80.5 (d, 169.7), 165.4	3419 1712
5	89 f	$C_5H_{10}N_2O_3$ ·HCl	147.07676 [(M-HCl) + 1]$^+$ (147.07697)	3.39 (3H, s), 4.26 (1H, br t, 9.3), 4.58 (1H, br dd, 8.7, 9.3), 4.77 (1H, br dd, 8.7, 9.3), 4.88 (1H, d, 12.6), 4.93 (1H, d, 12.6)	52.3, 57.7, 70.6, 76.8, 166.0	3416 1717
7	70 131-135	$C_6H_{12}N_2O_3$ ·HBF_4	161.09275 [(M-HBF_4) + 1]$^+$ (161.09262)	3.35 (3H, s), 3.60 (1H, t, 5.4), 3.61 (1H, t, 5.4), 3.79 (2H, t, 5.4), 4.22 (1H, br t, 9.3), 4.50 (1H, br dd, 8.4, 9.3), 4.73 (1H, br dd, 8.4, 9.3)	46.1, 52.6, 58.8, 68.5, 70.4, 165.1	3419 1703
8	65 g	$C_{12}H_{16}N_2O_3$ ·HCl	237.12346 [(M-HCl) + 1]$^+$ (237.12392)	3.68-3.89 (4H, m), 4.22 (1H, br t, 9.0), 4.46 (1H, br dd, 8.1, 9.0), 4.51 (1H, 1/2 ABq, 11.7), 4.56 (1H, 1/2 ABq, 11.7), 4.71 (1H, br dd, 8.1, 9.0), 7.28-7.35 (5H, m)	46.4, 52.6, 66.2, 70.3, 73.8, 128.9, 129.0[h], 129.5[h], 139.3, 165.2	3420 1705
9	86 144-147	$C_5H_{10}N_2O_3$ ·HCl	147.07694 [(M-HCl) + 1]$^+$ (147.07697)	3.70-3.74 (4H, m), 4.26 (1H, br t, 9.0), 4.50 (1H, br dd, 8.7, 9.0), 4.73 (1H, br dd, 8.7, 9.0)	47.7, 50.5, 56.5, 68.6, 164.0[i]	3413 1688
11	63 129-132	$C_6H_{10}N_2O_4$ ·HBF_4	175.07170 [(M-HBF_4) + 1]$^+$ (175.07188)	3.78 (3H, s), 4.30 (1H, br t, 9.3), 4.46 (2H, s), 4.56 (1H, br dd, 8.7, 9.3), 4.75 (1H, br dd, 8.7, 9.3)	47.9, 52.2, 53.4, 70.7, 167.4, 168.7	3423 1747 1706
12	96 78-82	$C_5H_8N_2O_4$	161.05561 [M + 1]$^+$ (161.05623)	4.31 (1H, br t, 8.7), 4.36 (2H, s), 4.54 (1H, br dd, 8.7, 9.0), 4.74 (1H, br dd, 8.7, 9.0)	52.4, 70.7, 167.3, 169.9	3407 1740 1715[j]
13	58 160-162	$C_7H_{12}N_2O_4$ ·HCl	189.08745 [(M-HCl) + 1]$^+$ (189.08753)	1.54 (3H, d, 7.5), 3.77 (3H, s), 4.31 (1H, br t, 8.7), 4.58 (1H, br dd, 8.7, 9.3), 4.74 (1H, br dd, 8.7, 9.3), 4.91 (1H, q, 7.5)	14.3, 52.4, 53.5, 55.7, 71.3, 166.6, 171.0	3458 1753 1716
14	95 j	$C_7H_{12}N_2O_4$ ·HCl	189.08674 [(M-HCl) + 1]$^+$ (189.08753)	1.54 (3H, d, 7.2), 3.75 (3H, s), 4.22 (1H, br t, 9.6), 4.53 (1H, br dd, 8.7, 9.6), 4.76 (1H, br dd, 8.7, 9.6), 4.92 (1H, q, 7.2)	13.9, 52.5, 53.5, 55.7, 70.3, 167.5, 170.6	3438 1746 1714
15	74 77-80	$C_5H_9N_3O_3$ ·HCl	160.07198 [(M-HCl) + 1]$^+$ (160.07222)	4.27 (1H, d, 17.4), 4.32 (1H, br t, 9.0), 4.34 (1H, d, 17.4), 4.56 (1H, br dd, 8.4, 9.0), 4.74 (1H, br dd, 8.4, 9.0)	48.2, 50.1, 68.6, 166.1, 166.7[i]	3377 1712 1678
16	65 89-92	$C_6H_{11}N_3O_4$ ·HCl	190.08331 [(M-HCl) + 1]$^+$ (190.08278)	3.72 (3H, s), 4.24 (2H, s), 4.29 (1H, br t, 9.0), 4.56 (1H, br dd, 8.4, 9.0), 4.73 (1H, br dd, 8.4, 9.0)	47.5, 52.2, 64.6, 70.6, 164.6, 167.7	3481 1712 1683
17	51 82-85	$C_6H_{11}N_3O_4$ ·HCl	190.08330 [(M-HCl) + 1]$^+$ (190.08278)	3.22 (3H, s), 4.33 (1H, br t, 9.0), 4.55 (1H, br dd, 8.4, 9.0), 4.56 (1H, d, 18.0), 4.63 (1H, d, 18.0), 4.72 (1H, br dd, 8.4, 9.0)	36.6, 48.3, 52.5, 70.6, 167.1, 168.3	3406 1719 1627
18[k]	77 g	$C_{10}H_{12}N_2O_2$ ·HCl	193.09734 [(M-HCl) + 1]$^+$ (193.09770)	4.21 (1H, br t, 9.0), 4.56 (1H, br dd, 8.7, 9.0), 4.71 (1H, br dd, 8.7, 9.0), 4.72 (1H, d, 17.4), 4.85 (1H, d, 17.4), 7.30-7.34 (5H, m)	50.0, 52.5, 70.5, 129.2, 129.3[h], 129.8[h], 135.8, 164.3	3354 1704
19	87 156-160	$C_{10}H_{11}FN_2O_2$ ·HCl	211.08782 [(M-HCl) + 1]$^+$ (211.08828)	4.22 (1H, br t, 9.0), 4.56 (1H, br dd, 8.4, 9.0), 4.72 (1H, br dd, 8.4, 9.0), 4.74 (1H, d, 15.9), 4.79 (1H, d, 15.9), 7.08 (2H, t, 8.7), 7.37 (2H, dd, 5.1, 8.7)	49.3, 52.5, 70.5, 116.5 (d, 22.2)[h], 131.4 (d, 8.0)[h], 131.9 (d, 3.5), 164.1 (d, 244.1), 164.3	3444 1713

[a]Melting points are uncorrected. [b]The value in parentheses is calculated value. [c]The number in each entry is the chemical shift value (δ) observed in ppm relative to Me_4Si, followed by the multiplicity of the signal and the coupling constant(s) in Hz. [d]The number in the entry is the chemical shift value (δ) observed in ppm relative to the solvent peak, followed in select cases by the multiplicity and the coupling constant in Hz. [e]IR were taken either neat or KBr pellets. [f]Solid was hygroscopic. [g]Compound existed as an oily soild. [h]Peak corresponded to two signals. [i]The solvent was used DMSO-d_6. [j]Data not available. [k]Reference 10.

Scheme 3. Proposed cycloserine (**1**) aromatization inactivation mechanism (**1** to **44**)[7] and potential pathway for *N*(2)-substituted cycloserine inactivation (**41** to **47**)



hydrogen bond donor/acceptor interactions with amino acid recipients within the alanine racemase substrate-binding cavity. However, other derivatives with similar hydrogen bonding capabilities, such as **8**, **10**, **15** and **17**, do not have activity in both assays. To understand the nature of the possible interaction of these side chains with alanine racemase, we are currently undertaking crystallographic studies of racemases with **5**, **9** and **16** bound to the active site.

We have learned that selective *N*(2) modification of cycloserine (**1**) led to a loss of inhibitory activity compared with **1** against alanine racemases from *E. coli*, *S. aureus*, and *M. tuberculosis*. Cycloserine (**1**) inhibition has been projected to proceed through the intermediacy of a C(4) amino cycloserine bound PLP aldimine **42**. In all our cases we retained the unsubstituted C(4) exocyclic amine necessary for PLP Schiff base formation. Why then do we observe a significant loss for *N*(2)-substituted cycloserine **41** activity? Recently, the SILVERMAN[7] and RINGE[8] research groups proposed a novel aromatization mechanism for inactivation of PLP-dependent aminotransferases by **1** (Scheme 3). Enzyme inactivation proceeds through a Schiff base and leads to the *loss* of the *N*(2) proton to give **44**. Utilization of this aromatization pathway for *N*(2)-substituted cycloserines **41** would lead to either **46** or **47**, intermediates that may be energetically less favorable than the aromatized adduct **44**, and less likely to form hydrogen bonds with nearby amino acid residues.[8]

Conclusions

We have synthesized a series of *N*(2)-substituted cycloserine derivatives and evaluated their inhibitory activity against alanine racemases from various bacterial organisms. Most analogues were devoid of significant activity and only compounds **5**, **9**, and **16** displayed modest inhibitory activity against *M. tuberculosis*, *E. coli*, and *S. aureus* alanine racemases. Our findings provide initial support for a recently proposed aromatization mechanism for cycloserine inhibition of PLP-dependent enzymes.[9,10]

Experimental Section

General Methods for Synthesis

Infrared spectra (IR) were run on a Mattson Genesis Series FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were taken on a Varian VXR 300 MHz NMR instrument. Low- and high-resolution (CI) mass spectral investigations were conducted at the University of Texas at Austin by Dr. M. MOINI. The low-resolution mass studies were run on a Finnegan MAT-TSQ-70 instrument and the high-resolution mass studies were conducted on a Micromass ZAB-E spectrometer. Thin-layer chromatography was run on precoated silica gel GHLF (10×20 cm; Aldrich No. Z27428-3). *N*-Methoxy-2-bromoethanamide[16] and *N*-hydroxy-*N*-methyl-2-bromoethanamide[17] were prepared according to literature procedures.

General Procedure for the Preparation of 2-Substituted-*N*-Cbz-DL-cycloserine Derivatives **21**~**38**

To an anhydrous DMF solution (1 ml) of **20**[10] (1 equiv) was added *tert*-BuOK (1.0~1.2 equiv). After the mixture was stirred at room temperature (30 minutes), the desired electrophile (1.5~5.0 equiv) was added to the mixture. The reaction mixture was stirred at 55~60°C (1~8 hours), filtered (Celite pad), and the solution was then concentrated *in vacuo* (Kugelrohr). The residue was purified by PTLC (1~10% MeOH-$CHCl_3$) to afford the desired product (Table 2).

2-Methylthiomethyl-*N*-Fmoc-DL-cycloserine (**40**)

To an anhydrous DME solution (2 ml) of **39**[13] (97 mg, 0.30 mmol) was added *tert*-BuOK (34 mg, 0.31 mmol). After the mixture was stirred at room temperature (30 minutes), chloromethyl methyl sulfide (29 μl, 0.33 mmol) was added. The mixture was stirred at room temperature (3 days), filtered, and the solution was then concentrated *in vacuo*. The residue was purified by PTLC (1% MeOH-$CHCl_3$) to give **40** as a white solid (44 mg, 38%): mp 100~102°C; Rf 0.67 (2.5% MeOH-$CHCl_3$); IR (neat) 3311, 1730, 1706 cm^{-1}; ^1H NMR (CD_3OD) δ 2.22 (3H, s, SCH_3), 4.09 (1H, dd, J=8.4, 9.0 Hz, C(5)*H*H′), 4.19 (1H, t, J=6.6 Hz, C(1′) H), 4.35 (2H, d, J=6.6 Hz, $CH_2OC(O)NH$), 4.57 (1H, t, J=8.4 Hz, C(5)H*H*′), 4.63 (2H, s, $N(CH_2SCH_3)$), 4.74 (1H, br dd, J=8.4, 9.0 Hz, C(4)H), 7.28 (2H, t, J=7.2 Hz, Ph), 7.37 (2H, t, J=7.2 Hz, Ph), 7.63 (2H, d, J=7.2 Hz, Ph), 7.77 (2H, d, J=7.2 Hz, Ph); ^{13}C NMR (CD_3OD) δ 15.9 (SCH_3), 48.4 ($CHCH_2O$), 49.6 ($N(CH_2SCH_3)$), 54.7 (C(4)), 68.3 ($CH_2OC(O)$), 72.0 (C(5)), 121.0, 126.2, 128.2, 128.8, 142.6, 145.2 (2 Ph), 158.3 (C(O)NH), 168.5 (C(3)); MS (+CI) 385 $[M+1]^+$; M_r (+CI) 385.121 23 $[M+1]^+$ (calcd for $C_{20}H_{21}N_2O_4S$ 385.122 20).

General Procedure for the Preparation of 2-Substituted-DL-cyloserine Acid Salts **2**~**5**, **7**~**9**, **11**~**19**

To a MeOH solution of the 2-substituted-*N*-Cbz-DL-cycloserine (1 equiv) was added 10% Pd-C (20 wt.%/equiv of Cbz derivative) and the mixture was hydrogenated under 1 atm of H_2 (1~8 hours). The catalyst was filtered (Celite pad), and the solvent was removed *in vacuo*. The residue was purified by PTLC (5~10% MeOH-$CHCl_3$) to give the desired amine. The amine was dissolved in MeOH (0.1~0.5 ml), and then the solution was cooled to 0~5°C and either 1 M HCl-Et_2O or 54 wt.% HBF_4-Et_2O (1.5 equiv) was added. The mixture was stirred at 0~5°C (30 minutes). Excess acid (HCl, HBF_4) was removed *in vacuo* and the product was precipitated by addition of isopropyl ether. The product was filtered, washed with isopropyl ether, and dried *in vacuo* to yield the desired product (Table 3).

2-Methylthiomethyl-DL-cycloserine Hydrochloride (**6**)

To a THF (0.57 ml) solution of **40** (29 mg, 0.07 mmol) was added Et_2NH (0.29 ml) at 0~5°C. The mixture was warmed to room temperature and then stirred (2.5 hours). The mixture was concentrated *in vacuo* to give a residue, which was purified by PTLC (2.5% MeOH-$CHCl_3$) to give the amine. The amine was dissolved in MeOH (0.1 ml), and then the solution was cooled to 0~5°C and 1 M HCl-Et_2O (111 μl, 0.11 mmol) was added. The mixture was stirred at 0~5°C (30 minutes). Excess HCl was removed *in vacuo* and the product was precipitated by addition of isopropyl ether, and dried to give **6** as a white hygroscopic solid (10 mg, 67%): Rf 0.28 (5% MeOH-$CHCl_3$); IR (neat) 3410, 1706 cm^{-1}; ^1H NMR (CD_3OD) δ 2.26 (3H, s, SCH_3), 4.26 (1H, br t, J=9.3 Hz, C(5)*H*H′), 4.55 (1H, br dd, J=8.7, 9.3 Hz, C(5)H*H*′), 4.68 (1H, d, J=15.0 Hz, $N(CHH'SCH_3)$), 4.74 (1H, d, J=15.0 Hz, $N(CHH'SCH_3)$), 4.78 (1H, br dd, J=8.7, 9.3 Hz, C(4)H); ^{13}C NMR (CD_3OD) δ 16.1 (SCH_3), 49.9 ($N(CH_2SCH_3)$), 52.3 (C(4)), 70.7 (C(5)), 164.6 (C(3)); MS (+CI) 163 $[(M-HCl)+1]^+$; M_r (+CI) 163.053 46 $[(M-HCl)+1]^+$ (calcd for $C_5H_{11}N_2O_2S$ 163.054 13).

2-Hydroxypropyl-DL-cycloserine Hydrochloride (**10**)

A MeOH (5.6 ml) solution of **28** (56 mg, 0.15 mmol) was hydrogenated at room temperature (1 hour) in the presence of 10% Pd-C (11 mg). The catalyst was removed by filtration (Celite pad) and the filtrate concentrated *in vacuo* to give a residue, which was purified by PTLC (7% MeOH-$CHCl_3$) to give the amine. To a MeOH (3.6 ml) solution of the amine (36 mg, 0.14 mmol) was added 10% Pd-C (7 mg) and the mixture was hydrogenated under 35 atm of H_2 (10 hours). The catalyst was filtered (Celite pad), and the solvent was removed *in vacuo*. The residue was dissolved in MeOH (0.1 ml), and then the solution was cooled to 0~5°C and 1 M HCl-Et_2O (216 μl, 0.22 mmol) was added. The mixture was stirred at 0~5°C (30 minutes) and then excess HCl was removed *in vacuo*. The product was precipitated by addition of isopropyl ether and dried *in vacuo* to give **10** as a pale yellow solid (11 mg, 38%): mp 110~113°C; Rf 0.16 (10% MeOH-$CHCl_3$); IR (KBr) 3345, 1700 cm^{-1}; ^1H NMR (CD_3OD) δ 1.86 (2H, quin, J=6.0 Hz, $N(CH_2CH_2CH_2OH)$), 3.62 (2H, t, J=6.0 Hz, $N(CH_2CH_2CH_2OH)$), 3.72 (1H, t, J=6.0 Hz, $N(CH_2CH_2CHH'OH)$), 3.73 (1H, t, J=6.0 Hz, $N(CH_2CH_2CHH'OH)$), 4.23 (1H, br t, J=9.0 Hz, C(5)*H*H′), 4.51 (1H, br dd, J=8.4, 9.0 Hz, C(5)H*H*′), 4.73 (1H, br dd, J=8.4, 9.0 Hz, C(4)H); ^{13}C NMR (CD_3OD) δ 30.7 ($N(CH_2CH_2CH_2OH)$), 43.7 ($N(CH_2CH_2CH_2OH)$),

52.7 (C(4)), 60.0 ($N(CH_2CH_2CH_2OH)$), 70.4 (C(5)), 164.1 (C(3)); MS (+CI) 161 $[(M-HCl)+1]^+$; M_r (+CI) 161.092 74 $[(M-HCl)+1]^+$ (calcd for $C_6H_{13}N_2O_3$ 161.092 62).

Preparation of Alanine Racemases

The alanine racemases from the *alr* genes of *M. tuberculosis* and *E. coli* were purified as described from *E. coli* strains harboring pET expression plasmids pMB1909 and pMB1556, respectively.[5] The sequence of the *alr* gene from *S. aureus* was initially obtained from a genomic sequence provided to us by Schering-Plough. Primers were made, and the gene was amplified and sequenced. The nucleotide sequence is identical with the subsequent GenBank Acc. No. Y16431 entry. Analogous to what was described for *M. tuberculosis*,[5] the gene was cloned in pET26, expressed in *E. coli* BL21(DE3)pLysS (Novagen, Madison, WI) to make strain MB1978, and the protein was purified to electrophoretic homogeneity using anion exchange chromatography.

Alanine Racemase Enzyme Assay

Alanine racemase activity was measured at room temperature in the D→L direction using a spectrophotometric assay.[14] The alanine racemase enzymes from *S. aureus* (0.1 μg), *E. coli* (0.1 μg) or *M. tuberculosis* (1.0 μg) were incubated with 100 mM Tricine, pH 8.5, 0.08 units L-alanine dehydrogenase (Sigma), 10 mM NAD and 2.5 mM inhibitor. After 30 minutes at room temperature, 10 mM D-alanine was added and the reaction was monitored in a Beckman spectrophotometer at 340 nm for 5 minutes. Enzyme activity was proportional to the amount of NADH produced in the reaction. Inhibition was expressed as the quotient of enzyme activity without inhibitor over enzyme activity in the presence of inhibitor. All assays were performed in duplicate (Table 1).

Antimicrobial Growth Assay

E. coli and *S. aureus* were both grown in cation-supplemented Mueller-Hinton broth (MHB) (Becton-Dickinson). Stationary overnight cultures were diluted 10^{-6} in MHB and aliquots of 100 μl each were added to the wells of a standard 96 well microtiterplate. Control wells were filled with MHB without bacteria. Each inhibitor (5 mM) was added to the top row of wells of the plate and two-fold serial dilutions were performed down each column of wells. The plates were incubated for 16 hours at 37°C and culture turbidity was monitored using a microplate reader at 560 nm. All assays were performed in duplicate (Table 1).

K_i Determination

Enzyme activity in the presence of different inhibitor concentrations was determined at various substrate concentrations.[18] For each inhibitor concentration a set of reciprocal substrate and velocity data was obtained. The slope derived from each double reciprocal plot was then replotted versus inhibitor concentration. The X-intercept of the line that fit these data was then used to calculate the K_i (Table 1).

Acknowledgments

This work was supported by the National Institutes of Health grant AI46340, the Robert A. Welch Foundation, and grants 3652-233 and 3652-272 from the Texas Advanced Research Program. We thank Schering-Plough for providing us with unpublished sequence data.

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