Synthesis and Testing of Heterocyclic Analogues of Diaminopimelic Acid (DAP) as Inhibitors of DAP Dehydrogenase and DAP Epimerase

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Abstract: Substrate analogues were synthesized and examined as inhibitors of diaminopimelic acid (DAP) dehydrogenase from *Bacillus sphaericus* and of DAP epimerase from *Escherichia coli*. These enzymes produce *meso*-DAP (**3**) (a precursor for L-lysine and for peptidoglycan) from L-tetrahydrodipicolinic acid (**1**) and LL-DAP (**2**), respectively. The epimerase was purified by an improved procedure and confirmed to require both carboxyl and both amino groups for substrate recognition using deuterium-exchange experiments with DAP isomers, L-lysine, D-lysine, L-α-aminopimelate, and racemic α-aminopimelate. An imidazole analogue of DAP, (2*S*)-2-amino-3-(4-carboxyimidazol-1-yl)propanoic acid (**4**), was synthesized by condensation of benzyl imidazole-4-carboxylate (**8**) with *N*-benzyloxycarbonyl(Cbz)-L-serine β-lactone (**9**) (product structure confirmed by X-ray analysis) followed by hydrogenolytic deprotection. Two other analogues, (2*S*,5′*R*)-2-amino-3-(3-carboxy-2-isoxazolin-5-yl)propanoic acid (**5**) and its 5′*S* diastereomer **6**, were prepared by condensation of methyl *N*-Cbz-L-allylglycinate (**13**) with methyl chlorooximidoacetate (**14**) followed by separation of isomers and deprotection with NaOH and Me_3SiCl/NaI. Similar condensation of ethyl chlorooximidoacetate with ethylene and of **14** with ethyl acrylate generated isoxazolines, which were saponified to 2-isoxazoline-3-carboxylic acid (**25**) and 2-isoxazoline-3,5-dicarboxylic acid (**26**), respectively. None of the compounds show significant inhibition of DAP epimerase or DAP dehydrogenase with the exception of **6**, which is a potent and specific inhibitor of DAP dehydrogenase. At pH 7.5 or 7.8, compound **6** shows competitive inhibition (K_i = 4.2 μM) with tetrahydrodipicolinic acid (**1**) for the forward reaction and noncompetive inhibition (K_i = 23 μM) with *meso*-DAP (**3**) for the reverse process. Preliminary tests for antimicrobial activity demonstrate that **6** inhibits the growth of *B. sphaericus*, which relies exclusively on DAP dehydrogenase to produce **3**.

Introduction

The diaminopimelic acid (DAP) pathway[1] to L-lysine in bacteria (Figure 1) and higher plants continues to attract interest from researchers in diverse areas. Since mammals lack this metabolic route and require L-lysine in their diet, inhibitors of the enzymes in this path possess potential as antibiotics or herbicides.[2] *meso*-DAP (**3**) is also a key constituent of the peptidoglycan cell wall layer[3] in Gram-negative bacteria and in physiologically active fragments (e.g. bacterial toxins, immunoadjuvants, sleep-inducing factors, antitumor agents) derived from it.[4] Hence considerable effort has focused on synthesis of DAP isomers[2,4b,5] and their analogues,[6] as well as on the function and genetic organization of DAP-related enzymes.[1a,7]

Among the most mechanistically interesting enzymes is DAP epimerase[8] (EC 5.1.1.7), which interconverts LL-DAP (**2**) and *meso*-DAP (**3**) (but not DD-DAP) without the aid of cofactors, metal ions, or reducible imine or keto functionalities. This unusual transformation, which is analogous to those catalyzed by proline racemase,[9] hydroxyproline racemase,[10] glutamate racemase,[11] and aspartate racemase,[12] appears to proceed via an intermediate having anionic character at the α-carbon.[6a] However, certain

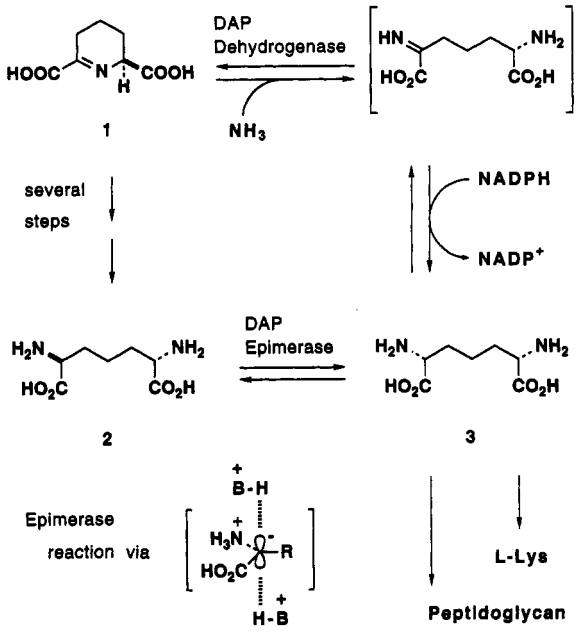

Figure 1. Portion of the DAP pathway showing the formation of *meso*-DAP (**3**) from THDP (**1**) by DAP dehydrogenase and from LL-DAP (**2**) by DAP epimerase. Possible intermediates for the transformations are indicated in brackets.

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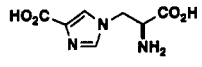
bacteria possess an alternative pathway for *meso*-DAP production and can transform an early precursor, tetrahydrodipicolinic acid (THDP) (**1**) directly to **3** using DAP dehydrogenase (EC 1.4.1.16).[13] Like other amino acid dehydrogenases (e.g. L-glutamate dehydrogenase[14]), this NADPH-dependent dehydrogenase reduces a primary imine functionality, which in this case probably results from cleavage of the THDP ring by transimination with ammonia. However, the stereochemistry of this reduction is opposite to the usual one and generates a D amino acid stereogenic center. Although their mechanisms and starting substrates are different, the epimerase and dehydrogenase share certain common features. Both enzymes produce *meso*-DAP (**3**), have an absolute requirement for an L amino acid center at the distal (nonreacting) site, and may proceed through intermediates with a planar α-carbon at the site involved in generation of the D center. These considerations suggest that conformationally restricted transition-state analogues having the DAP skeleton and a planar α-carbon bearing a basic nitrogen could be potent inhibitors of these enzymes. In the present study we describe the syntheses of a series of heterocyclic derivatives (including **4**, **5**, and **6**), their interaction with DAP dehydrogenase and epimerase, and a preliminary examination of their antibiotic activity.



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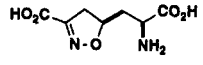

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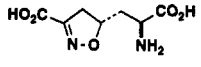

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Results and Discussion

Syntheses of DAP Analogues. The imidazole derivative **4** was synthesized as outlined in Figure 2 using nucleophilic ring opening of *N*-benzyloxycarbonyl(Cbz)-L-serine β-lactone (**9**) as a key step.[15] Conversion of imidazole-4-carboxylic acid (**7**)[16] to it benzyl ester (**8**) (42% yield) gives the required nucleophile. The ring

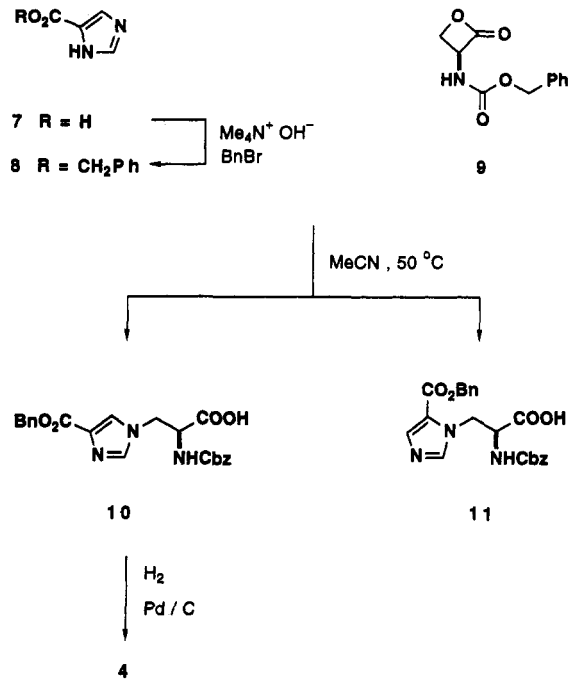

Figure 2. Synthesis of imidazole-containing DAP analogue **4**.

opening proceeds only in low yield because of poor regiospecificity with both nitrogens of the imidazole attacking the β-lactone to form **11** (18%) as well as **10** (11%),[17] but the route affords rapid access to the desired compound. Structural assignment of the regioisomers was confirmed by X-ray crystallographic analysis of **10** (see supplementary material). Hydrogenolytic deprotection of **10** produces the desired DAP analogue **4** in 81% yield.

To generate the framework of the isoxazoline analogues **5** and **6**, L-allylglycine (**12**) was protected as its *N*-Cbz methyl ester (**13**) and condensed with the nitrile oxide generated *in situ* by base treatment of methyl chlorooximidoacetate (**14**)[18] (Figure 3). This dipolar cycloaddition process generates primarily the expected[18b] diastereomers **17** (33%) and **18** (36%) along with small amounts (ca. 2%) of the regioisomers **15** and **16**. Direct stereochemical assignment at C-5 of the isoxazoline ring of **17** and **18** was not possible at this stage, so the separated compounds were individually saponified to the corresponding diacids **19** and **20** in quantitative yield. Vacuum pyrolysis of **19** and **20** induces the known thermal decarboxylation reaction of 2-isoxazoline-3-carboxylic acids[19] to generate corresponding cyano alcohols which cyclize spontaneously under the conditions to form the diastereomeric cyano lactones **21** (46%) and **22** (56%), respectively (Figure 4). Difference NOE experiments, supported by the values of coupling constants, elucidated the stereochemistry of the lactone substituents as depicted. Thus, irradiation at H-2 of the *trans* isomer **21** leads to enhancement of the resonances for H-3 and H-5, whereas irradiation of H-2 of the *cis* isomer **22** enhances the signals for the *pro-S* hydrogen at C-3 and for H-4.[20] The corresponding expected NOE effects are observed upon irradiation

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(17) It is likely that some attack by one or both of the imidazole nitrogens also proceeds on the carbonyl of the β-lactone, but the resulting imidazole amides hydrolyze very readily to the corresponding carboxylic acid (i.e. *N*-Cbz-L-serine) upon aqueous treatment of the reaction mixture.

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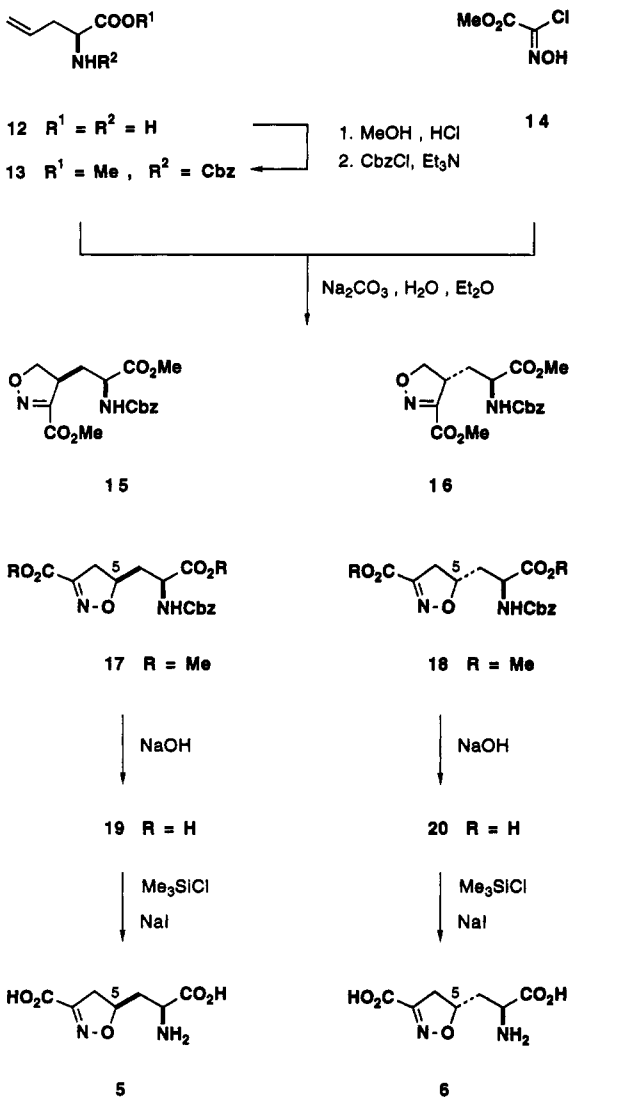

Figure 3. Synthesis of isoxazoline-containing DAP analogues **5** and **6**.

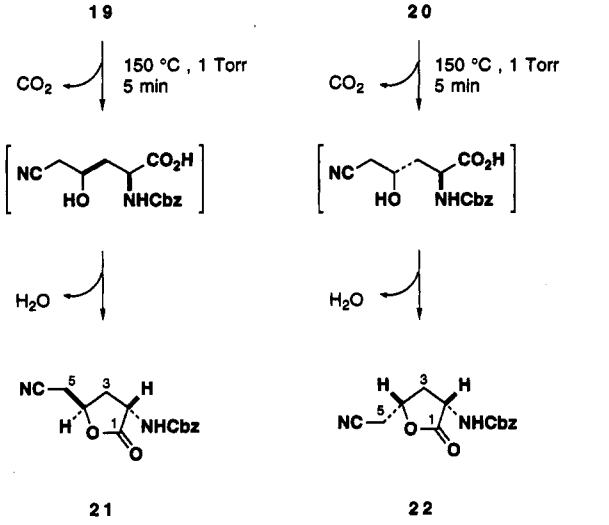

Figure 4. Conversion of **19** and **20** to lactones for stereochemical analysis.

of H-4 of **21** and **22**, thereby allowing unambiguous assignment of the stereochemistry of **19** and **20** (as well as of **17** and **18**) as 5*R* and 5*S*, respectively. Deprotection of the diacids **19** and **20** using trimethylsilyl chloride and sodium iodide affords the corresponding desired DAP analogues **5** and **6** (93% yield) (Figure 3).

Inhibition Studies with DAP Epimerase. Our earlier work on the interaction of DAP epimerase with pure β-fluorodiaminopimelate isomers suggests that the enzyme active site holds the flexible substrates quite rigidly and that it recognizes and binds the amino and carboxyl groups at both ends of the substrate.[6a] To confirm the latter idea, the epimerase was first purified from an overproducing strain of *Escherichia coli* using an improved procedure. Preliminary ammonium sulfate fractionation was followed by Matrex Green (Amicon) dye affinity column chromatography; the latter process removes over 90% of the contaminating proteins in a single step. Only a single dominant band could be seen by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Subsequent gel filtration MPLC on Q-Sepharose gives nearly pure enzyme. The measured K_m^{app} of 0.24 mM for *meso*-DAP (**3**) with this epimerase sample is in excellent agreement with the literature values of 0.16 and 0.26 mM.[8,21] Incubation of L-lysine, D-lysine, L-α-aminopimelate, and DL-α-aminopimelate with the dye-purified epimerase in deuterated buffer under conditions in which a statistical mixture of DAP isomers undergoes complete exchange[22] at the α-positions gave no incorporation (by NMR spectrometry) of deuterium at the α- or ε-carbons. These compounds also display very weak, if any, inhibition of the epimerase ($K_i \geq 2.9$ mM). This confirms that both carboxyls and both amino groups are required for substrate recognition by this enzyme.

Since pyrrole-2-carboxylic acid and 2-pyrroline-2-carboxylic acid strongly inhibit proline racemase,[9a,23] an enzyme whose mechanistic characteristics are similar to those of DAP epimerase, it seemed that **4**, **5**, and **6** could act as transition-state analogues and be potent inhibitors. However, these compounds are only very weak inhibitors of this enzyme (competitive inhibition: 18% at 1 mM for **4** and **5**; 4% at 0.5 mM for **6**). This may be due to steric interference caused by the extra substituent at the central carbon[24] or alternatively may reflect conformational requirements for recognition of the flexible DAP substrate, which cannot be accommodated with the restriction imposed by the five-membered ring. For example, if the facile elimination of HF from (2*R*,3*R*,6*S*)-3-fluoro-DAP (an LL-DAP analogue[25]) catalyzed by the epimerase occurs in *syn* coplanar arrangement to generate an *E* double bond rather than in *anti* periplanar arrangement to give a *Z* olefin,[6a] the required conformation is unattainable by compounds **4**, **5**, and **6**. Ongoing studies of DAP epimerase structure and mechanism may clarify the reasons for lack of effective binding by these heterocyclic DAP analogues.

Inhibition Studies with DAP Dehydrogenase. In order to examine the effects of the heterocyclic derivatives on DAP dehydrogenase, the enzyme was isolated and purified by literature procedures[13a] from *Bacillus sphaericus* IFO 3525. At neutral or slightly basic conditions (e.g. pH 7.8) this dehydrogenase normally catalyzes the reductive amination of THDP (**1**) to *meso*-DAP (**3**) with concomitant conversion of NADPH to $NADP^+$, which allows continuous spectrophotometric assay at 340 nm. In

(20) See supplementary material. Homonuclear decoupling experiments were employed to assist spectral assignment. The resonances of the diastereotopic hydrogens at H-3 of **21** were overlapped, as were the signals for the two hydrogens at H-5.

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(22) The epimerase eventually exchanges all of the α-hydrogens of both the meso and LL isomers of DAP with deuterated solvent, even though in any single transformation the distal (nonexchanging) site must always be L. The α-hydrogens of the DD isomer are not exchanged at all, and hence the final mixture consists of 75% of species having two deuteriums at the α-carbons and 25% of species (DD-DAP) having only hydrogens at those sites.

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(24) At least one isomer of *N*-hydroxy-DAP is a potent inhibitor ($K_i \leq 5.6$ μM) of DAP epimerase, but 3-methylene-DAP is a weak noncompetitive inhibitor ($K_i' \approx 1$ mM); see ref 21.

(25) The presence of fluorine at the β-position changes the priority of groups in nomenclature, and hence the fluorinated "LL isomer" has the 2*R*,6*S* configuration whereas the parent LL-DAP is 2*S*,6*S*.

contrast, pH 10.5 is the optimum for the reverse reaction, oxidative deamination of 3.[13a] However, the oxidative deamination reaction can also be forced to proceed at pH 7.8 by providing the enzyme with $NADP^+$ and *meso*-DAP in the absence of added ammonium ions. The substrate, tetrahydrodipicolinic acid (1), could be prepared only as a mixture of imine and enamine isomers together with the open form (α-keto-ε-aminopimelate)[26] using enzymatic oxidation of 3 with DAP dehydrogenase followed by purification.[27] Addition of the heterocyclic DAP analogues showed that neither the imidazole derivative 4 nor the isoxazoline compound 5 display significant inhibition of the DAP dehydrogenase reaction at millimolar concentrations (≤7% and 13%, respectively, at 1 mM). In contrast, the isomeric isoxazoline 6 is a potent inhibitor of the dehydrogenase with an inhibition constant (K_i) of 23 μM for the oxidative deamination reaction at pH 7.8. No time-dependent behavior could be detected. To the best of our knowledge, this is the most powerful substrate-based inhibitor reported for DAP dehydrogenase thus far.

Initially the inhibition by 6 would seem explicable based on its structural similarity to *meso*-DAP (3) and to the presumed imine intermediate in the normal reaction (Figure 1). However, more detailed kinetic studies quickly showed that at pH 7.8 isoxazoline 6 is a *noncompetitive* inhibitor with respect to 3 and *uncompetitive* with respect to $NADP^+$ (K_{ii} = 9.2 μM) for the oxidative deamination (reverse) reaction. Surprisingly, it is a simple *competitive* inhibitor of tetrahydrodipicolinic acid (1) (K_i = 4.2 μM) for the reductive amination (forward) reaction.[28] Thus, at pH 7.8 isoxazoline 6 binds at the same site(s) as 1 but at a different site than *meso*-DAP (3). The uncompetitive inhibition with $NADP^+$ demonstrates that 6 does not compete for its site on the enzyme. This latter result is expected, since earlier work by Misono and Soda[13a] had shown the enzyme mechanism is ordered binary–ternary, with $NADP^+$ binding first in the oxidative deamination reaction, followed by 3, and with NADPH being the last product released. In the reductive amination reaction, 1 binds to the enzyme after NADPH but before ammonia.[13a] Interestingly, inhibition by isoxazoline 6 decreases dramatically at the more basic conditions which are optimal for the reverse reaction (i.e. oxidative deamination of 3 to 1); at pH 10.5 the IC_{50} is only 4.1 mM. Tetrahydrodipicolinic acid (1) itself shows noncompetitive product inhibition of DAP dehydrogenase at pH 10.5,[13a] but this is not detectable at pH 7.8. Thus, at neutral pH isoxazoline 6 strongly retards both the normal forward reaction (1 → 3) and the reverse reaction (3 → 1), but it has little effect at high pH. One explanation for these unusual results may be that, like glutamate dehydrogenases,[14,29] DAP dehydrogenase possesses one or more allosteric sites and that THDP (1) and isoxazoline 6 compete with each other to bind at those regulatory locations. Change of conformation or ionization states of enzyme groups in this allosteric site may inhibit binding of 6 at high pH. Although the active form of DAP dehydrogenase from *B. sphaericus* is known to be a dimer of two identical subunits (each of *ca.* 40 000 Da),[13a] the three-dimensional structure of the enzyme has not been reported and the nature and location of the postulated allosteric site(s) are unknown.[30] In addition, the possibility exists that isoxazoline 6 does bind in the dehydrogenase active site and that some proportion of the intercept effect indicating noncompetitive inhibition in the Michaelis–Menten plots for the oxidative deamination of 3 may result from binding of the potent inhibitor to appreciable steady-state concentrations of enzyme–NADPH complex which may be generated by the reaction.

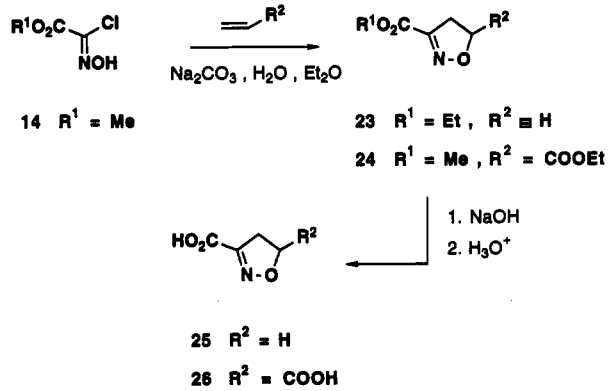

Figure 5. Synthesis of isoxazolines 25 and 26.

Additional heterocycles were synthesized to determine whether the functionalized amino acid side chain of 6 was important for binding or whether the isoxazoline moiety alone was a sufficient mimic of the THDP ring system and simple steric interactions account for the lack of inhibition by the diastereomeric isoxazoline 5. Compounds 23 (77% yield) and 24 (45% yield) are readily accessible by condensation of ethyl chlorooximidoacetate[18b] with ethylene and reaction of 14 with ethyl acrylate, respectively (Figure 5). Saponification of these esters affords 25 (90%) and racemic 26 (80%), both of which have no significant effect (at 7.75 mM: 7.5% and <2% inhibition, respectively) on DAP dehydrogenase. The isoxazoline-3,5-dicarboxylic acid (26) can also be viewed as a glutamic acid analogue, but preliminary tests with commercially available glutamate dehydrogenase (EC 1.4.1.4), a specific $NADP^+$-dependent enzyme from *Proteus* sp., showed only modest inhibition (IC_{50} = 0.64 mM). Compound 6 is an even weaker inhibitor (16% at 1 mM) of glutamate dehydrogenase. Hence, it is clear that the side chain of 6 makes it a specific inhibitor for DAP dehydrogenase and that its correct stereochemical attachment is required for the potent binding to this enzyme.

Antibacterial Activity of 6. In order to test in a preliminary manner whether isoxazoline 6 has a detectable effect on bacterial cells which use the DAP dehydrogenase route for manufacture of *meso*-DAP (3), three organisms were chosen. *B. sphaericus* IFO 3525 (Gram-positive) lacks DAP epimerase entirely and employs exclusively the DAP dehydrogenase pathway to make 3.[13b,31] In contrast, *Cornybacterium glutamicum* A5019 PR56 (Gram-positive) utilizes both the dehydrogenase and the epimerase pathways.[32] The Gram-negative bacterium *Proteus vulgaris* X-19-0 also has DAP dehydrogenase,[13c] but it is quite resistant to both tetracycline and ampicillin at 10 mM concentration. As might be expected, compound 6 inhibits the growth of *B. sphaericus*, which depends exclusively on the dehydrogenase for *meso*-DAP. However, a 100-fold higher concentration of 6 is necessary to have an antimicrobial effect equal to that of tetracycline or ampicillin. No antibacterial effect could be detected with *C. glutamicum* or with *P. vulgaris* at reasonable concentrations. These very preliminary experiments suggest that Gram-positive organisms which rely exclusively on the DAP

(26) The chemical synthesis of the potassium salt of 1 as a mixture of imine, enamine, and open form (α-keto-ε-aminopimelate) has recently been reported: Couper, L.; Robins, D. J.; Chrystal, E. J. T. *Tetrahedron Lett.* **1992**, *33*, 2717–2720.

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(28) The K_m^{app} of $NADP^+$ at pH 7.8 was determined as 8.2 μM, since only a value at pH 10.5 (83 μM) is available in the literature.[13a,27] Using the literature assay procedure,[13a,27] the K_m^{app} for 1 was determined as 0.12 mM, in reasonable agreement with the literature value 0.24 mM.[27] The literature values of Michaelis constants for NADPH and NH_3 are 0.2 and 12.5 mM, respectively.[13a]

(29) Stevenson, R. M.; LéJohn, H. B. *J. Biol. Chem.* **1971**, *246*, 2127–2135.

(30) DAP dehydrogenase from *Cornybacterium glutamicum* has been cloned, sequenced, and overexpressed; see ref 7c and also: Ishino, S.; Mizukami, T.; Yamaguchi, K.; Katsumata, R.; Araki, K. *Nucleic Acids Res.* **1987**, *15*, 3917.

(31) White, P. J. *J. Gen. Microbiol.* **1983**, *129*, 739–749.

(32) Ishino, S.; Yamaguchi, K.; Shirahata, K.; Araki, K. *Agric. Biol. Chem.* **1984**, *48*, 2557–2560.

(33) Gram-negative bacteria generally use *meso*-DAP (3) in their peptidoglycan, whereas Gram-positive organisms frequently employ L-lysine (derived by decarboxylation of 3).[3]

dehydrogenase for production of *meso*-DAP (**3**) for formation of L-lysine, and ultimately peptidoglycan,[33] may be the most vulnerable to inhibitors such as **6**.

Conclusions

The present work describes an efficient synthesis of optically pure isoxazoline **6** and its action as a potent and selective inhibitor of DAP dehydrogenase. Although it is noncompetitive with *meso*-DAP (**3**) at neutral pH, it does compete directly with the key precursor THDP (**1**). This suggests binding of **6** at an allosteric regulatory site, although some interaction at the active site may occur. Experiments with other isoxazolines demonstrate that the amino acid side chain and the stereochemistry of its attachment are critical for this effect. The inability of **6** to replace DAP in the active site of DAP epimerase and its behavior with DAP dehydrogenase reaffirms the concept that enzymes in the DAP pathway have evolved exquisite specificity and can accommodate little variation in substrate (or inhibitor) structure. Deuterium incorporation studies with DAP epimerase on modified DAP analogues further confirm that both amino and both carboxyl groups are required in correct stereochemical arrangement, even at the distal site. Preliminary examination of antimicrobial activity shows that **6** can inhibit growth of *B. sphaericus*, which relies exclusively on DAP dehydrogenase for production of the critical metabolite **3**. Current studies on the interaction of heterocyclic DAP analogues with other enzymes in the pathway to L-lysine, as well as on the route to peptidoglycan, will be reported in the future.

Experimental Section

General Methods. Most general experimental procedures and instrumentation have been described previously except as noted below.[15a,21] All reagents were purchased from Sigma or Aldrich and were used without further purification unless otherwise stated. Matrex Green affinity chromatography dye was purchased from Amicon (W. R. Grace Inc., Danzers, MA), and DEAE cellulose was purchased from Whatman. All other ion-exchange chromatography and electrophoresis reagents were obtained from BioRad. All solvents were dried and distilled prior to use according to standard procedures.[34] Proton NMR spectra were obtained on Bruker WH-200, WM-360, AM-400, and Varian Unity 500 spectrometers. ^{13}C NMR spectra were measured on Bruker WH-200 and AM-400 instruments. Mass spectral analysis employed Kratos A.E.I. MS50, MS12, or MS9 spectrometers for EI, CI, and FAB respectively. The term Cleland, used as a matrix in FAB MS, refers to a 5:1 mixture of dithiothreitol and dithioerythritol. Infrared spectra were measured on NICOLET 7199 or 205X Fourier transform spectrometers. Elemental analyses were performed on Carlo Erba 1108 and Perkin Elmer 240B elemental analyzers. Optical rotations were measured at the sodium D line on a Perkin Elmer 241 polarimeter using a 10-cm cell. Reverse-phase HPLC separations were done on a Bio-Rad Model 400 system using the following conditions unless otherwise specified: solid phase—C_{18} Microbondapak 10 μm, two 8 mm × 10 cm columns in tandem; mobile phase—solvent A = 0.1% aqueous TFA, solvent B = 0.1% TFA in 70% aqueous MeCN. Enzyme activity units for DAP dehydrogenase and DAP epimerase are defined such that 1 unit (U) of enzyme will turnover 1 μmol of substrate per minute under the assay conditions. Results of enzyme inhibition studies (run in triplicate) were calculated by computer using *Enzyme Kinetics v1.11* from Trinity Software or the *Enzfitter* program (Elsevier Biosoft, Cambridge, U.K.) using Michaelis–Menten kinetics and nonlinear regression analysis.

Enzyme Substrates: THDP (1), LL-DAP (2), and *meso*-DAP (3). LL-DAP (**2**) and *meso*-DAP (**3**) were first purified from the commercially available statistical mixture of isomers by literature procedures (isomeric purity: 75% for **2** with no detectable **3**; ca. 80–90% for **3**).[35] Pure *meso*-DAP (**3**) (isomeric purity 97–98%) was prepared by the method of Kolodziejczyk *et al.*[4b] Stereochemical purity was determined by literature procedure using bis Marfey derivatives.[36] THDP (**1**) was generated enzymatically from **3** and partially purifed to a mixture of imine, enamine, and open forms (20% by mass as determined by ^1H NMR) according to previously described methods.[13a]

(*S*)-2-Amino-3-(4-carboxy-1-imidazolyl)propanoic Acid (4). A solution of **10** (21 mg, 50 μmol) in MeOH (1 mL) was treated with Pd/C (10%, 4 mg), thoroughly degassed, and stirred under 1 atm of H_2 for 1 h. The mixture was filtered through Celite, concentrated *in vacuo*, and purified by reverse-phase HPLC (100% A, then up to 40% B) to give **4** as a white solid (8.0 mg, 81%) (retention time 4.1 min, retention coefficient 1.05): IR (KBr) 3590–3220, 1675, 1204, 1140 cm^{-1}; ^1H NMR (D_2O, 360 MHz) δ 4.46 (t, 1 H, *J* = 5.5 Hz, H-2), 4.86 (d, 2 H, *J* = 5.5 Hz, C(3)H_2), 8.08 (br s, 1 H, H-5′), 8.92 (br s, 1 H, H-2′); FAB MS (HCOOH/glycerol) *m/z* 200.13 (9%, MH^+).

(2*S*,5′*R*)-2-Amino-3-(3-carboxy-2-isoxazolin-5-yl)propanoic Acid (5). A solution of diacid **19** (84 mg, 0.25 mmol) and TMSCl (128 μL, 1.00 mmol) in dry MeCN (1 mL) under argon was treated with NaI (152 mg, 1.00 mmol), and the mixture was stirred at 20 °C for 90 min. Water (5 mL) was added, and the mixture was washed with CH_2Cl_2 (equal volume) and then freeze-dried. Purification by flash chromatography (SiO_2; 7:3 *i*-PrOH/concentrated NH_3) gave **5** as a white solid (20 mg, 40%): mp dec to solid foam at 110–115 °C; foam dec at 180–184 °C; $[\alpha]^{20}_D$ = +98.0° (*c* 0.80 H_2O); IR (KBr) 3600–3300, 3250–2500, 1620 cm^{-1}; ^1H NMR (D_2O, 400 MHz) δ 2.17 (A of ABXY, 1 H, *J* = 15.0, 8.0, 2.5 Hz, 1 H of C(1′)H_2), 2.30 (B of ABXY, 1 H, *J* = 15.0, 5.5, 3.0 Hz, 1 H of C(1′)H_2), 2.98 (A of ABX, 1 H, *J* = 18.0, 7.0 Hz, 1 H of C(4)H_2), 3.45 (B of ABX, 1 H, *J* = 18.0, 10.5 Hz, 1 H of C(4)H_2), 4.04 (dd, 1 H, *J* = 8.0, 5.5 Hz, H-2′), 5.06–5.14 (m, 1 H, H-5); FAB MS (Cleland) *m/z* 203.06 (74%, MH^+).

(2*S*,5′*S*)-2-Amino-3-(3-carboxy-2-isoxazolin-5-yl)propanoic Acid (6). The procedure used to transform **19** to **5** was employed to convert diacid **20** (85 mg, 0.25 mmol) to **6** as a white solid (48 mg, 93%): mp 134–137 °C dec to residue with mp > 250 °C; $[\alpha]^{20}_D$ = −38° (*c* 1.09 H_2O); IR (KBr) 3560–3300, 3250–2840, 1630, 1402 cm^{-1}; ^1H NMR (D_2O, 400 MHz) δ 2.20 (A of ABXY, 1 H, *J* = 15.5, 6.5, 4.0 Hz, 1 H of C(1′)H_2), 2.27 (B of ABXY, 1 H, *J* = 15.5, 9.5, 4.0 Hz, 1 H of C(1′)H_2), 2.97 (A of ABX, 1 H, *J* = 18.0, 7.0 Hz, 1 H of C(4)H_2), 3.43 (B of ABX, 1 H, *J* = 18.0, 10.5 Hz, 1 H of C(4)H_2), 3.95 (dd, 1 H, *J* = 6.5, 4.0 Hz, H-2′), 4.92 (dddd, 1 H, *J* = 10.5, 9.5, 7.0, 4.0 Hz, H-5); FAB MS (Cleland) *m/z* 203.11 (29%, MH^+).

Benzyl Imidazole-4-carboxylate (8). A solution of imidazolecarboxylic acid (**7**)[16] (224 mg, 2.00 mmol) and $Me_4NOH{\cdot}5H_2O$ (363 mg, 2.00 mmol) in DMF (5 mL) was treated with benzyl bromide (240 μL, 2.02 mmol) and was stirred at 20 °C for 16 h. It was then partitioned between H_2O and EtOAc. The aqueous phase was extracted with EtOAc, and the combined organic layers were dried and evaporated *in vacuo*. Purification by flash chromatography (SiO_2; EtOAc/AcOH 95:5) gave **8** as a white solid (168 mg, 42%): mp 159–163 °C; IR (KBr) 3600–3320, 1723, 1162 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.61 (br s, 1 H, NH), 5.32 (s, 2 H, CH_2), 7.29–7.41 (m, 5 H, Ph), 7.70 (s, 1 H, CH), 7.74 (s, 1 H, CH); CI-MS (NH_3) *m/z* 203 (100%, MH^+).

(*S*)-2-(*N*-(Benzyloxycarbonyl)amino)-3-(4-benzyloxycarbonyl-1-imidazolyl)propanoic Acid (10) and (*S*)-2-(*N*-(Benzyloxycarbonyl)amino)-3-(5-benzyloxycarbonyl-1-imidazolyl)propanoic Acid (11). A solution of **8** (101 mg, 0.50 mmol) and *N*-Cbz-L-serine β-lactone (**9**)[15] (221 mg, 1.00 mmol) in dry MeCN (5 mL) was stirred at 50 °C under argon for 17 h. The solvent was evaporated *in vacuo*, and the residue was purified by reverse-phase HPLC (eluting with 45% B to 100% B) to give **10** (23 mg, 11%) (retention time 22.9 min, retention coefficient 4.6) and **11** (38 mg, 18%) (retention time 27.6 min, retention coefficient 6.0) as solids. The structure of **10** was confirmed by X-ray crystallographic analysis (see supplementary material). For **10**: mp 165–171 °C; IR (MeOH cast) 3570–3090, 1714 cm^{-1}; ^1H NMR (acetone-d_6, 200 MHz) δ 4.51–4.68 (m, 1 H, H-2), 4.71–4.85 (m, 2 H, H-3), 5.04 (s, 2 H, CH_2Ph), 5.32 (s, 2 H, CH_2Ph), 6.95 (d, 1 H, *J* = 7.5 Hz, NH), 7.27–7.50 (m, 10 H, 2 × Ph), 7.73 (s, 1 H, H-5′), 7.90 (s, 1 H, H-2′); FAB MS (Cleland) *m/z* 446.23 (27%, MNa^+), 424.24 (100%, MH^+).

For **11**: mp 147 °C; IR (MeOH cast) 3520–3250, 1723 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) 4.67–4.73 (m, 1 H, H-2), 4.85–4.92 (m, 1 H, 1 H of C(3)H_2), 5.00 (s, 2 H, CH_2Ph), 5.17–5.23 (m, 1 H, 1 H of C(3)H_2), 5.38 (s, 2 H, CH_2Ph), 6.89 (d, 1 H, *J* = 8.5 Hz, NH), 7.28–7.49 (m, 10 H, 2 × Ph), 8.04 and 8.46 (2 × br s, 2 H, H-2′, H-4′); FAB MS (Cleland) *m/z* 446.23 (19%, MNa^+), 424.26 (100%, MH^+).

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Methyl (*S*)-2-(*N*-(Benzyloxycarbonyl)amino)-4-pentenoate (13). A suspension of (*S*)-2-amino-4-pentenoic acid (L-allylglycine) (1.00 g, 8.7 mmol) in methanol was converted to its methyl ester hydrochloride using HCl gas by the standard procedure.[37] Evaporation of solvent and excess HCl *in vacuo* yielded pure methyl (*S*)-2-amino-4-pentenoate as a white crystalline solid (1.38 g, 96%): mp 77.5–79 °C (lit[38] mp 73–74 °C for racemate); $[\alpha]^{20}_D$ = +6.4° (*c* 1.05 MeOH); IR (KBr) 3650–3300, 1748 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.58–2.82 (m, 2 H, H-3), 3.83 (s, 3 H, OMe), 4.20–4.32 (m, 1 H, H-2), 5.21–5.34 (m, 2 H, CH=C*H*$_2$), 5.79 (ddt, 1 H, *J* = 17.0, 10.0, 7.0 Hz, C*H*=CH$_2$), 8.52 (br s, 3 H, NH$_3^+$); ^{13}C NMR (CDCl$_3$, 100.6 MHz) δ 35.59 (*C*H$_2$C=CH$_2$), 53.52 (OMe), 53.68 (C$_\alpha$), 121.57 (CH=*C*H$_2$), 131.66 (*C*H=CH$_2$), 170.31 (*C*O$_2$Me); CI MS *m/z* (NH$_3$) 130 (100%, [M − Cl]$^+$), 88 (30%, [H-NCHCO$_2$Me]$^+$), 70 (44%). Anal. Calcd for C$_6$H$_{12}$ClNO$_2$: C, 43.51; H, 7.30; Cl, 21.41; N, 8.46. Found: C, 43.23; H, 7.49; Cl, 21.32; N, 8.29.

A literature procedure for *N*-CBz protection was adapted to convert the ester to **13**.[39] A cooled (−5 °C) solution of methyl (*S*)-2-amino-4-pentenoate hydrochloride (331 mg, 2.00 mmol), 4-(*N*,*N*-dimethylamino)-pyridine (1 crystal), and Et$_3$N (560 μL, 4.02 mmol) in CHCl$_3$ (7 mL) under argon was treated dropwise (5 min) with CBzCl (314 μL, 2.20 mmol). The mixture was then allowed to warm to 20 °C and was stirred for 19 h. Excess CBzCl was removed by washing with 10% aqueous citric acid (10 mL), and the organic phase was diluted with CH$_2$Cl$_2$ (20 mL). It was washed with saturated NaHCO$_3$ (10 mL) and saturated NaCl (10 mL), dried, and evaporated *in vacuo*. The crude product was purified by flash chromatography (SiO$_2$; gradient elution CH$_2$Cl$_2$ to 5% EtOAc in CH$_2$Cl$_2$) to give **13** as a colorless oil (239 mg, 45%): bp 120–130 °C at 2.5 Torr; $[\alpha]^{20}_D$ = +15.3° (*c* 1.63 CHCl$_3$); IR (CHCl$_3$ cast) 3450–3300, 1724 cm^{-1}; ^{1}H NMR (200 MHz, CDCl$_3$) δ 2.38–2.62 (m, 2 H, NCHC*H*$_2$), 3.68 (s, 3 H, OMe), 4.42 (ddd, 1 H, *J* = 7.5, 7.5, 7.5 Hz, H$_\alpha$), 5.03–5.13 (m, 4 H, CH=C*H*$_2$ + PhC*H*$_2$), 5.53–5.77 (m, 2 H, NH, C*H*=CH$_2$), 7.28–7.33 (m, 5 H, Ph); ^{13}C NMR (100.6 MHz, CDCl$_3$) δ 36.46 (C$_\alpha$*C*H$_2$), 52.13 (Me), 53.17 (C$_\alpha$), 66.76 (Ph*C*H$_2$), 119.07 (CH=*C*H$_2$), 127.90, 127.96 and 128.31 (aromatic CH's), 131.96 (*C*H=CH$_2$), 136.11 (quaternary aromatic C), 155.60 (OCON), 172.03 (*C*O$_2$Me); exact mass 263.1158 (6%, M$^+$) (263.1158 calcd for C$_{14}$H$_{17}$NO$_4$). Anal. Calcd for C$_{14}$H$_{17}$NO$_4$: C, 63.87; H, 6.51; N, 5.32%. Found: C, 63.54; H, 6.38; N, 5.39.

Methyl Chlorooximidoacetate (14). The literature procedure for the ethyl ester[18b] was adapted. A cooled (0 °C) solution of methyl glycinate hydrochloride (2.00 g, 16 mmol) in MeOH (10 mL) was treated with concentrated HCl and then dropwise (10 min) with an aqueous solution (5 mL) of NaNO$_2$ (1.10 g, 16 mmol). The resulting solution was stirred at 0 °C for 10 min and then treated with a second equivalent each of concentrated HCl and aqueous NaNO$_2$. After 45 min at 0 °C, the mixture was treated with saturated aqueous NaCl (25 mL) and then extracted with Et$_2$O (3 × equal volume). The combined organic extracts were dried and evaporated. The solid residue was recrystallized from Et$_2$O/pentane to give **14** as colorless prisms (1.31 g, 60%): mp 61–63 °C (lit[18a] mp 57–60 °C); IR (KBr) 3500–3150, 1725, 1605, 1302, 1077, 1038 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 3.90 (s, 3 H, Me), 10.27 (s, 1 H, NOH); ^{13}C NMR (CDCl$_3$, 50.3 MHz) δ 54.01 and 54.35 (Me of *Z* and *E* oximes), 132.32 (C=N), 159.40 (CO); exact mass 136.9878 and 138.9850 (73% and 23%, M$^+$) (136.9880 calcd for C$_3$H$_4$^{35}ClNO$_3$, 138.9850 calcd for C$_3$H$_4$^{37}ClNO$_3$). Anal. Calcd for C$_3$H$_4$ClNO$_3$: C, 26.20; H, 2.93; Cl, 25.78; N, 10.18. Found: C, 26.30; H, 2.72; Cl, 25.65; N, 9.97.

Formation of Methyl (2*S*,4*S*)-2-(*N*-(Benzyloxycarbonyl)amino)-3-(3-(methoxycarbonyl)-2-isoxazolin-4-yl)propanoate (15), Its 2*S*,4*R* Isomer (16), Methyl (2*S*,5*R*)-2-(*N*-(Benzyloxycarbonyl)amino)-3-(3-(methoxycarbonyl)-2-isoxazolin-5-yl)propanoate (17), and Its 2*S*,5*S* Isomer (18). A solution of **13** (500 mg, 1.90 mmol) and **14** (780 mg, 5.68 mmol) in Et$_2$O (5 mL) was treated dropwise (3 h, syringe pump) with a solution of Na$_2$CO$_3$ (603 mg, 5.69 mmol) in H$_2$O (4 mL) with vigorous stirring. The mixture was then stirred at 20 °C for a further 4 h. It was diluted with Et$_2$O (5 mL), the phases were separated, and the aqueous phase was extracted with Et$_2$O (3 × equal volume). The combined Et$_2$O layers were dried and evaporated *in vacuo*. Separation of the residue by flash chromatography [SiO$_2$ (200:1); 3:1 Et$_2$O/petroleum ether (35–60 °C)] gave four isomeric products [*R*$_f$'s for SiO$_2$ TLC; 3:1 Et$_2$O/petroleum ether (35–60 °C)]: (a) *R*$_f$ 0.40, 3,4-disubstituted isoxazoline **15** or **16** (7 mg, 1.4%); (b) *R*$_f$ 0.30, 3,4-disubstituted isoxazoline **16** or **15** (3 mg, 0.6%); (c) *R*$_f$ 0.25, isoxazoline **17** (235 mg, 34%); and (d) *R*$_f$ 0.20, **18** (270 mg, 39%). The stereochemistry at C-4′ of the 3,4-disubstituted isoxazolines **15** and **16** was not determined. Data for isomer a (*R*$_f$ 0.40, **15** or **16**): IR (CH$_2$Cl$_2$ cast) 3500–3250, 1719, 1523, 1441 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 1.93–2.01 and 2.12–2.20 (2 × m, 2 H, CHC*H*$_2$CH), 3.51–3.59 (m, 1 H, N=CCH), 3.73 (s, 3 H, OMe), 3.85 (s, 3 H, OMe), 4.36–4.45 (m, 2 H, CH$_2$O), 4.53–4.59 (m, 1 H, H$_\alpha$), 5.10 (s, 2 H, PhC*H*$_2$), 5.40–5.43 (m, 1 H, NH), 7.31–7.35 (m, 5H, Ph); exact mass 364.1287 (0.3%, M$^+$) (364.1271 calcd for C$_{17}$H$_{20}$N$_2$O$_7$).

For isomer b (*R*$_f$ 0.30, **16** or **15**): IR (CHCl$_3$ cast) 3500–3250, 1720, 1520, 1440 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 1.82–1.91 and 2.40–2.47 (2 × m, 2 H, CHC*H*$_2$CH), 3.63–3.70 (m, 1 H, N=CCH), 3.75 (s, 3 H, OMe), 3.84 (s, 3 H, OMe), 4.31–4.46 (m, 2 H, CH$_2$O), 4.47–4.56 (m, 1 H, H$_\alpha$), 5.08–5.14 (m, 2 H, PhC*H*$_2$), 5.38–5.44 (m, 1 H, NH), 7.30–7.36 (m, 5H, Ph); CI MS *m/z* (NH$_3$) 365 (MH$^+$).

For **17**: $[\alpha]^{20}_D$ = +95.8° (*c* 0.98 CH$_2$Cl$_2$); IR (CH$_2$Cl$_2$ cast) 3440–3275, 1722, 1524, 1440, 1257 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.17 (dd, 2 H, *J* = 6.0, 6.0 Hz, CHC*H*$_2$CH), 2.87 (A of ABX, 1 H, *J* = 18.0, 7.5 Hz, 1 H of N=CC*H*$_2$), 3.32 (B of ABX, 1 H, *J* = 17.5, 11.0 Hz, 1 H of N=CC*H*$_2$), 3.74 (s, 3 H, OMe), 3.85 (s, 3 H, OMe), 4.43 (dt, 1 H, *J* = 6.5, 6.0 Hz, H$_\alpha$), 4.89 (ddt, 1 H, *J* = 11.0, 7.5, 7.0 Hz, CH$_2$C*H*CH$_2$), 5.08 (s, 2 H, PhC*H*$_2$), 5.66 (d, 1 H, *J* = 7.0 Hz, NH), 7.32 (s, 5H, Ph); ^{13}C NMR (CDCl$_3$, 100.6 MHz) δ 37.30 (CH*C*H$_2$CH), 38.77 (N=C*C*H$_2$), 51.10 (NHCH), 52.59 (2 × OMe), 66.91 (Ph*C*H$_2$), 80.04 (CHO), 127.88, 128.04 and 128.34 (aromatic CH's), 135.89 (quaternary aromatic C), 151.08 (C=N), 155.75 (OCON), 160.72 (*C*OC=N), 171.57 (CH*C*O$_2$Me); exact mass 364.1246 (0.1%, M$^+$) (364.1271 calcd for C$_{17}$H$_{20}$N$_2$O$_7$). Anal. Calcd for C$_{17}$H$_{20}$N$_2$O$_7$: C, 56.04; H, 5.53; N, 7.69. Found: C, 56.26; H, 5.37; N, 7.72.

For **18**: mp 82–83 °C; $[\alpha]^{20}_D$ = −100.9° (*c* 1.04 CH$_2$Cl$_2$); IR (CH$_2$Cl$_2$ cast) 3450–3270, 1724, 1525, 1440, 1258 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.00 (A of ABXY, 1 H, *J* = 14.5, 8.0, 4.5 Hz, 1 H of C(1′)H$_2$), 2.21 (B of ABXY, 1 H, *J* = 14.5, 9.0, 4.5 Hz, 1 H of C(1′)H$_2$), 2.84 (A of ABX, 1 H, *J* = 17.5, 8.0 Hz, 1 H of C(4)H$_2$), 3.29 (B of ABX, 1 H, *J* = 17.5, 11.0 Hz, 1 H of C(4)H$_2$), 3.72 (s, 3 H, OMe), 3.85 (s, 3 H, OMe), 4.53 (ddd, 1 H, *J* = 8.5, 8.0, 4.5 Hz, H-2′), 4.88 (dddd, 1 H, *J* = 11.0, 9.0, 8.0, 4.5 Hz, H-5), 5.08 (s, 2 H, PhC*H*$_2$), 5.61 (d, 1 H, *J* = 8.5 Hz, NH), 7.32 (s, 5H, Ph); ^{13}C NMR (CDCl$_3$, 100.6 MHz) δ 37.16 (CH*C*H$_2$CH), 38.72 (N=C*C*H$_2$), 51.29 (NHCH), 52.41 (2 × OMe), 66.83 (Ph*C*H$_2$), 80.32 (CHO), 127.82, 127.94 and 128.26 (aromatic CH's), 135.91 (quaternary aromatic C), 151.17 (C=N), 155.83 (OCON), 160.65 (*C*OC=N), 171.78 (CH*C*O$_2$Me); exact mass 364.1279 (0.1%, M$^+$) (364.1271 calcd for C$_{17}$H$_{20}$N$_2$O$_7$). Anal. Calcd for C$_{17}$H$_{20}$N$_2$O$_7$: C, 56.04; H, 5.53; N, 7.69. Found: C, 55.83; H, 5.45; N, 7.60.

(2*S*,5*R*)-2-(*N*-(Benzyloxycarbonyl)amino)-3-(3-carboxy-2-isoxazolin-5-yl)propanoic Acid (19). A mixture of **17** (75.5 mg, 0.21 mmol) and 10% aqueous NaOH (660 μL, 1.65 mmol) was stirred vigorously for 4 h and then diluted with H$_2$O (2 mL) and stirred for a further 17 h. The mixture was diluted with H$_2$O (3.5 mL), washed with EtOAc (equal volume), acidified to pH 1 (2 M HCl), and extracted with EtOAc (3 × equal volume). Combined extracts were dried and evaporated *in vacuo* to give **19** as an oil (69 mg, 99%): IR (acetone cast) 3460–3150, 1719, 1529, 1432, 1244 cm^{-1}; ^{1}H NMR (acetone-*d*$_6$, 200 MHz) δ 2.21 (dd, 2 H, *J* = 7.0, 7.0 Hz, C(1′)H$_2$), 3.01 (A of ABX, 1 H, *J* = 17.5, 8.0 Hz, 1 H of C(4)H$_2$), 3.38 (B of ABX, 1 H, *J* = 17.5, 11.0 Hz, 1 H of C(4)H$_2$), 4.40 (dt, 1 H, *J* = 8.0, 7.0 Hz, H-2′), 5.00 (ddt, 1 H, *J* = 11.0, 8.0, 7.0 Hz, H-5), 5.09 (s, 2 H, PhC*H*$_2$), 6.73 (d, 1 H, *J* 8.0 Hz, NH), 7.25–7.40 (m, 5H, Ph), 10.51 (br s, 2 H, 2 × CO$_2$H); ^{13}C NMR (acetone-*d*$_6$, 100.6 MHz) δ 37.35 (C-1′), 39.18 (C-4), 51.80 (C-2′), 66.72 (Ph*C*H$_2$), 81.60 (C-5), 128.32, 128.39 and 128.92 (aromatic CH's), 137.65 (quaternary aromatic C), 152.46 (C-3), 156.56 (OCON), 161.74 (*C*OC=N), 172.96 (CH*C*O$_2$H); FAB MS (Cleland) *m/z* 359.19 (2.6%, MNa$^+$), 337.12 (70%, MH$^+$).

(2*S*,5*S*)-2-(*N*-Benzyloxycarbonyl)amino)-3-(3-carboxy-2-isoxazolin-5-yl)propanoic Acid (20). The procedure for the conversion of **17** to **19** was employed to transform **18** (120 mg, 0.33 mmol) to **20**, which is an oil (111 mg, 99%): IR (MeOH cast) 3500–3120, 1718, 1530, 1428, 1257, 1235 cm^{-1}; ^{1}H NMR (acetone-*d*$_6$, 200 MHz) δ 2.05 (A of ABXY, 1 H, *J* = 14.5, 10.5, 4.0 Hz, 1 H of C(1′)H$_2$), 2.31 (B of ABXY, 1 H, *J* = 14.5, 9.0, 4.0 Hz, 1 H of C(1′)H$_2$), 2.96 (A of ABX, 1 H, *J* = 17.5, 8.0 Hz, 1 H of C(4)H$_2$), 3.36 (B of ABX, 1 H, *J* = 17.5, 11.0 Hz, 1 H of C(4)H$_2$), 4.47 (ddd, 1 H, *J* = 10.5, 8.5, 4.0 Hz, H-2′), 4.97 (dddd, 1 H, *J* = 11.0, 9.0, 8.0, 4.0 Hz, H-5), 5.09 (s, 2 H, PhC*H*$_2$), 6.70 (d, 1 H, *J* = 8.5 Hz, NH), 7.24–7.39 (m, 5H, Ph), 10.51 (br s, 2 H, 2 × CO$_2$H);

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^{13}C NMR (acetone-d_6, 100.6 MHz) δ 37.28 (C-1′), 39.18 (C-4), 51.84 (C-2′), 66.71 (Ph*C*H$_2$), 80.90 (C-5), 128.33, 128.39 and 128.91 (aromatic CH's), 137.60 (quaternary aromatic C), 152.70 (C-3), 156.97 (OCON), 161.69 (*C*OC=N), 173.32 (CH*C*O$_2$H); FAB MS (Cleland) *m/z* 336.76 (57%, MH$^+$).

(2*S*,4*R*)-2-(*N*-(Benzyloxycarbonyl)amino)-5-cyano-4-hydroxypentanoic Acid γ-Lactone (21). Neat **19** (33 mg, 98 μmol) was placed under vacuum (1 Torr) and heated at 150 °C for 5 min. The residue was purified by flash chromatography (SiO$_2$; 1:1 EtOAc/CH$_2$Cl$_2$) to give **21** as a white solid (12.4 mg, 46%): mp 105–106 °C; $[\alpha]^{20}_D$ = −15.0° (*c* 1.17 CHCl$_3$); IR (CH$_2$Cl$_2$ cast) 3440–3240, 2255, 1791, 1716 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 2.56 (dd, 2 H, *J* = 9.0, 6.0 Hz, C(3)H$_2$), 2.72 (A of ABX, 1 H, *J* = 17.0, 4.5 Hz, 1 H of C(5)H$_2$), 2.80 (B of ABX, 1 H, *J* = 17.0, 5.5 Hz, 1 H of C(5)H$_2$), 4.41 (ddd, 1 H, *J* = 9.5, 9.5, 6.5 Hz, H-2), 4.74–4.95 (m, 1 H, H-4), 5.10 (s, 2 H, C*H*$_2$Ph), 5.49 (d, 1 H, *J* = 9.5 Hz, NH), 7.28–7.37 (m, 5H, Ph); difference NOE experiment—irradiating H-2 gives NOE's at C(3)H$_2$ (5 %), C(5)H$_2$ (3%), C*H*$_2$Ph (2%), NH (6%), and Ph (6%); irradiating H-4 gives NOE's at C(3)H$_2$ (6%), C(5)H$_2$ (6%), and Ph (5%); exact mass 274.0954 (15%, M$^+$) (274.0954 calcd for C$_{14}$H$_{14}$N$_2$O$_4$).

(2*S*,4*S*)-2-(*N*-(Benzyloxycarbonyl)amino)-5-cyano-4-hydroxypentanoic Acid γ-Lactone (22). The procedure used to convert **19** to **21** was employed to transform **20** (33 mg, 98 μmol) to **22** as an oil (15.0 mg, 56%): $[\alpha]^{20}_D$ = +18.6° (*c* 1.45 CHCl$_3$); IR (CH$_2$Cl$_2$ cast) 3470–3270, 2256, 1789, 1716 cm^{-1}; ^{1}H NMR (C$_6$D$_6$, 500 MHz) δ 1.26 (A of ABXY, 1 H, *J* = 11.5, 11.5, 11.5 Hz, *proR* H-3), 1.56 (A of ABX, 1 H, *J* = 17.0, 5.5 Hz, 1 H of C(5)H$_2$), 1.64 (B of ABX, 1 H, *J* = 17.0, 5.0 Hz, 1 H of C(5)H$_2$), 1.77–1.82 (m, 1 H, *proS* H-3), 3.29–3.35 (m, 1 H, H-4), 3.92–3.98 (m, 1 H, H-2), 4.93 and 5.00 (AB, 2 H, *J* = 12.0 Hz, C*H*$_2$Ph), 5.06 (d, 1 H, *J* = 7.5 Hz, NH), 7.19 (s, 5H, Ph); difference NOE experiment—irradiating H-2 gives NOE's at *proS* H-3 (2.5%), NH (2.5%), and H-4 (2.5%); irradiating H-4 gives NOE's at *proS* H-3 (3.5%), C(5)H$_2$ (4%), and H-2 (2%); exact mass 274.0951 (17%, M$^+$) (274.0954 calcd for C$_{14}$H$_{14}$N$_2$O$_4$).

Ethyl 2-Isoxazoline-3-carboxylate (23). The procedure of Drefahl and Hörhold[40] was adapted. A solution of ethyl chlorooximidoacetate[18b] (3.03 g, 20 mmol) in a large volume of Et$_2$O (300 mL) in a 1-L flask was fitted with a pressure-equalized dropping funnel containing a solution of distilled Et$_3$N (2.95 mL, 21 mmol) in Et$_2$O (40 mL). The flask was cooled to 0 °C, and the apparatus was thoroughly degassed and placed under 1 atm of ethylene. The Et$_3$N solution was added dropwise over 90 min, and the solution was stirred at 0 °C for a further 1 h. Water (50 mL) was added, the layers were separated, and the Et$_2$O layer was dried, filtered, and concentrated carefully *in vacuo* so as to avoid loss of volatile product. The residual oil was purified by flash chromatography [SiO$_2$ (100:1), gradient elution (9:1 to 4:1) petroleum ether (35–60 °C)/EtOAc] to give **23** as a colorless liquid (2.21 g, 77%): R_f 0.30 [4:1]; bp 120–125 °C, 2.8 Torr (lit[40] bp 78 °C, 0.5 Torr); λ$_{max}$ 246 nm (ε = 5250 AU·L·mol^{-1}·cm^{-1}); IR (CH$_2$Cl$_2$ cast) 1739, 1588, 1260, 1122 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 1.31 (t, 3 H, *J* = 7.0 Hz, Me), 3.15 (t, 2 H, *J* = 10.5 Hz, H-4), 4.29 (q, 2 H, *J* = 7.0 Hz, C*H*$_2$CH$_3$), 4.48 (t, 2 H, *J* = 10.5 Hz, H-5); ^{13}C NMR (CDCl$_3$, 100.6 MHz) 13.66 (Me), 33.57 (C-4), 61.77 (CH$_2$CH$_3$), 71.17 (C-5), 151.85 (C-3), 160.41 (*C*O$_2$-Et); CI MS *m/z* (NH$_3$) 161 (100%, MNH$_4^+$), 144 (84%, MH$^+$). Anal. Calcd for C$_6$H$_9$NO$_3$: C, 50.35; H, 6.34; N, 9.79. Found: C, 50.59; H, 6.46; N, 9.67.

A side product of isoxazoline formation, the nitrile oxide dimer, 3,4-bis(ethoxycarbonyl)-2,5-furazan 2-(*N*-oxide), could often also be isolated in varying amounts (TLC: SiO$_2$; 4:1 petroleum ether (35–60 °C)/EtOAc; R_f 0.45): bp 125–135 °C, 2.5 Torr (lit[41] bp 113 °C, 2 Torr); λ$_{max}$ 269 nm (ε = 4100 AU·L·mol^{-1}·cm^{-1}); IR (CH$_2$Cl$_2$ cast) 1751, 1625, 1247, 1067 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 1.33 (t, 3 H, *J* = 7.0 Hz, Me), 1.38 (t, 3 H, *J* = 7.0 Hz, Me), 4.39 (q, 2 H, *J* = 7.0 Hz, C*H*$_2$), 4.44 (q, 2 H, *J* = 7.0 Hz, C*H*$_2$); ^{13}C NMR (CDCl$_3$, 100.6 MHz) δ 13.59 and 13.63 (2 × Me), 63.38 (2 × *C*H$_2$), 106.53 (C=N$^+$—O$^-$), 148.24 (C=N), 154.88 (*C*O$_2$Et), 156.45 (*C*O$_2$Et); exact mass 230.0541 (3%, M$^+$) (230.0539 calcd for C$_8$H$_{10}$N$_2$O$_6$). Anal. Calcd for C$_8$H$_{10}$N$_2$O$_6$: C, 41.75; H, 4.38; N, 12.17. Found: C, 41.90; H, 4.39; N, 12.10.

(*RS*)-5-(Ethoxycarbonyl)-3-(methoxycarbonyl)-2-isoxazoline (24). A solution of methyl chlorooximidoacetate (**14**) (275 mg, 2.00 mmol) and ethyl acrylate (600 mg, 5.99 mmol) in Et$_2$O (2.5 mL) under argon was treated dropwise (4 h, syringe pump) with a solution of Na$_2$CO$_3$ (212 mg, 2.00 mg) in H$_2$O (2.5 mL) with vigorous stirring. The mixture was stirred for a further 20 h and was diluted with Et$_2$O (10 mL), and the aqueous layer was decanted. The Et$_2$O layer was dried and evaporated *in vacuo* to give **24** as an oil (181 mg, 45%): IR (CHCl$_3$ cast) 2990–2940, 1737, 1259, 1212 cm^{-1}; ^{1}H NMR (CDCl$_3$, 200 MHz) δ 1.23 (t, 3 H, *J* = 7.0 Hz, CH$_2$*Me*), 3.42 (d, 2 H, *J* = 9.5 Hz, N=CCH$_2$), 3.81 (s, 3 H, OMe), 4.18 (q, 2 H, *J* = 7.0 Hz, OCH$_2$), 5.12 (t, 1 H, *J* = 9.5 Hz, OCH); exact mass 201.0631 (2%, M$^+$) (201.0637 calcd for C$_8$H$_{11}$NO$_5$).

2-Isoxazoline-3-carboxylic Acid (25). The procedure used for the hydrolysis of **17** to **19** was employed to transform **23** (716 mg, 5.00 mmol) to pure crystalline **25** (517 mg, 90%), which was recrystallized from EtOAc/pentane: mp 109–110 °C (dec with softening at 104–106 °C) (lit[42] mp 105–105.5 °C); IR (KBr) 3600–3170, 2720–2450, 1719, 1585, 1276, 930 cm^{-1}; ^{1}H NMR (acetone-d_6, 200 MHz) δ 3.20 (t, 2 H, *J* = 10.5 Hz, H-4), 4.50 (t, 2 H, *J* = 10.5 Hz, H-5), 11.04 (br s, 1 H, CO$_2$H); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 34.33 (C-4), 72.00 (C-5), 152.97 (C-3), 161.97 (*C*O$_2$H); CI MS *m/z* (NH$_3$) 133 (86%, MNH$_4^+$), 116 (40%, MH$^+$). Anal. Calcd for C$_4$H$_5$NO$_3$: C, 41.75; H, 4.38; N, 12.17. Found: C, 41.31; H, 4.22; N, 11.90.

(*RS*)-2-Isoxazoline-3,5-dicarboxylic Acid (26).[43] The hydrolysis procedure used to convert **17** to **19** was employed to hydrolyze **24** (150 mg, 0.75 mmol). Recrystallization of the crude product from EtOAc/petroleum ether (35–60 °C) gave **26** as a white solid (95 mg, 80%): mp 147–148 °C dec (lit[43] mp 135 °C); IR (KBr) 3600–2400, 1717 cm^{-1}; ^{1}H NMR (acetone-d_6, 200 MHz) δ 3.48 (A of ABX, 1 H, *J* = 18.0, 7.5 Hz, 1 H of CH$_2$), 3.60 (B of ABX, 1 H, *J* = 18.0, 11.5 Hz, 1 H of CH$_2$), 5.30 (dd, 1 H, *J* = 11.5, 7.5 Hz, CHO), 11.23 (br s, 2 H, 2 × CO$_2$H); ^{13}C NMR (acetone-d_6, 100.6 MHz) δ 38.17 (CH$_2$), 80.48 (CH), 152.32 (C=N), 161.26 (conj CO$_2$H), 170.74 (CO$_2$H); FAB MS (Cleland) *m/z* 159.91 (MH$^+$). Anal. Calcd for C$_5$H$_5$NO$_5$: C, 37.75; H, 3.17; N, 8.80. Found: C, 37.91; H, 3.21; N, 8.71.

Purification of DAP Epimerase. Cells (354 g wet mass) of the strain of *E. coli* JM109 containing plasmid pDF6, which overexpresses DAP epimerase (obtained from Dr. William Higgins, Merrell-Dow Research Institute, Strasbourg, France), were washed in buffer A (20 mM potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0), resuspended in buffer A (1.2 L), and ruptured by sonication. Cell debris was removed by centrifugation (16 300*g* for 30 min and then 25 400*g* for 1 h) to give a cloudy brown supernatant (1.6 L). Ammonium sulfate fractionation (15, 30, and 45%) gave most DAP epimerase activity in the 15–30% pellet and no activity in the final supernatant. The pellet was resuspended in buffer A and dialyzed against buffer A to give a solution of specific activity 13.6 U/mg. This solution was loaded onto a Matrex Green dye affinity column, washed with buffer A, and eluted with a gradient of 0–2 M KCl in buffer A. Over 90% of the protein washed straight through the column, whereas >90% of DAP epimerase activity was bound. Active fractions of similar specific activity were combined and concentrated by ultrafiltration (giving some loss of activity) to give three 60-mL fractions of specific activities 34, 36, and 37 U/mg, respectively. This enzyme was one major band by SDS PAGE, with minor contaminating proteins of higher molecular weight. This protein was used in subsequent experiments. Further purification using a MPLC column packed with Q-Sepharose (Pharmacia) and pre-equilibrated with buffer A, eluting with buffer A and then a gradient of 0.2–2.0 M NaCl in buffer A, gave DAP epimerase which was essentially a single band by SDS PAGE, but at this level of purity, it was found that enzyme stability was markedly reduced.

Interaction of DAP Epimerase with Substrate Analogues. Inhibition studies with DAP epimerase employed a previously described[21] coupled enzyme assay with LL-DAP (**2**) and DAP dehydrogenase for compounds which did not show significant interaction with the latter enzyme (e.g. **4** and **5**). Alternatively, a direct tritium release assay with [G-^{3}H]DAP (Amersham) was used for compounds which substantially inhibit the dehydrogenase (e.g. **6**).[21] Results of enzyme inhibition studies (run in triplicate) were calculated by computer using *Enzyme Kinetics v1.11* from Trinity Software or the *Enzfitter* program (Elsevier Biosoft, Cambridge, U.K.) using Michaelis–Menten kinetics and nonlinear regression analysis. Heterocyclic DAP analogues were examined for time-dependent inhibition by preincubation of the epimerase in both the presence and absence of the potential inhibitor (ca. 3.0 mM). In a typical

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experiment, aliquots were removed and assayed at 0, 30, 60, and 120 min. No time-dependent inhibition was detected with any of the substrates tested.

Analogues were tested as substrates for the epimerase by incubation with enzyme (ca. 700 mU) in deuterated assay buffer (0.5 mL) for 6 h at 25 °C. The incubations were quenched by freezing to −78 °C, and the potential substrates were recovered by ion-exchange chromatography (BioRad AG1 X8 anion exchanger, OH^- form, 100–200 mesh, 0.5 mL; elution with H_2O and then 1 N HCl) and analyzed by ^1H and ^2H NMR. Under these conditions the statistical mixture of DAP isomers was determined to undergo complete expected (75%) exchange.[22] None of the other compounds tested showed any deuterium incorporation.

Interaction of DAP Dehydrogenase with Substrate Analogues. DAP dehydrogenase was isolated and purified by literature procedure[13a] from the organism *B. sphaericus* IFO 3525. Inhibition studies with DAP dehydrogenase were done at pH 7.5 (reductive amination of THDP (**1**) to *meso*-DAP (**3**)), pH 10.5 (optimal conditions for oxidative deamination of **3** to **1**), and pH 7.8 (forced oxidative deamination of **3** to **1** using $NADP^+$ in the absence of NH_3) by the modification[21] of the literature procedure.[13a] Unless noted below, the reductive amination reaction employed 0.2 M Tris-HCl, 0.2 M NH_4Cl, pH 7.5, 0.1 mM NADPH. The oxidative deamination reaction at pH 7.8 used saturating conditions of *meso*-DAP (**3**) and $NADP^+$ (0.25 mM) with no added NH_3 (i.e. no NH_4^+ in 0.1 M Tris-HCl buffer containing 1 mM EDTA). Preincubation both in the presence and absence of $NADP^+$, and with or without turnover, showed that inhibition by heterocyclic analogues was reversible, with no evidence of slow binding. In the absence of substrate, enzyme solution was incubated in assay buffer at 25 °C with potential inhibitor (e.g. 100 μM of **6**) for up to 2 h, using 100-μL portions to initiate standard spectrophotometric assays. No time-dependent loss of activity was observed. To investigate any effects during turnover, parallel enzyme reactions were run with 0 and 10 μM inhibitor (e.g. **6**) for 6.5 min. After this time, 100 μL of each solution was used to initiate assays containing 1.0 and 0 μM of inhibitor (e.g. **6**), respectively (i.e., in each case a final inhibitor concentration of 1.0 μM was achieved). The initial rates for both assays were identical. The inhibition kinetics of **6** were further examined by obtaining initial rate data at [**6**] = 5.0, 20, 35, 50, 65, and 80 μM for the following: (a) [**3**] = 50, 62, 81, 117, 212, and 1100 μM at saturating [$NADP^+$]; (b) [$NADP^+$] = 4.0, 4.9, 6.5, 9.3, 16.7, and 80 μM at saturating [**3**] (for [**6**] = 0, the K_m^{app} for $NADP^+$ was determined from a direct linear plot as 8.2 μM). From data array a, computer analyses of direct linear plots give a value for **6** of K_i = 23 μM, and inspection of a Lineweaver–Burk plot (supplementary material) showed the inhibition to be noncompetitive with respect to *meso*-DAP (**3**). A similar analysis of data array b showed uncompetitive inhibition by **6** with respect to $NADP^+$ (supplementary material), with K_{ii} = 9.2 μM. Inhibition by **6** was found to decrease to an insignificant level at pH 10.5 (IC_{50} = 4.1 mM).

For the reductive-amination reaction for the conversion of THDP (**1**) to **3**, the inhibition kinetics of **6** were determined by obtaining initial rate data at [**6**] = 0, 4.0, 23, 42, 61, and 80 μM for [**1**] = 60, 75, 95, 140, 240, and 500 μM at saturating [NADPH]. Substrate inhibition was observed at or above [**1**] = 1.0 mM. Computer analysis of this data array gives a value for **6** of K_i = 4.2 μM from direct linear plots, and inspection of a Lineweaver–Burk plot (supplementary material) showed the inhibition to be simple competitive with respect to **1**.

Interaction of Isoxazoline Analogues with Glutamate Dehydrogenase. Commercially available (Sigma) L-glutamate dehydrogenase (EC 1.4.1.4), a specific $NADP^+$-dependent enzyme from *Proteus* sp., was used without further purification and was assayed and tested with inhibitors by literature procedure.[44] Compound **26** was found to be a poor, but measurable, inhibitor. Under saturating conditions of α-ketoglutarate and NADPH, an IC_{50} value of 0.64 mM was determined. Compound **6** showed no significant inhibition (16% at 1.0 mM) of this L-glutamate dehydrogenase.

Antibacterial Assays. Preliminary tests for antimicrobial activity employed the agar diffusion hole plate method.[45] Each solution to be tested (100 μL) was placed in 10-mm wells cut from agar plates inoculated with one of tests organisms, *B. sphaericus* IFO 3525, *C. glutamicum* A5019 PR56, or *P. vulgaris* X-19-0. The plates were incubated at 37 °C for 18 h. Inhibition of growth was measured as a halo around the well containing the test compound. The results obtained were as follows: (A) *B. sphaericus*: tetracycline (1.0 mM), 19-mm inhibition zone; ampicillin (1.0 mM), 17-mm zone; isoxazoline **6** (10 mM), 12-mm zone; **6** (100 mM), 18-mm zone. (B) *C. glutamicum*: tetracycline (1.0 mM), 25-mm inhibition zone; ampicillin (1.0 mM), 23-mm zone; isoxazoline **6** (10 or 100 mM), no inhibition zone. (C) *P. vulgaris*: tetracycline (10 mM), ampicillin (10 mM), isoxazoline **6** (10 or 100 mM), no inhibition zones.

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Supplementary Material Available: DAP epimerase purification and kinetic data for inhibition studies, X-ray structure and listing of parameters for **10**, and NMR spectra for NOE experiments (31 pages); listing of observed and calculated structure factors for **10** (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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