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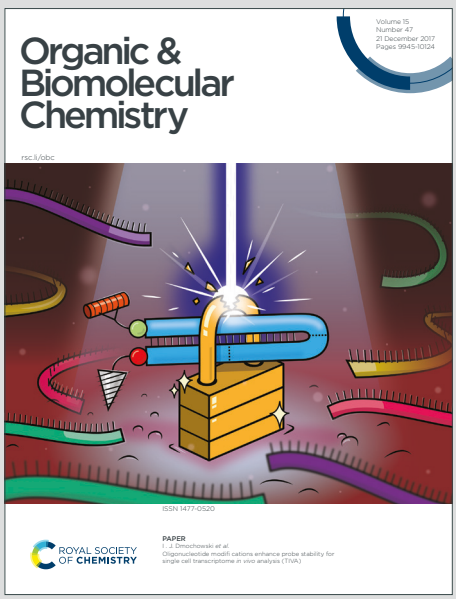

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ARTICLE

Substrate-Product Analogue Inhibitors of Isoleucine 2-Epimerase from *Lactobacillus buchneri* by Rational Design

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A rational approach that may be applied to a broad class of enzyme-catalyzed reactions to design enzyme inhibitors affords a powerful strategy, facilitating the development of drugs and/or molecular probes of enzyme mechanisms. A strategy for the development of substrate-product analogues (SPAs) as inhibitors of racemases and epimerases is elaborated using isoleucine 2-epimerase from *Lactobacillus buchneri* (*Lb*IleE) as a model enzyme. *Lb*IleE catalyzes the PLP-dependent, reversible, racemization or epimerization of nonpolar amino acids at the C-2 position. The enzyme plays an important role in the biosynthesis of branched-chain D-amino acids and is a potential target for the development of antimicrobial agents. 3-Ethyl-3-methyl-L-norvaline (K_i = 2.9 ± 0.2 mM) and 3-ethyl-3-methyl-D-norvaline (K_i = 1.5 ± 0.2 mM) were designed as SPAs based on the movement of the *sec*-butyl side chain of the substrate L-Ile during catalysis, and were competitive inhibitors with binding affinities exceeding that of L-Ile by 1.3- and 2.5-fold, respectively. Surprisingly, these compounds were not substrates, but the corresponding compounds lacking the 3-methyl group were substrates. Unlike serine, glutamate, and proline racemases, which exhibit stringent steric requirements at their active sites, the active site of *Lb*IleE was amenable to binding bulky SPAs. Moreover, *Lb*IleE bound the SPA 2,2-di-*n*-butylglycine (K_i = 11.0 ± 0.2 mM) as a competitive inhibitor, indicating that the hydrophobic binding pocket at the active site was sufficiently plastic to tolerate *gem*-dialkyl substitution at the α-carbon of an amino acid. Overall, these results reveal that amino acid racemases/epimerases are amenable to inhibition by SPAs provided that they possess a capacious active site.

Introduction

Inhibitor design, through a single, rational approach applied to a broad class of enzymes sharing similar active site architectures, furnishes a powerful strategy for the development of lead compounds for the development of drugs and/or molecular probes of enzyme mechanisms. Recently, we described a new strategy for designing inhibitors of racemases and epimerases with capacious and plastic active sites.[1,2] Since enzymes catalyzing racemization and epimerization reactions bind their enantiomeric or epimeric substrates in a mirror-image orientation,[3,4] the inversion of stereochemistry may be accompanied by a movement of two groups attached to the stereocenter through the active site between the so-called *R*- and *S*-pockets during catalysis, particularly in the absence of specific polar interactions.[5-7] Hence, substrate-product analogues (SPAs) that incorporate structural features of both the substrate and product of enzyme-catalyzed racemization or epimerization reactions may serve as inhibitors. Previously, we reported that *gem*-disubstituted SPA inhibitors may be designed that present binding determinants simultaneously to the *R*- and *S*-pockets, as demonstrated for mandelate racemase[8,9] and α-methylacyl-coenzyme A (CoA) racemase (AMACR).[1,2] Such SPAs combine the structural attributes of the two ligands and enhanced binding affinity is anticipated due to the additional binding determinants, provided that the added steric bulk is tolerated. Unfortunately, application of this inhibitor design strategy to glutamate,[10] serine,[11] and proline[11] racemases yielded inhibitors that exhibited either poor or only modest binding affinities relative to their substrates due to the compact nature of their active sites.

Recently, kinetic and structural studies on a pyridoxal 5'-phosphate (PLP)-dependent isoleucine 2-epimerase from *Lactobacillus buchneri* (E.C. 5.1.1.21, *Lb*IleE) were reported.[12-14] This enzyme participates in the production of branched-chain D-amino acids (D-BCAAs) in lactic acid bacteria by catalyzing the reversible racemization or epimerization of nonpolar L-amino acids at the C-2 position via a 2-base mechanism.[15] While *Lb*IleE preferentially catalyzes the epimerization of L-Ile and D-*allo*-Ile with Lys 280 and Asp 222 acting as the Brønsted base catalysts that abstract the C2-proton from L-Ile and D-*allo*-Ile, respectively (**Scheme 1**),[14] it also catalyzes the racemization or epimerization of norvaline, norleucine, Val, 2-aminobutanoic acid, Leu, Phe, Met, and L-*allo*-Ile, but with varying levels of reduced specific activity.[12] The broad substrate specificity of *Lb*IleE, along with X-ray crystallographic studies,[14] suggested that the binding pocket for the nonpolar side chain was quite roomy, and hence the enzyme might be amenable to inhibition by SPAs. Indeed, structures of *Lb*IleE with the bound reaction-intermediate

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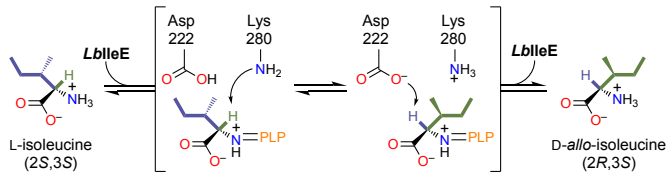
† Electronic Supplementary Information (ESI) available: ^{1}H NMR, ^{13}C NMR, and mass spectra, HPLC chromatograms, enzyme purification SDS-PAGE, and kinetic plots. See DOI: 10.1039/x0xx00000x



Scheme 1 Reaction catalyzed by *Lb*IleE

analogues (*i.e.*, reduced imines) PLP-L-Ile (PDB 5WYA)[14] and PLP-D-*allo*-Ile (PDB 5WYF)[14] revealed that the nonpolar side chain is bound in a large hydrophobic cavity and moves through this cavity during catalysis (**Fig. 1A**), while the imino-PLP moiety and the carboxylate group of the substrate remain bound at their respective binding sites. Recognizing that the entire *sec*-butyl side chain of the substrate does not move entirely to a new location within the hydrophobic cavity, but is only slightly displaced, we designed SPAs that would effectively fill the hydrophobic cavity (**Fig. 1B**).

Over the past decade, it has become evident that D-amino acids play important regulatory roles in bacteria.[16,17] Indeed, D-BCAAs such as D-Ile, D-Leu, and D-Val are involved in regulating remodeling of the cell wall in *Vibrio cholera*,[18] and D-Leu plays a role in biofilm disassembly in *Bacillus subtilis*.[19] Consequently, enzymes catalyzing the biosynthesis of D-BCAAs are attractive targets for the development of antimicrobial agents.[16,20]

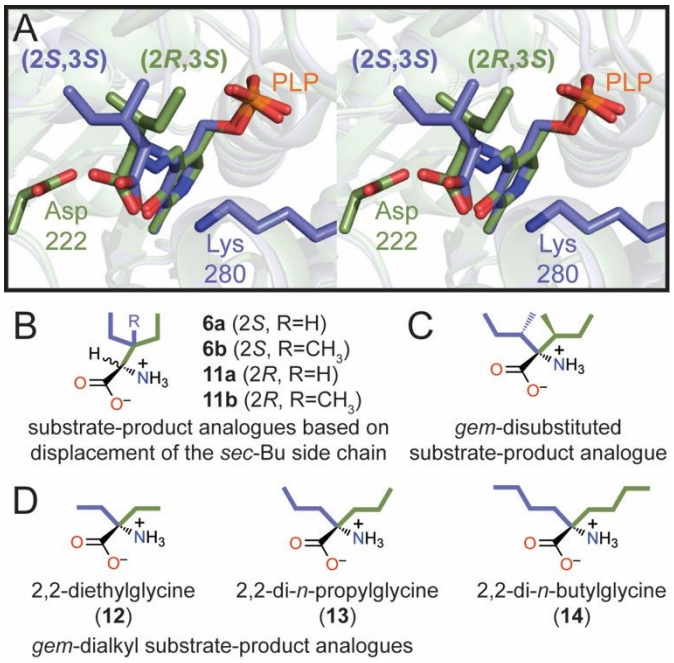
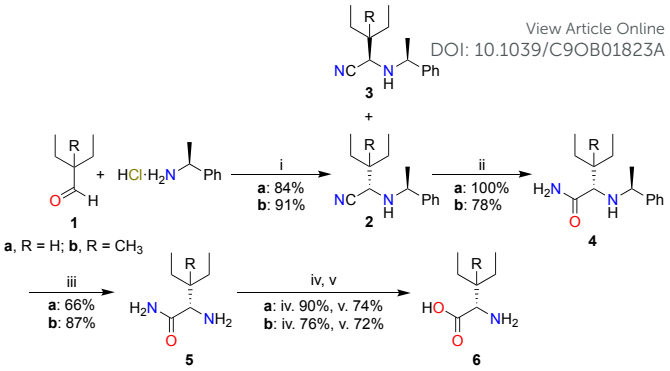
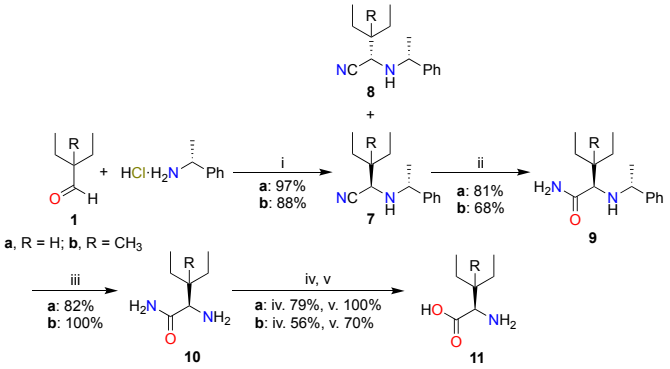


Fig. 1 Design of substrate-product analogues (SPAs). (A) Stereoview showing the superposition of the reaction-intermediate analogues PLP-L-Ile (2*S*,3*S*; purple) and PLP-D-*allo*-Ile (2*R*,3*S*; olive) (stick representation) bound at the active site of *Lb*IleE (PDB 5WYA and 2WYF, respectively).[14] The carboxylate group and the imino-PLP moiety remain fixed, while the nonpolar side chain is free to move within its hydrophobic binding pocket during catalysis. The 2*S*- and 2*R*-specific Brønsted acid-base catalysts Lys 280 and Asp 222, respectively, are also shown in stick representation. (B) The structures of the SPAs, based on the observed change in location of the nonpolar side chain accompanying catalysis, are shown. Note the similarity between the superposed structures in panel A and the structures of **6a,b** and **11a,b**. (C) The *gem*-disubstituted SPA, designed based on movement of the entire nonpolar side chain, is shown. (D) Examples of *gem*-disubstituted SPAs, based on movement of the entire nonpolar side chain, are shown.



Scheme 2 Synthesis of the (2*S*)-substrate-product analogues (**6a,b**). Key: (i) NaCN, MeOH:H_2O (1:1); (ii) conc. H_2SO_4; (iii) H_2 (60 psi), Pd/C, MeOH, rt, 2 days; (iv) conc. HCl, reflux; and (v) SPE, lyophilization.



Scheme 3 Synthesis of the (2*R*)-substrate-product analogues (**11a,b**). Key: (i) NaCN, MeOH:H_2O (1:1); (ii) conc. H_2SO_4; (iii) H_2 (60 psi), Pd/C, MeOH, rt, 2 days; (iv) conc. HCl, reflux; and (v) SPE, lyophilization.

Herein, we report the inhibition of an amino acid racemase responsible for the production of D-BCAAs by the first rationally designed SPAs that exceed the binding affinities of the corresponding substrates.

Results and discussion

Syntheses

Based on the superposition of the X-ray crystal structures of *Lb*IleE with the bound reaction-intermediate analogues PLP-L-Ile (PDB 5WYA) and PLP-D-*allo*-Ile (PDB 5WYF) (**Fig. 1A**),[14] compounds **6a**, **6b**, **11a**, and **11b** (**Fig. 1B**) were designed to present alkyl groups simultaneously to the (*S*)- and (*R*)-hydrophobic pockets that are occupied by the alkyl side chain of L-Ile or D-*allo*-Ile, respectively. The synthesis of the SPAs **6a,b** and **11a,b** are outlined in **Schemes 2** and **3**, respectively. Starting from either 2-ethylbutyraldehyde (**1a**) or 2-ethyl-2-methylbutyraldehyde (**1b**), a chiral Strecker reaction was conducted using (*S*)-(–)-α-methylbenzylamine to selectively yield crystals of the nitrile diastereomers **2a** and **2b**, respectively, as described by Resnick and Galante.[21] Alternatively, use of (*R*)-(+)-α-methylbenzylamine selectively yielded crystals of the nitrile diastereomers **7a** and **7b**, respectively. Diastereomers **3a,b** and **8a,b** were removed with the solvent upon filtration. Subsequently, the nitriles **2a,b** and **7a,b** were converted to their corresponding amides **4a,b** and

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9a,b, respectively, by treatment with concentrated H_2SO_4 for 24 – 48 h. Generally, longer reaction times were required for the compounds with the bulkier amino acid side chain (*i.e.*, **b** series). Removal of the chiral auxiliary was effected by catalytic hydrogenation over 5% Pd/C for 24 – 48 h to yield the amino acid amides **5a,b** and **10a,b**. Finally, the amino acid amides were refluxed in concentrated HCl, followed by solid phase extraction (SPE) and lyophilization to furnish the pure amino acids **6a,b** and **11a,b**. The yields for each of the steps ranged between 56% to ~100% and were similar to those reported by Resnick and Galante[21] for both the **a** and **b** series of compounds. All synthesized compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (MS). Details are presented in **ESI,† Figs. S1 – S41**.

The enantiomeric excess (e.e.) was estimated for **6a,b** and **11a,b** using HPLC to separate the isoindole derivatives prepared by reacting the compounds with *o*-phthaldialdehyde in the presence of either *N*-acetyl-L-cysteine (**6a** and **11a**) or *N*-isobutyryl-L-cysteine (**6b** and **11b**) as shown in **ESI,† Figs. S42** and **S43**. Overall, e.e. values of 96.3%, 100%, 99.1%, and 99.5% were obtained for **6a**, **6b**, **11a**, and **11b**, respectively, with the pairs **6a** and **11a**, and **6b** and **11b**, giving equal and opposite circular dichroism spectra (**ESI,† Fig. S44**).

Substrate studies

Following an approach similar to that described by Awad *et al*.,[13] we inserted a codon-optimized synthetic open reading frame encoding *Lb*IleE into a pET-28a(+) plasmid. The resulting pET28a(+)-wt*Lb*IleE plasmid encodes an *Lb*IleE fusion protein bearing an N-terminal His_6-tag, which allowed for purification to ≥97% using immobilized metal ion affinity chromatography (**ESI,† Fig. S45**).

Previously, Mutaguchi *et al*.[12] characterized the activity of *Lb*IleE with L-Ile and D-*allo*-Ile as substrates. Our His_6-tagged variant of *Lb*IleE exhibited similar affinities for these substrates, but the k_{cat} values were ~2-fold greater than those reported by Mutaguchi *et al*.[12] (**Table 1**). Consequently, the efficiency (k_{cat}/K_m) of our *Lb*IleE was also increased by ~2-fold. Mutaguchi *et al*.[12] also demonstrated that *Lb*IleE exhibits a broad substrate specificity, catalyzing the racemization of a variety of amino acids bearing linear and branched alkyl side chains such as norvaline, norleucine, Val, 2-aminobutanoic acid, Leu, and *allo*-Ile, although with ~20-60% reduced specific activity relative to L-Ile. To explore the ability of the hydrophobic pocket to accommodate larger alkyl groups, we explored the ability of the *Lb*IleE to act on the enantiomers of 2-cyclohexylglycine. We found that *Lb*IleE was able to catalyze the racemization of L- and D-2-cyclohexylglycine with catalytic efficiencies of approximately 30 $mM^{-1}s^{-1}$ (**Table 1**), which was not surprising since *Lb*IleE also exhibits a low racemase activity with Phe.[12] Moreover, this observation suggests that *Lb*IleE should be able to accommodate the additional steric bulk of compounds **6a,b** and **11a,b** within its hydrophobic binding pocket. Indeed, both compounds **6a** and **11a** were substrates for *Lb*IleE, exhibiting catalytic efficiencies similar to those of L- and D-2-cyclohexylglycine and D-*allo*-Ile (**Table 1**).

Table 1 Alkyl-based substrates of *Lb*IleE[a]

substrate	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $mM^{-1}s^{-1}$
L-isoleucine	3.8 ± 0.2	253 ± 14	67 ± 4
	(5.00 ± 0.08)[b]	(126 ± 4)[b]	(25 ± 1)[b]
D-*allo*-isoleucine	11 ± 1	447 ± 42	40 ± 2
	(13.2 ± 0.6)[b]	(235 ± 7)[b]	(18 ± 1)[b]
3-ethyl-L-norvaline (**6a**)	7.0 ± 0.8	215 ± 31	31 ± 3
3-ethyl-D-norvaline (**11a**)	4.3 ± 0.6	137 ± 14	32 ± 3
L-2-cyclohexylglycine	4.9 ± 0.7	148 ± 12	31 ± 6
D-2-cyclohexylglycine	7 ± 1	202 ± 16	29 ± 3

[a] Values of the kinetic constants are the means, and errors are the SD from 3 experiments. [b] Kinetic parameters reported by Mutaguchi *et al*.[12] The reported k_{cat} and k_{cat}/K_m values were per tetramer, but have been adjusted here to reflect the turnover number per active site.

Representative Michaelis-Menten plots for all substrates examined are shown in **ESI,† Fig. S46**.

Unlike L-Ile and D-*allo*-Ile, which are diastereomers, **6a** and **11a**, as well as L- and D-2-cyclohexylglycine, are enantiomers. Consequently, the overall equilibrium constant (K_{eq}) for interconversion of the enantiomeric pairs should be unity. Indeed, from the Haldane relationship (K_{eq} = [D]/[L] = $(k_{cat}/K_m)^{L\rightarrow D}/(k_{cat}/K_m)^{D\rightarrow L}$),[22] the values of K_{eq} for the interconversion of **6a** and **11a**, and L- and D-2-cyclohexylglycine, are 1.0 ± 0.1 and 1.1 ± 0.2, respectively.

Inhibition by substrate-product analogues 6b and 11b

Compounds **6b** and **11b** were designed to mimic the structure suggested from the superposition of the alkyl side chains of the PLP-L-Ile and PLP-D-*allo*-Ile complexes observed in the X-ray crystal structures (**Fig. 1A** & **1B**).[14] Surprisingly, the addition of a single methyl group to the alkyl side chain of the substrates **6a** and **11a** resulted in **6b** and **11b** acting as competitive inhibitors of *Lb*IleE with K_i values of 2.9 mM and 1.5 mM, respectively (**Table 2** and **ESI,† Figs. S47** and **S48**). SPAs are expected to exhibit enhanced binding affinity due to their additional binding determinants, provided that the added steric bulk does not compromise binding. Apparently, the additional methyl group in **6b** and **11b** affords additional van der Waals interactions to promote binding so that these compounds bind slightly better than their corresponding substrates L-Ile and D-*allo*-Ile, respectively. Many amino acid racemases, including *Lb*IleE, exhibit rather weak binding of their substrates, often with K_m values in the millimolar range. Hence, it is not surprising that the binding affinities of the SPAs are also in the low millimolar range.

Table 2 Alkyl-based substrate-product analogue inhibitors of *Lb*IleE[a]

inhibitor	IC_{50}, mM	K_i, mM
3-ethyl-3-methyl-L-norvaline (**6b**)	–	2.9 ± 0.2
3-ethyl-3-methyl-D-norvaline (**11b**)	–	1.5 ± 0.2
2,2-diethylglycine (**12**)	334 ± 18	144 ± 8[b]
2,2-di-*n*-propylglycine (**13**)	43 ± 5	19 ± 2[b]
2,2-di-*n*-butylglycine (**14**)	18 ± 2	11.0 ± 0.2

[a] Values are the means, and errors are the SD from 3 experiments. [b] Calculated from the observed IC_{50} value using eqn. 4 with K_m = 3.8 mM and [L-Ile] = 5.0 mM.

Examination of 6b and 11b as potential substrates

Since **6a** and **11a** were both substrates for *Lb*IleE, we tested **6b** and **11b** as potential substrates. Even when the concentration of the enzyme was increased from 2 μg/mL to 20 μg/mL (*i.e.*, 10-fold above its regular assay concentration), no turnover was observed using the HPLC-based assay. This observation suggested that the introduction of the additional methyl group in **6b** and **11b** (*cf*. **6a** and **11a**) either resulted in a binding orientation wherein the α-proton of **6b** and **11b** was no longer correctly positioned for abstraction by the enantiospecific Brønsted base, or abstraction of the α-proton could occur but congestion of the hydrophobic pocket at the active site arising from the additional steric bulk of the side chain obviated the ability of the compound to undergo a Walden inversion. To distinguish between these two possibilities, we conducted a hydrogen-deuterium (H-D) exchange experiment. Using ^{1}H NMR spectroscopy to follow H-D exchange of the α-proton in deuterated assay buffer in the presence of 362 μg/mL enzyme, we observed almost complete replacement of the α-proton in **6a** (8.5 (± 0.1)% H remaining) and **11a** (6.6 (± 0.2)% H remaining) over 1 h as evidenced by the marked reduction in the area under the doublet at 3.76 ppm, corresponding to the α-proton (**ESI,† Fig. S49**). On the other hand, under identical conditions, the integral of the singlet at 3.57 ppm, corresponding to the α-proton in **6b** and **11b**, remained at 100 (±6)% and 98 (±5)%, respectively, indicating that no H-D exchange with the α-proton was detected for the 3-methyl-containing compounds over the same time period, during which the enzyme was fully active. Thus, **6b** and **11b** are not substrates for *Lb*IleE (or they are extremely unreactive) since the enzyme is unable to catalyze their deprotonation. This remarkable observation suggests that, unlike the substrates **6a** and **11a**, the orientation of **6b** and **11b** at the active site is altered by the additional methyl group such that neither Asp 222 nor Lys 280 (**Scheme 1**) can interact with the α-proton and/or that formation of a Schiff base with bound PLP does not occur. Similarly, Mutaguchi *et al*.[12] observed that *tert*-Leu was not a substrate. These observations suggest that the presence of a quaternary β-carbon is not compatible with catalysis. An alternative possibility is that deprotonation occurs, but internal return occurs so rapidly that H-D exchange with the conjugate acid of the Brønsted base cannot occur. However, this seems unlikely since Lys 280 is a polyprotic base and, upon abstraction of the α-proton, would exist as Lys-ND_2H^+, thereby having a 2/3 probability of incorporating a deuterium upon internal return.[23]

Inhibition by *gem*-dialkyl glycines

Typically, the design of SPAs involves the geminal substitution on the stereogenic center undergoing inversion.[1,2,8-11] The corresponding analogue for *Lb*IleE is shown in **Fig. 1C**. We initially avoided this type of analogue because of our previous observations that amino acid racemases seem to have sterically restrictive active sites that may not tolerate *gem*-dialkyl substitutions at the α-carbon of the amino acid substrate. To explore the feasibility of such SPAs as inhibitors of *Lb*IleE, we examined the ability of several *gem*-disubstituted glycines to inhibit *Lb*IleE activity (see **Fig. 1D**). Representative IC_{50} determination curves are shown in **ESI,† Fig. S50**. 2,2-Diethylglycine (**12**) proved to be an extremely weak inhibitor of *Lb*IleE, while 2,2-di-*n*-propylglycine (**13**) was a modest inhibitor (**Table 2**). The fact that *Lb*IleE bound 2,2-di-*n*-propylglycine suggested that the very weak binding of 2,2-diethylglycine was not due to geminal substitution of the ethyl groups on the α-carbon, but arose from the lack of van der Waals interactions with the larger hydrophobic binding pocket. 2,2-Di-*n*-butylglycine (**14**) was shown to be a competitive inhibitor (see **ESI,† Fig. S51**) and, interestingly, bound to *Lb*IleE with an affinity similar to that observed for the substrate D-*allo*-Ile (*cf*. K_m value). These observations suggest that there is sufficient plasticity and/or room at the active site of *Lb*IleE to accommodate amino acid analogues with *gem*-dialkyl substitution on the α-carbon. This behavior differs markedly from that observed previously for the PLP-dependent serine racemase from *Saccharomyces pombe*, which was not able to bind the *gem*-disubstituted SPA α-(hydroxymethyl)serine.[11] Consequently, better binding affinity might be obtained with bulkier aliphatic side chains, however, the low solubility of such inhibitors becomes an impediment.

Concluding remarks

The rational design of SPA inhibitors of racemases and epimerases generally assumes that two groups on the carbon undergoing stereoinversion remain fixed in orientation through their interactions with binding determinants at the active site, while the hydrogen and a motile group formally switch positions. Consequently, this motile group travels between the *R*- and *S*-binding pockets that recognize the motile group of the corresponding enantiomers. *Gem*-disubstituted SPAs may be designed that simultaneously present the motile group to both of these binding pockets.[1,2,8-11] The additional binding interactions should result in an inhibitor binding affinity that exceeds the binding affinity of the substrate, provided that the active site can tolerate the additional steric bulk. In the present study, the 2,2-dialkylglycines conform to this design strategy with 2,2-di-*n*-butylglycine binding with an affinity similar to that of the substrates. However, superposition of the structures of the *Lb*IleE complexes[14] with the bound PLP-L-Ile and PLP-D-*allo*-Ile analogues clearly indicated that stereoinversion is accompanied by only partial motion of the *sec*-butyl side chain of L-Ile or D-*allo*-Ile during catalysis (**Fig. 1A**). Therefore, we modified the SPA inhibitor design strategy and prepared analogues that mimicked the partial displacement of the *sec*-butyl group (**Fig. 1B**). Indeed, *Lb*IleE bound compounds **6b** and **11b** with affinities that slightly exceeded those exhibited for the corresponding substrates, thereby validating this new variation of the SPA inhibitor design strategy. The inhibitors described in the present work may serve as tools for studying the metabolism of D-BCAAs or their regulatory roles in bacteria.[16,20] Furthermore, they may furnish a route to lead compounds for the development of antimicrobial agents[16,20] or agents to control bacterial biofilm formation.[19]

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Experimental

General information

2-Ethylbutyraldehyde, D-*allo*-isoleucine, and L-isoleucine were purchased from TCI America (Portland, OR). L-2-Cyclohexylglycine and D-2-cyclohexylglycine were purchased from Oakwood Products, Inc. (Estill, SC). 2-Amino-2-ethylbutanoic acid (2,2-diethylglycine) and 2-propyl-D,L-norvaline (2,2-di-*n*-propylglycine) were purchased from Acrotein ChemBio Inc. (Hoover, AL). 5-Aminononane-5-carboxylic acid hydrochloride (2,2-di-*n*-butylglycine) was purchased from Toronto Research Chemicals (Toronto, ON). All other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Dichloromethane (DCM) was dried and distilled over calcium hydride prior to use. Concentration under reduced pressure refers to the removal of solvent using a rotary evaporator. All NMR spectra were obtained using a Bruker AVANCE 300 or 500 MHz spectrometer. Chemical shifts (δ in ppm) for proton (^{1}H) spectra are reported relative to the residual solvent signal for $CDCl_3$ (δ 7.26), DMSO-d_6 (δ 2.50), or HOD (δ 4.79).[24] Chemical shifts (δ in ppm) for carbon (^{13}C) spectra are reported relative to the residual solvent signal for $CDCl_3$ (δ 77.16) or DMSO-d_6 (δ 39.52).[24] High resolution (HR) electrospray ionization (ESI) mass spectra (MS) were collected using a Bruker microTOF Focus orthogonal ESI-TOF mass spectrometer instrument operating in either negative or positive ion mode as indicated in the experimental procedures below. Abbreviations used for reporting NMR spectral data are: bm, broad multiplet; bs, broad singlet; bt, broad triplet; d, doublet; dd, doublet of doublets; m, multiplet; q, quartet; s, singlet; and t, triplet. For high-performance liquid chromatography (HPLC) analyses, a Waters 510 pump and 680 controller were used for solvent delivery. Injections were made using a Rheodyne 7725i sample injector fitted with a 20-µL injection loop. Analytes were detected using a Waters 474 scanning fluorescence detector.

*Lb*IleE production

The open reading frame (ORF) encoding isoleucine 2-epimerase from *Lactobacillus buchneri* JCM 1115 (Genbank KC413940.1) (*Lb*IleE) was synthesized and codon-optimized by Bio Basic Canada Inc. (Markham, ON, Canada) and inserted into a pET-28a(+) plasmid (Novagen/Millipore (Canada) Ltd., ON) between the SacI and XhoI sites of the multiple cloning region to generate the pET-28a(+)-wt*Lb*IleE plasmid. This construct encodes wild-type *Lb*IleE with an N-terminal $(His)_6$-tag (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSEFELM$_1$–*Lb*IleE), where M$_1$ denotes the initial Met of wild-type *Lb*IleE. The stop codon of the ORF was utilized so that the C-terminal $(His)_6$-tag was not present. *Lb*IleE was overexpressed in and purified from *Escherichia coli* BL21(DE3) cells transformed with the plasmid following a procedure modified from that described by Awad *et al*.[13] Briefly, two disposable sterile culture tubes (10 mL) containing lysogeny broth (LB, 5 mL) and kanamycin (15 µg/mL) were inoculated with a glycerol stock solution (10 µL each) and incubated overnight at 37 °C with continuous shaking at 250 rpm. The culture (10 mL) from both tubes was then used to inoculate LB broth (1.0 L) containing kanamycin (15 µg/mL) in a 2-L Erlenmeyer flask. The culture was incubated at 37 °C with continuous shaking at 250 rpm until the O.D. was ~0.6. IPTG was added (final concentration 1.0 mM) and the culture was incubated overnight at 20 °C. The cells were then harvested by centrifugation (3000 × *g*, 10 min, 4 °C) and the cell pellet was re-suspended in ice-cold binding buffer [Tris-HCl buffer (20 mM, pH 7.9) containing NaCl (500 mM) and imidazole (5 mM)]. The cells were lysed using sonication with 5 × 10 s bursts with 1 min cooling intervals, at a constant power setting of 5.5 using a Branson Sonifier 250. The soluble cell extract was clarified by ultracentrifugation (110 000 × *g*, 30 min, 4 °C) and the supernatant was loaded onto a column containing His·Bind resin (2.5 mL) (Novagen/Millipore (Canada) Ltd., ON). The column was then eluted with binding buffer (25 mL) followed by wash buffer [Tris-HCl buffer (20 mM, pH 7.9) containing NaCl (500 mM) and imidazole (70 mM)] (15 mL). The enzyme was eluted with strip buffer [Tris-HCl buffer (20 mM, pH 7.9) containing NaCl (500 mM) and EDTA (100 mM)] (6 mL). Upon elution, the enzyme was dialyzed (MWCO 10 kDa) against citrate buffer (200 mM, pH 5.5) containing PLP (0.10 mM)] and DTT (1.0 mM) and then stored at –20 °C. The purity of the enzyme (≥ 97%) was assessed using SDS-PAGE (8% acrylamide) with staining by Coomassie blue R-250.[25] Protein concentrations were determined from the intrinsic enzyme absorbance at 280 nm using an extinction coefficient of ε = 49 280 $M^{-1}cm^{-1}$ (assuming all Cys reduced), which was estimated using the ProtParam program from ExPasy (web.expasy.org/protparam/).[26] Alternatively, protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON) with bovine serum albumin (BSA) standards. The $(His)_6$-tag was not removed from the enzyme.

Enzyme assays

The epimerization of L-Ile and D-*allo*-Ile was followed using a reversed-phase (RP) HPLC-based assay similar to that described by Mutaguchi *et al*.[12] Assay solutions (total volume of 850 µL) contained L-Ile or D-*allo*-Ile (2.0 – 50.0 mM) and *Lb*IleE (2.0 µg/mL) in citrate (assay) buffer (200 mM, pH 5.5) containing PLP (0.1 mM). The reaction was incubated for 4 min at 37 °C and terminated by removing a 500-µL aliquot and mixing it with 50% trichloroacetic acid (125 µL). Following centrifugation (15 min, 10 000 × *g*, 4 °C) of this solution, the supernatant (500 µL) was added to NaOH (1 M, 300 µL). The neutralized samples (20 µL) were added to 140 µL of borate-NaOH buffer (0.1 M, pH 10.4) followed by 40 µL of the freshly prepared derivatization solution (20 mg *N*-acetyl-L-cysteine and 16 mg *o*-phthaldialdehyde in 2 mL methanol) and the reaction allowed to proceed for 1 min. An aliquot (10 µL) was then transferred to 990 µL of sodium acetate buffer (100 mM, pH 5.9) and analyzed using RP-HPLC (20-µL injection volume) on a Kinetex C18 column (5 µm; 100 Å; 250 × 4.6 mm; Phenomenex, Torrance, CA) equipped with a guard column. The isoindole derivatives were eluted under isocratic conditions at a flow rate of 0.45 mL/min with sodium acetate buffer (50 mM, pH 5.9):methanol (49:51) and detected using fluorescence detection (λ_{ex}, 337 nm; λ_{em}, 454 nm). The

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chromatograms were generated and integrated using PeakSimple software v. 4.32 (Mandel Scientific, Guelph, ON, Canada). Initial velocities were calculated using standard curves with the product of the reaction being monitored, *i.e.*, L-Ile (0.5 – 6.0 mM), D-*allo*-Ile (0.5 – 6.0 mM), L-2-cyclohexylglycine (0.4 – 1.4 mM), D-2-cyclohexylglycine (0.4 – 1.4 mM), **6a** (0.2 – 2.4 mM), and **11a** (0.2 – 2.2 mM) derivatized in the same manner as described above.

Kinetic studies

The Michaelis-Menten equation (eqn. 1) was fit to the initial velocity data using nonlinear regression analysis and the program *KaleidaGraph* v. 4.02 from Synergy Software (Reading, PA), where v_i is the initial velocity, V_{max} is the apparent maximal velocity at saturating concentrations of substrate, K_m is the apparent Michaelis constant, and [S] is the substrate concentration.

$$v_i = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

The values of k_{cat} were calculated by dividing the observed V_{max} values by the enzyme concentration (protomer MW = 53 487 Da). The concentration ranges of L-Ile (20.0 – 50.0 mM), D-*allo*-Ile (20.0 – 50.0 mM), L-2-cyclohexylglycine (2.0 – 10.0 mM), D-2-cyclohexylglycine (2.0 – 10.0 mM), 3-ethyl-L-norvaline (**6a**) (2.0 – 20.0 mM), and 3-ethyl-D-norvaline (**11a**) (2.0 – 50.0 mM) were as indicated. Inhibition experiments with 3-ethyl-3-methyl-L-norvaline (**6b**) (0, 5.0, and 10.0 mM) and 3-ethyl-3-methyl-D-norvaline (**11b**) (0, 5.0, and 10.0 mM) were conducted using concentrations of L-Ile between 5.0 and 50.0 mM and a concentration of *Lb*IleE of 2.0 μg/mL. Inhibition experiments with 2,2-di-*n*-butylglycine (**14**) (0, 5.4, and 10.8 mM) were conducted using concentrations of L-Ile between 2.5 and 20.0 mM and a concentration of *Lb*IleE of 2.0 μg/mL. The apparent K_m/V_{max} values were obtained from direct fits of eqn. 1 to initial velocity data (*i.e.*, Michaelis-Menten plots) using nonlinear regression analysis and the *KaleidaGraph* software. Competitive inhibition constants (K_i) were determined by re-plotting these apparent K_m/V_{max} values against inhibitor concentration in accord with eqn. 2.

$$v_i = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (2)$$

The IC_{50} values for the inhibition of the *Lb*IleE by the *gem*-dialkylglycines **12**, **13**, and **14**, relative to L-Ile (5.0 mM), were determined by fitting the relative velocities (v_i/v_o) obtained at various concentrations of **12** (50.0 – 400.0 mM), **13** (5.0 – 40.0 mM), and **14** (2.0 – 13.0 mM) to eqn. 3, where the IC_{50} value is the concentration of inhibitor that yields $v_i/v_o = 0.5$.[27]

$$\frac{v_i}{v_o} = \frac{IC_{50}}{IC_{50} + [I]} \quad (3)$$

The IC_{50} values were used to estimate the values for the inhibition constants using the Cheng-Prusoff equation and assuming competitive inhibition (eqn. 4).[28]

$$K_i = \frac{IC_{50}}{1 + \left([S]/[K_m]\right)} \quad (4)$$

Hydrogen-deuterium exchange

Water was removed from the assay buffer or solutions of assay buffer containing either **6a**, **11a**, **6b**, or **11a** (10.0 mM in 10 mL) by lyophilization. The dry powder was then dissolved in D_2O (7.5 mL) and the solvent was removed by lyophilization. This procedure was then repeated. The resulting powder was then dissolved in D_2O, the solution was adjusted to pD 5.5 using DCl (pH 5.1 on the pH meter),[29] and the solution was brought to a volume of 10 mL in a volumetric flask by addition of D_2O. Solutions (650 μL) of deuterated assay buffer alone, or of **6a**, **11a**, **6b**, or **11a** (10.0 mM) in deuterated assay buffer were placed in an NMR tube. *Lb*IleE (100 μL of a 2714 μg/mL stock solution in assay buffer containing H_2O) was added and the tube was incubated at 37 °C for 1 h in a water bath. The samples were then analyzed using ^{1}H NMR spectroscopy. The extent of H-D exchange was estimated by comparing the integration of the doublets at 3.76 ppm arising from the α-proton of **6a** and **11a** with the integration of the overlapping triplets of the CH_3 groups centered at 0.90 ppm. Similarly, the integration of the singlet at 3.57 ppm arising from the α-proton of **6b** and **11b** were compared with the integration of the singlet corresponding to the CCH_3 at 0.9 ppm and the overlapping triplets arising from the two CH_2CH_3 groups centered at 0.80 ppm.

Syntheses

Compound **1a**[30] and **2a – 6a**[21] were synthesized as described in the literature. In general, compounds **2** – **6** and **7** – **11** were prepared following the protocols adapted from Resnick and Galante.[21]

3-Ethyl-L-norvaline (6a).[21] Compound **5a** (2.85 g, 20 mmol), prepared as described by Resnick and Galante,[21] was mixed with concentrated HCl (50 mL) and the mixture was refluxed for 24 h. Upon cooling to room temperature, white crystals formed. After 30 min, the white crystals (3.23 g, 90%) were collected by suction filtration and air-dried. The hydrochloride salt was then converted to the free amino acid by dissolving the crystals (1.52 g, 8.4 mmol) in water (4 mL) with gentle heating followed by passage through a reversed-phase, solid-phase extraction column (60 mL/10 g, C18-E, 55 μm, 70 Å, Phenomenex, Torrance, CA). Fractions (3 mL) containing the amino acid (yellow spots on a silica TLC sheet developed with a potassium dichromate dip) were combined and the aqueous solvent was removed by lyophilization to yield a white powder: yield 0.90 g (74%); m.p. 200 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.43 (br s, NH_2, 2H), 3.81 (d, J = 3.5 Hz, $CHNH_2$, 1H), 1.69-1.78 (m, $CHCHNH_2$, 1H), 1.19-1.30 (m), 1.30-1.46 (m) and 1.46-1.57 (m) (2 × CH_2, 4H), 0.90 (t, J = 7.4 Hz, CH_3) and 0.88 (t, J = 7.4 Hz CH_3) (6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.54 (COOH), 53.78 (CHCOOH), 42.29 ($CHCHNH_2$), 21.80 (CH_2), 21.59 (CH_2), 11.60 (CH_3), 11.44 (CH_3) ppm. HRMS-ESI$^+$ (m/z): $[M+H]^+$ calcd for

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$C_7H_{16}NO_2$, 146.1181, found 146.1171; $[M+Na]^+$ calcd for $C_7H_{15}NO_2Na$, 168.1000, found 168.0999.

(2S)-3-Ethyl-3-methyl-2-{[(1*S*)-1-phenylethyl]amino}-pentanenitrile (2b). Aqueous HCl (2.9 M, 13.8 mL) was added to a solution of methanol (200 mL) and water (186 mL) followed by (*S*)-(–)-α-methylbenzylamine (5.1 mL, 40 mmol, 1.0 equiv.). 2-Ethyl-2-methylbutanal (5.66 mL, 40 mmol, 1.0 equiv.) was then added followed by NaCN (1.96 g, 40 mmol, 1.0 equiv.). After stirring for 48-60 h to allow for adequate asymmetric crystallization to occur, white crystals were collected by suction filtration: yield 8.90 g (91%); m.p. 51 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.32 (m, ArH, 4H), 7.25 (m, ArH, 1H), 3.86 (qd, J = 6.4, 1.9 Hz, CHPh, 1H), 2.87 (d, J = 12.3 Hz, CHCN, 1H), 2.70 (dd, J = 12.3, 1.5 Hz, NH, 1H), 1.49 – 1.27 (m, CH_3CH and CH_2, 6H), 1.31 – 1.12 (m, CH_2, 1H), 0.85 (s, CH_3C, 3H), 0.59 (m, 2 × CH_3CH_2, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 144.54 (C_{Ph}), 128.86 (2 × C_{Ph}), 127.80 (C_{Ph}, 127.40 (2 × C_{Ph}), 120.27 (C_{CN}), 56.98 (CHPh), 55.97 (CHCN), 38.99 (CCH_2), 28.05 (CH_2), 27.09 (CH_2), 24.69 (CH_3CH), 20.75 (CH_3C), 7.91 (CH_3CH_2), 7.76 (CH_3CH_2) ppm. HRMS-ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{25}N_2$, 245.2018, found 245.2003.

3-Ethyl-3-methyl-*N*^{2}-[(1*S*)-1-phenylethyl]-L-norvalinamide (4b). Concentrated H_2SO_4 (4 mL per gram of reactant; 655 mmol, 18.2 equiv.) was added to the amino nitrile **2b** (8.90g, 36 mmol, 1.0 equiv.) and stirred for 72 h. The reaction mixture was then pipetted onto ice (75 mL) followed by addition of concentrated NH_4OH (aq.) (1 mL aliquots with cooling by additional ice) until the pH was 8. The resulting white gummy product and opaque white solution were poured into a separatory funnel with EtOAc used to transfer the gummy product. The solution was then extracted with EtOAc (3 ×100 mL) and the combined organic layers were dried over anhydrous Na_2SO_4. In some preparations, a minor amount of reactant remained, which was removed by column chromatography on silica using hexanes:EtOAc (8:2). The reactant (**2b**) and product (**4b**) had R_f values of 0.88 and 0.19, respectively. Removal of the solvent *in vacuo* yielded a white solid: yield 7.46 g (78%); m.p. 114 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.36 – 7.24 (m, ArH and CONH, 5H), 7.20 (m, ArH, 1H), 6.99 (br s, CONH, 1H), 3.47 (q, J = 6.5 Hz, CHPh, 1H), 2.54 (br s, $CHCONH_2$, 1H), 2.01 (d, J = 8.9 Hz, NH, 1H), 1.54 – 1.36 (m, CH_2) and 1.31 – 1.07 (m, CH_3CH and CH_2) (7H), 0.74 (s, CH_3C, 3H), 0.57 (t, J = 7.5 Hz, CH_3CH_2, 3H), 0.47 (t, J = 7.4 Hz, CH_3CH_2, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 175.48 ($CONH_2$), 145.77 (C_{Ph}), 127.96 (2 × C_{Ph}), 127.00 (2 × C_{Ph}), 126.61 (C_{Ph}), 63.75 ($CHCONH_2$), 56.03 (CH_{Ph}), 37.84 (CCH_2), 27.14 (CH_2), 26.93 (CH_2), 25.47 (CH_3CH), 19.75 (CH_3C), 7.67 (CH_3CH_2), 7.56 (CH_3CH_2) ppm. HRMS-ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{27}N_2O$, 263.2133, found 263.2126.

3-Ethyl-3-methyl-L-norvalinamide (5b). MeOH (75 mL) was carefully added to 5% Pd on charcoal (1.87 g) under a flow of argon. Compound **4b** (7.46 g, 28 mmol) was added and the mixture was shaken (Parr Shaker) for 72 h under an atmosphere of hydrogen (60 psi). The reaction mixture was then filtered through a pad of Celite and the Celite was washed with MeOH (50 mL). After the solvent was removed *in vacuo*, the resulting solid was dissolved in DCM (10 mL) and the solvent was again removed *in vacuo* to yield a white solid: yield 0.94 g (96%); m.p. 87 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.17 (s, $CONH_2$, 1H), 6.80 (s, $CHNH_2$, 1H), 2.96 (s, $CHNH_2$, 1H), 1.45 – 1.16 (m, CH_2, 4H), 0.83 – 0.67 (m, CH_3C and 2 × CH_3CH_2, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 176.53 ($CONH_2$), 59.03 ($CHNH_2$), 38.24 (CCH_2), 27.24 (CH_2), 26.73 (CH_2), 19.61 (CH_3C), 7.94 (CH_3CH_2), 7.70 (CH_3CH_2) ppm. HRMS-ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_8H_{19}N_2O$, 159.1497, found 159.1489.

3-Ethyl-3-methyl-L-norvaline (6b). Compound **5b** (3.70 g, 23 mmol) was mixed with concentrated HCl (50 mL) and the mixture was refluxed for 24 h. Upon cooling to room temperature, white crystals formed. After 30 min, the product was collected by suction filtration and air-dried to yield white crystals: yield 3.46 g (76%). The hydrochloride salt was then converted to the free amino acid by dissolving the crystals (0.7028 g, 3.6 mmol) in water (4 mL) with gentle heating followed passage through a reversed-phase, solid-phase extraction column (60 mL/10 g, C18-E, 55 µm, 70 Å). Fractions (3 mL) containing the amino acid (yellow spots on a silica TLC sheet developed with a potassium dichromate dip) were combined and the aqueous solvent was removed by lyophilization to yield a white powder: yield 0.41 g (72%); m.p. 183 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.36 (br s, NH_2, 2H), 3.60 (s, $CHNH_2$, 1H), 1.35-1.48 (2 × CH_2, 4H), 0.90 (s, CH_3C, 3H), 0.793 (t, J = 7.4 Hz, CH_2CH_3) and 0.789 (t, J = 7.5 Hz CH_2CH_3) (6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.94 (COOH), 57.99 (CHCOOH), 37.72 (CCHCOOH), 27.27 (CH_2), 26.84 (CH_2), 19.85 (CH_3C), 7.51 (CH_3), 7.47 (CH_3) ppm. HRMS-ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_8H_{18}NO_2$, 160.1338, found 160.1336.

(2R)-3-Ethyl-2-{[(1*R*)-1-phenylethyl]amino}pentanenitrile (7a). Aqueous HCl (2.9 M, 6.9 mL) was added to a solution of methanol (100 mL) and water (93 mL) followed by (*S*)-(–)-α-methylbenzylamine (2.55 mL, 20 mmol, 1.0 equiv.). 2-Ethyl-butanal (2.46 mL, 20 mmol, 1.0 equiv.) was then added followed by NaCN (0.98 g, 20 mmol, 1.0 equiv.). After stirring for 48 h to allow for adequate asymmetric crystallization to occur, the white crystals were collected by suction filtration: yield 4.45 g (97%); m.p. 58 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.39 – 7.27 (m, ArH, 4H), 7.32 – 7.19 (m, ArH,1H), 3.88 (qd, J = 6.5, 2.2 Hz, CHPh, 1H), 3.01 (dd, J = 11.6, 5.5 Hz, CHCN 1H), 2.92 (dd, J = 11.6, 2.3 Hz, NH, 1H), 1.65 – 1.35 (m, $CHCH_2$ and CH_2CH_3) and 1.34 – 1.18 (m, CH_3CHPh and CH_2CH_3) (8H), 0.73 (m, 2 × CH_2CH_3, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 144.68 (C_{Ph}), 128.93 (2 × C_{Ph}), 127.71 (C_{Ph}), 127.14 (2 × C_{Ph}), 120.67 (C_{CN}), 56.71 (CHPh), 51.25 (CHCN), 43.83 (CHCHCN), 24.92 (CH_3CHPh), 22.22 (CH_2CH_3), 21.93 (CH_2CH_3), 11.26 (CH_2CH_3), 10.92 (CH_2CH_3). HRMS-ESI$^+$ (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{23}N_2$, 231.1861, found 231.1865.

3-Ethyl-*N*^{2}-[(1*R*)-1-phenylethyl]-D-norvalinamide (9a). Concentrated H_2SO_4 (2 mL per gram of reactant; 166 mmol, 8.7 equiv.) was added to the amino nitrile **7a** (4.45 g, 19 mmol, 1.0 equiv.) and stirred for 36 h. The reaction mixture was then pipetted onto ice (75 mL) followed by addition of concentrated NH_4OH (aq.) (1 mL aliquots with cooling by additional ice) until the pH was 8. The resulting white gummy product and opaque white solution were poured into a separatory funnel with EtOAc used to transfer the gummy product. The solution was then

extracted with EtOAc (3 × 75 mL) and the combined organic layers were dried over anhydrous Na_2SO_4. Removal of the solvent *in vacuo* gave a clear oil: yield 1.64 g (81%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.36 – 7.26 (m, ArH and CONH, 5H), 7.24 – 7.17 (m, ArH, 1H), 7.02 – 6.98 (m, CONH, 1H), 3.55 (q, J = 6.5 Hz, CHPh, 1H), 2.62 (m, CHCONH$_2$, 1H), 2.06 (br d, J = 5.4 Hz, NH, 1H), 1.47 (m, CHCH$_2$CH$_3$, 1H), 1.32 – 1.16 (m, 2 × CH$_3$CH$_2$ and CH$_3$CHPh, 9H – 2H for overlapping t of EtOAc = 7H), 0.72 (t, J = 7.1 Hz, CH$_2$CH$_3$, 3H), 0.61 (t, J = 7.0 Hz, CH$_2$CH$_3$, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 176.57 (CONH$_2$), 145.87 (C_{Ph}), 128.01 (2 × C_{Ph}), 126.84 (2 × C_{Ph}), 126.59 (C_{Ph}), 61.07 (CHCONH$_2$), 56.15 (CHPh), 44.24 (CHCH$_2$), 25.24 (CH$_3$CHPh), 21.78 (CH$_2$), 20.92 (CH$_2$), 11.35 (CH$_2$CH$_3$), 10.97 (CH$_2$CH$_3$) ppm. HRMS-ESI$^+$ (m/z): [M+H]$^+$ calcd for $C_{15}H_{25}N_2O$, 249.1967, found 249.1970.

3-Ethyl-D-norvalinamide (10a). MeOH (40 mL) was carefully added to 5% Pd on charcoal (0.53 g) under a flow of argon. Compound **9a** (3.51 g, 14 mmol) was added and the mixture was shaken (Parr Shaker) for 72 h under an atmosphere of hydrogen (60 psi). The reaction mixture was then filtered through a pad of Celite and the Celite was washed with MeOH (50 mL). After the solvent was removed *in vacuo*, the resulting solid was dissolved in DCM (10 mL) and the solvent was again removed *in vacuo* to give a white solid: yield 1.68 g (82%); m.p. 115 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.33 (s, CONH$_2$, 1H), 6.93 (s, CHNH$_2$, 1H), 3.12 (d, J = 4.7 Hz, CHNH$_2$, 1H), 1.41-1.50 (m, CHCH$_2$, 1H), 1.25-1.38 (m) and 1.10-1.22 (m) (2 × CH$_2$, 4H), 0.86 (t, J = 7.4 Hz, CH$_3$, 3H), 0.82 (t, J = 7.4 Hz, CH$_3$, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 177.30 (CONH$_2$), 55.79 (CHNH$_2$), 44.24 (CHCH$_2$), 22.20 (CH$_2$), 20.83 (CH$_2$), 11.65 (CH$_3$), 11.58 (CH$_3$). HRMS-ESI$^+$ (m/z): [M+H]$^+$ calcd for $C_7H_{17}N_2O$, 145.1341, found 145.1334.

3-Ethyl-D-norvaline (11a). Compound **10a** (1.68 g, 12 mmol) was mixed with concentrated HCl (50 mL) and the mixture was refluxed for 24 h. Upon cooling to room temperature, white crystals formed. After 30 min, the crystals were collected by suction filtration and air-dried: yield 1.68 g (79%). The hydrochloride salt was then converted to the free amino acid by dissolving the crystals (0.80 g, 4.4 mmol) in water (4 mL) with gentle heating followed passage through a reversed-phase, solid-phase extraction column (60 mL/10 g, C18-E, 55 µm, 70 Å). Fractions (3 mL) containing the amino acid (yellow spots on a silica TLC sheet developed with a potassium dichromate dip) were combined and the aqueous solvent was removed by lyophilization to give a white powder: yield 0.64 g (100%); m.p. 200 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.42 (br s, NH$_2$, 2H), 3.80 (d, J = 3.5 Hz, CHNH$_2$, 1H), 1.68-1.78 (m, CHCHNH$_2$, 1H), 1.18-1.30 (m), 1.30-1.45 (m), and 1.45-1.57 (m) (2 × CH$_2$, 4H), 0.90 (t, J = 7.4 Hz, CH$_3$) and 0.88 (t, J = 7.4 Hz CH$_3$) (6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.56 (COOH), 53.81 (CHCOOH), 42.30 (CHCHCOOH), 21.79 (CH$_2$), 21.59 (CH$_2$), 11.60 (CH$_3$), 11.45 (CH$_3$) ppm. HRMS-ESI$^+$ (m/z): [M+H]$^+$ calcd for $C_7H_{16}NO_2$, 146.1181, found 146.1172.

(2*R*)-3-Ethyl-3-methyl-2-{[(1*R*)-1-phenylethyl]amino}-pentanenitrile (7b). Aqueous HCl (2.9 M, 6.9 mL) was added to a solution of methanol (100 mL) and water (93 mL) followed by (*S*)-(–)-α-methylbenzylamine (2.55 mL, 20 mmol, 1.0 equiv.). 2-Ethyl-2-methylbutanal (2.83 mL, 20 mmol, 1.0 equiv.) was then added followed by NaCN (0.98 g, 20 mmol, 1.0 equiv.). After stirring for 48-60 h to allow for adequate asymmetric crystallization to occur, the product was collected by suction filtration giving white crystals: yield 4.28 g (88%); m.p. 51 °C; ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.42 – 7.22 (m, ArH, 5H), 4.06 (q, J = 6.5 Hz, CHPh, 1H), 3.01 (s, CHCN, 1H), 1.25-1.57 (m, CH$_3$CHPh, 2 × CH$_2$, and NH, 8H), 0.94 (s, CH$_3$CH, 3H), 0.69 (m, 2 × CH$_3$CH$_2$, 6H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 143.64 (C_{Ph}) 128.74 (2 × C_{Ph}), 127.81 (C_{Ph}), 127.41 (2 × C_{Ph}), 120.33 (C_{CN}), 56.88 (CHPh), 56.21 (CHCN), 39.22 (CCHCN), 28.21 (CH$_2$), 27.41(CH$_2$), 25.16 (CH$_3$CHPh), 20.58 (CH$_3$C), 7.88 (CH$_3$CH$_2$), 7.70 (CH$_3$CH$_2$) ppm. HRMS-ESI$^+$ (m/z): [M+H]$^+$ calcd for $C_{16}H_{25}N_2$, 245.2018, found 245.2014.

3-Ethyl-3-methyl-*N*^{2}-[(1*R*)-1-phenylethyl]-D-norvalinamide (9b). Concentrated H_2SO_4 (4 mL per gram of reactant; 315 mmol, 18.2 equiv.) was added to the amino nitrile **7b** (4.28 g, 18 mmol, 1.0 equiv.) and stirred for 72 h. The reaction mixture was then pipetted onto ice (75 mL) followed by addition of concentrated NH_4OH (aq.) (1 mL aliquots with cooling by additional ice) until the pH was 8. The resulting white gummy product and opaque white solution were poured into a separatory funnel with EtOAc used to transfer the gummy product. The solution was then extracted with EtOAc (3 ×100 mL) and the combined organic layers were dried over anhydrous Na_2SO_4. Note that in some preparations, a minor amount reactant remained, which was removed by column chromatography on silica using hexanes:EtOAc (8:2). The reactant (**2b**) and product (**4b**) had R_f values of 0.88 and 0.19, respectively. Removal of the solvent *in vacuo* gave a white solid: yield 3.15 g (68%); m.p. 111 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.36 – 7.14 (m, ArH, 5H and CONH, 1H), 6.98 (s, CONH, 1H), 3.47 (q, J = 6.5 Hz, CHPh, 1H), 2.54 (s, CHCONH$_2$, 1H), 2.01 (d, NH, 1H), 1.54 – 1.36 (m, CH$_2$) and 1.30 – 1.08 (m, CH$_3$CHPh and CH$_2$) (6H), 0.74 (s, CH$_3$C, 3H), 0.57 (t, J = 7.4 Hz, CH$_3$CH$_2$, 3H), 0.47 (t, J = 7.5 Hz, CH$_3$CH$_2$, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 175.46 (CONH$_2$), 145.76 (C_{Ph}), 127.95 (C_{Ph}), 126.99 (C_{Ph}), 126.60 (C_{Ph}), 63.74 (CHCONH$_2$), 56.02 (CHPh), 37.83 (CCHCONH$_2$), 27.13 (CH$_2$), 26.92(CH$_2$), 25.46 (CH$_3$CHPh), 19.74 (CH$_3$C), 7.66 (CH$_3$CH$_2$), 7.55(CH$_3$CH$_2$) ppm. HRMS-ESI$^+$ (m/z): [M+H]$^+$ calcd for $C_{16}H_{27}N_2O$, 263.2123, found 263.2121.

3-Ethyl-3-methyl-D-norvalinamide (10b). MeOH (35 mL) was carefully added to 5% Pd on charcoal (0.79 g) under a flow of argon. Compound **9b** (3.15 g, 12 mmol) was added and the mixture was shaken (Parr Shaker) for 72 h under an atmosphere of hydrogen (60 psi). The reaction mixture was then filtered through a pad of Celite and the Celite was washed with MeOH (50 mL). After the solvent was removed *in vacuo*, the resulting solid was dissolved in DCM (10 mL) and the solvent was again removed *in vacuo* to give a white solid: yield 1.90 g (100%); m.p. 91 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.17 (s, CONH, 1H), 6.80 (s, CHNH, 1H), 2.96 (s, CHNH$_2$, 1H), 1.47 – 1.16 (m, 2 × CH$_2$, 4H), 0.83 – 0.67 (m, CH$_3$C and 2 × CH$_3$CH$_2$, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 177.01 (CONH$_2$), 59.52 (CHNH$_2$), 38.73 (CCH$_3$), 27.72 (CH$_2$), 27.22 (CH$_2$), 20.09 (CH$_3$C), 8.43 (CH$_3$CH$_2$), 8.18 (CH$_3$CH$_2$). HRMS-ESI$^+$ (m/z): [M+H]$^+$ calcd for $C_8H_{19}N_2O$, 159.1497, found 159.1492; [M+Na]$^+$ calcd for $C_8H_{18}N_2ONa$, 181.1317, found 181.1312.

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3-Ethyl-3-methyl-D-norvaline (11b). Compound **10b** (1.91 g, 12 mmol) was mixed with concentrated HCl (50 mL) and the mixture was refluxed for 24 h. Upon cooling to room temperature, white crystals formed. After 30 min, the crystals were collected by suction filtration and air-dried: yield 1.32 g (56%). The hydrochloride salt was then converted to the free amino acid by dissolving the crystals (0.70 g, 3.6 mmol) in water (4 mL) with gentle heating followed passage through a reversed-phase, solid-phase extraction column (60 mL/10 g, C18-E, 55 µm, 70 Å). Fractions (3 mL) containing the amino acid (yellow spots on a silica TLC sheet developed with a potassium dichromate dip) were combined and the aqueous solvent was removed by lyophilization to give a white powder: yield 0.40 g (70%); m.p. 183 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.34 (br s, NH_2, 2H), 3.59 (s, $CHNH_2$, 1H), 1.31-1.47 (m, 2 × CH_2, 4H), 0.90 (s, CH_3C, 3H), 0.794 (t, J = 7.4 Hz, CH_3) and 0.790 (t, J = 7.4 Hz CH_3) (6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.97 (COOH), 58.03 (CHCOOH), 37.70 (CCH_2), 27.27 (CH_2), 26.84 (CH_2), 19.85 (CCH_3), 7.51 (CH_3CH_2), 7.47 (CH_3CH_2) ppm. HRMS-ESI$^+$ (m/z): $[M+H]^+$ calcd for $C_8H_{18}NO_2$, 160.1338, found 160.1331.

Quantification, enantiomeric excess, and circular dichroism (CD)

Compounds **6a**, **6b**, **11a**, and **11b** were quantified using ^{1}H NMR spectroscopy with pyrazine as an internal standard. Each amino acid (20.0 mg) was dissolved in DMSO-d_6 (1.000 mL). To this solution (525 µL), a solution of pyrazine (175 µL, 0.400 M in DMSO-d_6) was added to yield a final concentration of pyrazine equal to 0.100 M. The ratio of peak areas corresponding to the ^{1}H NMR signals arising from the protons of pyrazine and the CH_3CH_2 protons of compounds **6a**, **6b**, **11a**, and **11b** were used to calculate the concentration.

The enantiomeric excess was determined using HPLC analysis of the *o*-phthaldialdehyde-derivatized amino acids as described for the kinetic assays (*vide supra*). Compounds **6a** and **11a** were derivatized in the presence of *N*-acetyl-L-cysteine and compounds **6b** and **11b** were derivatized in the presence of *N*-isobutyryl-L-cysteine.

For CD analyses, compounds **6a**, **6b**, **11a**, and **11b** were each dissolved in 5.0 M HCl to yield a final concentration of 5.0 mg/mL and CD spectra were recorded between 200 and 260 nm using a quartz cuvette with a 0.1-cm light path. In addition, the spectrum of the 5.0 M HCl was recorded. The spectrum for each compound was recorded in triplicate, averaged, and then the averaged spectrum of the 5.0 M HCl solution was subtracted.

Conflicts of interest

There are no conflicts to declare.

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TOC Figure

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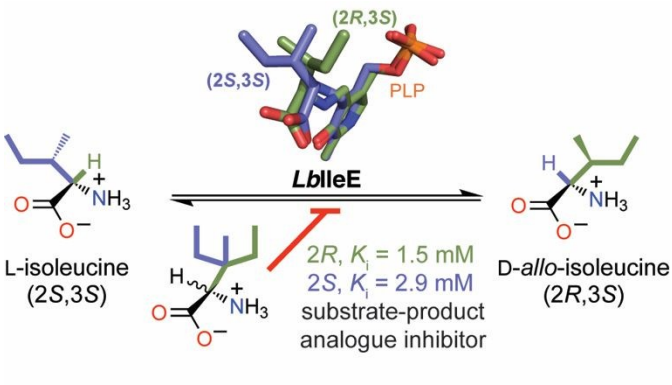

TOC Statement

Substrate-product analogues, designed based on partial movement of the *sec*-butyl side chain during catalysis, inhibit isoleucine 2-epimerase.

By Noa T. Sorbara, Joshua W.M. MacMillan, Gregory D. McCluskey, and Stephen L. Bearne