Substrate-Product Analogue Inhibitors of Ile 2-Epimerase

Sorbara, N., et al.

Electronic Supplementary Information

for

Substrate-Product Analogue Inhibitors Isoleucine 2-Epimerase from *Lactobacillus buchneri* by Rational Design[†]

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Figure S1. ¹H NMR spectrum of 3-ethyl-L-norvaline (6a)



Figure S1

Figure S2. ¹³C NMR spectrum of 3-ethyl-L-norvaline (6a)



Figure S2



Figure S3. ¹H NMR spectrum of (2S)-3-ethyl-3-methyl-2-{[(1S)-1-phenylethyl]amino}pentanenitrile (2b)

Figure S3





Figure S4

Figure S5. ¹H NMR spectrum of 3-ethyl-3-methyl- N^2 -[(1*S*)-1-phenylethyl]-L-norvalinamide (4b)



Figure S5

Figure S6. 13 C NMR spectrum of 3-ethyl-3-methyl- N^2 -[(1S)-1-phenylethyl]-L-norvalinamide (4b)



Figure S6





Figure S7





Figure S8





Figure S9

Figure S10. ¹³C NMR spectrum of 3-ethyl-3-methyl-L-norvaline (6b)



Figure S10





Figure S11



Figure S12. ¹³C NMR spectrum of (2R)-3-ethyl-2-{[(1R)-1-phenylethyl]amino}pentanenitrile (7a)







Figure S13

Figure S14. ¹³C NMR spectrum of 3-ethyl- N^2 -[(1*R*)-1-phenylethyl]-D-norvalinamide (9a)



Figure S14





Figure S15

Figure S16. ¹³C NMR spectrum of 3-ethyl-D-norvalinamide (10a)



Figure S16





Figure S17

Figure S18. ¹³C NMR spectrum of 3-ethyl-D-norvaline (11a)



Figure S18





Figure S19





Figure S20

Figure S21. ¹H NMR spectrum of 3-ethyl-3-methyl- N^2 -[(1*R*)-1-phenylethyl]-D-norvalinamide (9b)



Figure S21





Figure S22





Figure S23



Figure S24. ¹³C NMR spectrum of 3-ethyl-3-methyl-D-norvalinamide (10b)



Figure S25. ¹H NMR spectrum of 3-ethyl-3-methyl-D-norvaline (11b)



Figure S25

Figure S26. ¹³C NMR spectrum of 3-ethyl-3-methyl-D-norvaline (11b)



Figure S26

Figure S27. High resolution mass spectrum of 3-ethyl-L-norvaline $(6a) - H^+$ adduct



Figure S27

Figure S28. High resolution mass spectrum of 3-ethyl-L-norvaline $(6a) - Na^+$ adduct



Figure S28

Figure S29. High resolution mass spectrum of (2*S*)-3-ethyl-3-methyl-2-{[(1*S*)-1-phenylethyl]amino}pentanenitrile (**2b**)

Analysis Info								Acquisition Date	8/17/2018 10:02:	42 AM
Analysis Name Method Sample Name Comment	D:\Data\Xiao\Aug 17 2018000001.d Xiao 1.m 2b							Operator Instrument		57
Acquisition Para Source Type Scan Range Scan Begin Scan End	ameter ESI n/a 50 m/z 1500 m/z		lon Polari Capillary Hexapole Skimmer Hexapole	ity Exit RF 1		Positive 80.0 V 125.0 V 50.0 V 22.5 V		Set Corrector Fill Set Pulsar Pull Set Pulsar Push Set Reflector Set Flight Tube Set Detector TOF	45 V 400 V 400 V 1300 V 9000 V 2200 V	
Sum Formula C 16 H 25 N 2	Sigma m/z 0.04 245.2012	Err [ppm] 3.85	Mean Err [ppm] 3.62	rdb 5.50	N Rule ok	e even				
ens. :10 ⁴				245.	2003				+MS, 0.6	-1.2min #(31-67
6- 4-										
2-							246.2037			
0	243	244		245	. L	24		247	248	,,

Figure S29

Figure S30. High resolution mass spectrum of 3-ethyl-3-methyl- N^2 -[(1*S*)-1-phenylethyl]-L-norvalinamide (4b)



Figure S30

Figure S31. High resolution mass spectrum of 3-ethyl-3-methyl-L-norvalinamide (5b)



Figure S31

Figure S32. High resolution mass spectrum of 3-ethyl-3-methyl-L-norvaline (6b)



Figure S32

Figure S33. High resolution mass spectrum of (2R)-3-ethyl-2-{[1R)-1-phenylethyl]amino}pentanenitrile (7a)



Figure S33

Figure S34. High resolution mass spectrum of 3-ethyl- N^2 -[(1*R*)-1-phenylethyl]-D-norvalinamide (9a)





Figure S35. High resolution mass spectrum of 3-ethyl-D-norvalinamide (10a)

Analysis Name D:\Data\Xiao\Aug Method Xiao 1.m Sample Name 10a Comment	17 2018000011.d		Operator Instrument	Administrator	
Acquisition Parameter Source Type ESI Scan Range n/a Scan Begin 50 m/z Scan End 1500 m/z				microror	57
Source Type ESI Scan Range n/a Scan Begin 50 m/z Scan End 1500 m/z			Set Corrector Fill	45 V	
	Ion Polarity Capillary Exit Hexapole RF Skimmer 1 Hexapole 1	Positive 80.0 V 125.0 V 50.0 V 22.5 V	Set Pulsar Pull Set Pulsar Push Set Reflector Set Flight Tube Set Detector TOF	400 V 400 V 1300 V 9000 V 2200 V	
Sum Formula Sigma m/z C 7 H 17 N 2 O 1 0.03 145.1335	Err [ppm] Mean Err [ppm] rdb 1.08 0.94 0.50	NRule e ok even			
	145.1334			+MS, 0.6	·1.4min #(35-74)
1.25-					
1.00					
0.75-					
0.50-					
0.25-					
0.00		146.1366 , / ,			

Figure S35

Figure S36. High resolution mass spectrum of 3-ethyl-D-norvaline (11a)



Figure S36

Figure S37. High resolution mass spectrum of (2R)-3-ethyl-3-methyl-2-{[(1R)-1-phenylethyl]amino}pentanenitrile (7b)

Analysis Info								Acc	uisition Date	8/17/2018 10:1	3:11 AM	
Analysis Name Method Sample Name Comment	D:∖Data\Xia Xiao 1.m 7b	o\Aug 17 20	Aug 17 2018000002.d					Operator Instrument		Administrator micrOTOF	57	
Acquisition Pa Source Type Scan Range Scan Begin Scan End	rameter ESI n/a 50 m/z 1500 m/z		lon Polarit Capillary E Hexapole Skimmer ⁄ Hexapole	y Exit RF I 1		Positive 80.0 V 125.0 V 50.0 V 22.5 V			Set Corrector Fill Set Pulsar Pull Set Pulsar Push Set Reflector Set Flight Tube Set Detector TOF	45 V 400 V 1300 V 9000 V 2200 V		
Sum Formula C 16 H 25 N 2	a Sigma m/z 0.06 245.2012	Err [ppm]	Mean Err [ppm] -0.49	rdb 5.50	N Rule ok	e ⁻ even						_
ens. 10 ⁴⁻			245.2	014						+MS, 0).6-1.3min #(35	5-69)
2.5-												
2.0-												
1.5-												
1.0-												
0.5-												
-			J			:	246.2043	5				
0.0	3.5 244.0	244.5	245.0	24	45.5	246.0	,,,)	246.5	247.0	247.5 24	18.0	m/z

Figure S37

Figure S38. High resolution mass spectrum of 3-ethyl-3-methyl- N^2 -[(1*R*)-1-phenylethyl]-D-norvalinamide (9b)





Figure S39. High resolution mass spectrum of 3-ethyl-3-methyl-D-norvalinamide (10b) – H+ adduct



Figure S39

Figure S40. High resolution mass spectrum of 3-ethyl-3-methyl-D-norvalinamide $(10b) - Na^+$ adduct



Figure S40

Figure S41. High resolution mass spectrum of 3-ethyl-3-methyl-D-norvaline (11b)



Figure S41



Figure S42

Figure S42. Representative HPLC chromatograms of derivatized **6a** (A) and **11a** (B). Compounds **6a** and **11a** were derivatized with *N*-acetyl-L-cysteine and OPA, and were separated on the reversed-phase column using isocratic elution with sodium acetate buffer (50 mM, pH 5.9):methanol (49:51). The %ee values for **6a** and **11a** are 96.3% and 99.1%, respectively. RT = retention time. See the Experimental section for additional details.



Figure S43

Figure S43. Representative HPLC chromatograms of derivatized **6b** (A) and **11b** (B). Compounds **6b** and **11b** were derivatized with *N*-acetylcysteine and OPA, and were separated on the reversed-phase column using isocratic elution with sodium acetate buffer (50 mM, pH 5.9):methanol (49:51). The %ee values for **6b** and **11b** are 100.0% and 98.0%, respectively. RT = retention time. See the Experimental section for additional details.



Figure S44. Representative circular dichroism spectra of the enantiomers of **6** and **11**. Spectra are shown for **6a** (---) and **11a** (—) in panel A, and for **6b** (---) and **11b** (—) in panel B. See the Experimental section for additional details.



Figure S45. SDS-PAGE gel (8%) showing the purification of *Lb*IleE. Lane 1, molecular weight marker; lane 2, crude cell lysate; lane 3, binding buffer eluant; lane 4, wash buffer eluant; and lane 5, strip buffer eluant (purified *Lb*IleE post-dialysis). The calculated MW of the His₆-tagged *Lb*IleE fusion protein, based on the amino acid sequence, is 53 487 Da.



Figure S46

Figure S46. Representative Michaelis-Menten plots for the determination of kinetic parameters. The dependence of the initial velocities (v_i) on the concentration of substrate is shown for L-isoleucine (A), D-*allo*-isoleucine (B), L-2-cyclohexylglycine (C), D-2-cyclohexylglycine (D), 3-ethyl-L-norvaline (**6a**) (E), and 3-ethyl-D-norvaline (**11a**) (F). The curves are fits of eqn. 1 to the initial velocity data. See the Experimental section for additional details.



Figure S47. Competitive inhibition of *Lb*IleE by **6b**. A representative Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) showing the competitive inhibition of *Lb*IleE by **6b** are shown. The concentrations of **6b** were 0 (\bullet), 5.0 (\blacktriangle), and 10.0 mM (\blacktriangledown). The concentrations of the L-isoleucine substrate were 5.0, 10.0, 15.0, 25.0, and 50.0 mM, and the concentration of *Lb*IleE was 2.0 µg/mL. A representative replot (C) of the apparent K_m/V_{max} (values from direct fits of the Michaelis-Menten eqn. 2 to the initial velocity data) vs. inhibitor concentration is shown. The K_i value (determined in triplicate) for inhibition of *Lb*IleE by **6b** is 2.9 ± 0.2 mM.



Figure S48. Competitive inhibition of *Lb*IleE by **11b**. A representative Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) showing the competitive inhibition of *Lb*IleE by **11b** are shown. The concentrations of **11b** were 0 (\bullet), 5.0 (\blacktriangle), and 10.0 mM (\blacktriangledown). The concentrations of the L-isoleucine substrate were 5.0, 10.0, 15.0, 25.0, and 50.0 mM, and the concentration of *Lb*IleE was 2.0 µg/mL. A representative replot (C) of the apparent K_m/V_{max} (values from direct fits of the Michaelis-Menten eqn. 2 to the initial velocity data) vs. inhibitor concentration is shown. The K_i value (determined in triplicate) for inhibition of *Lb*IleE by **11b** is 1.5 ± 0.2 mM.



Figure S49

Figure S49. *Lb*IleE catalyzes hydrogen-deuterium exchange with **6a** and **11a**, but not **6b** and **11b**. Selected regions of representative ¹H NMR spectra are shown. Deuterated assay buffer (200 mM citrate, pD 5.5, containing 0.1 mM PLP) (A) showed the appearance of three signals at 3.82, 3.69, and 3.58 ppm (denoted by *) upon addition of enzyme (B). Over 1 h in the absence of enzyme, the doublet arising from the α -proton of **6a** (C) and **11a** (E) at 3.76 ppm showed no H-D exchange. However, in the presence of enzyme, the doublets showed a marked decrease in signal intensity for both **6a** (D) and **11a** (F), indicating that the α -proton of both compounds had undergone H-D exchange. The integrals are shown above the doublets (3.76 ppm) relative to the overlapping triplets (centered at 0.90 ppm) corresponding to the signal arising from both CH₃ groups (*i.e.*, 6H). Over 1 h in the absence of enzyme, the singlet arising from the α -proton of **6b** (G) and **11b** (I) at 3.57 ppm showed no H-D exchange. In the presence of enzyme, the singlets showed no significant decrease in signal intensity for both **6b** (H) and **11b** (J), relative to the CCH₃ (0.90 ppm, 3H) and the overlapping triplets (centered at 0.80 ppm, 6H) corresponding to the signal arising from both CH₂CH₃ groups, indicating that no H-D exchange occurred at the α -position of both compounds. See the Experimental section for additional details.



Figure S50. Representative v_i/v_o vs. [inhibitor] plots (IC₅₀ curves) for the inhibition of *Lb*IleE by *gem*-dialkylglycines. The curves shown are the nonlinear regression fits of eqn. 3 to the relative velocity values (v_i/v_o). The IC₅₀ values for inhibition by 2,2-diethylglycine (**12**), 2,2-di-*n*-propylglycine (**13**), and 2,2-di-*n*-butylglycine (**14**) were 334 ± 18 mM, 43 ± 5 mM, and 16 ± 2 mM, respectively (from three determinations). For compounds **13** and **14**, the IC₅₀ values are estimated by extrapolation due to the limited solubility of the compounds in the assay buffer. The concentration of L-isoleucine was 5.0 mM and the concentration of *Lb*IleE was 2.0 µg/mL. See the Experimental section for additional details.



Figure S51. Competitive inhibition of *Lb*IleE by 2,2-di-*n*-butylglycine (14). A representative Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) showing the competitive inhibition of *Lb*IleE by 14 are shown. The concentrations of 14 were 0 (\bullet), 5.4 (\blacktriangle), and 10.8 mM (\triangledown). The concentrations of the L-isoleucine substrate were 2.5, 5.0, 10.0, 15.0, and 20.0 mM, and the concentration of *Lb*IleE was 2.0 µg/mL. A representative replot (C) of the apparent K_m/V_{max} (values from direct fits of the Michaelis-Menten eqn. 2 to the initial velocity data) vs. inhibitor concentration is shown. The K_i value (determined in triplicate) for inhibition of *Lb*IleE by 14 is $11.0 \pm 0.2 \text{ mM}$.