Chemoenzymatic Synthesis of Enantiomerically Pure (2*S*,3*R*,4*S*)-4-Hydroxyisoleucine, an Insulinotropic Amino Acid Isolated from Fenugreek Seeds

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A short six-step synthesis of (2*S*,3*R*,4*S*)-4-hydroxyisoleucine (**1a**) with total control of stereochemistry is reported, the last step being the enzymatic resolution by hydrolysis of a *N*-phenylacetyl lactone derivative using the commercially available penicillin acylase G immobilized on Eupergit C (E-PAC).

Introduction

4-Hydroxyisoleucine was first isolated as a new amino acid lactone from γ-aminitin hydrolysate[1] and later from ε-amanitin,[2] one of the cyclic octapeptides found in the highly poisonous mushroom, *Amanita phalloides*.[4] In 1973 Fowden et al.[3] reported the isolation and identification of free 4-hydroxyisoleucine from fenugreek seeds, *Trigonella foenum graecum* L. (Leguminosae).

Their results suggested that 4-hydroxyisoleucine in fenugreek and in γ-amanitin have the same absolute configurations at the three chiral carbon atoms. Gieren et al.[4] had correctly determined the configurations which were 2*S*,3*R*,4*S*; this result was confirmed by Alcock[5] who carried out an X-ray crystal structure determination of 4-hydroxyisoleucine. In fenugreek, (2*R*,3*R*,4*S*)-4-hydroxyisoleucine is also present as a minor component. Dardenne et al.[6] reported the presence of the same isomer in the fruit bodies of *Lactarius camphoratus*. Ribes et al.[7] showed that (2*S*,3*R*,4*S*)-4-hydroxyisoleucine extracted from fenugreek seeds, known for their antidiabetic properties in traditional medicine, was an insulinostimulant compound which could be used in diabetes mellitus treatment. More recently they tested synthetic analogues on insulin secretion in comparison with the natural one: (2*S*,3*R*,4*S*)-4-hydroxyisoleucine and its minor isomer (2*R*,3*R*,4*S*)-4-hydroxyisoleucine: (2*S*,4*R*)- and (2*S*,4*S*)-4-hydroxynorvalines as well as (2*S*,3*S*)- and (2*S*,3*R*)-3-hydroxynorvalines prepared in our laboratory.[8a,8b] They proved that insulinotropic properties were shown by only the major isomer (2*S*,3*R*,4*S*)-4-hydroxyisoleucine in the micromolar range. By 2010,[9] it has been estimated that more than 200 million of the world's population will have diabetes mellitus and it is obvious that the search for new improved antidiabetic drugs is of real interest. A few stereoselective syntheses of 4-hydroxyisoleucine have been reported in the literature, including the enantioselective synthesis of the (3*R*,4*R*,5*R*)- and the (3*R*,4*S*,5*S*)-lactone diastereomers by Schöllkopf[10] using a reaction between a chiral glycine anion equivalent and epoxybutane. However the stereochemical assignment of the (3*R*,4*R*,5*R*)-lactone was erroneous as demonstrated by Fredj[11] et al. who achieved the enantiospecific synthesis of the 3*R*,4*R*,5*R* and 3*S*,4*R*,5*R* diastereomers starting from benzyl 2,3-anhydro-4-*O*-(*tert*-butyldimethylsilyl)-β-L-ribopyranoside.

We described in 2001[12] the synthesis of (2*R*,3*R*,4*R*)-4-hydroxyisoleucine lactone with good yields and total control of the stereochemistry. The key intermediate was the didehydroamino acid derivative arising from an aldol dehydration reaction between a glycine anion equivalent and butan-2,3-dione using an optically pure oxazinone as the chiral auxiliary. X-ray structures of each intermediate have been obtained and the stereochemistry of each asymmetric center clearly identified.[13]

Very recently, Wang et al.[14] described a practical eight-step synthesis of (2*S*,3*R*,4*S*)-4-hydroxyisoleucine. The key steps involved the biotransformation of ethyl 2-methylacetoacetate to (2*S*,3*S*)-3-hydroxy-2-methylbutanoate with *Geotrichum candidum* and an asymmetric Strecker synthesis.

In 2001, Sauvaire et al. published a patent concerning the use of amino acids for making medicines for treating insulin resistance.[15]

In 1989, Englisch-Peters[16] described the synthesis of 4-hydroxy analogues of leucine and isoleucine via 1,4-Michael addition and 4-hydroxy valine by a modified Erlenmeyer strategy. The diastereomers of 4-hydroxy valine were separated by column chromatography and the 4-hydroxy-Leu and 4-hydroxy-Ile by reversed-phase HPLC. The enantiomers of allo- and iso-4-hydroxyvaline were enzymatically

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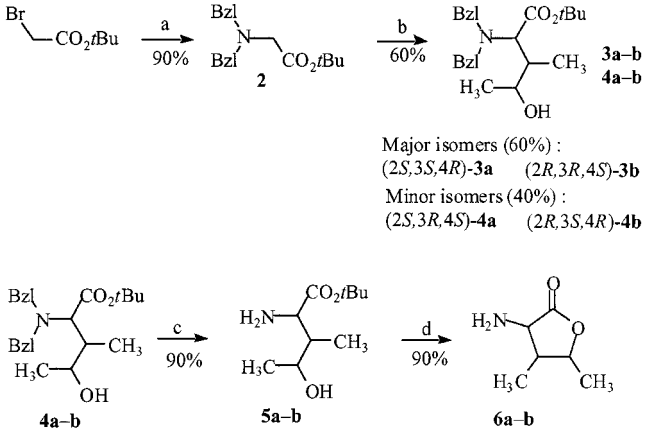
separated using D- or L-amino acid oxidase which selectively oxidized the α-amino group of the D- or L- enantiomer to the corresponding α-keto acid.

We describe in this paper a new strategy in six steps combining a glycine enolate approach with the *cis*-2,3-dimethyloxirane ring opening and a final enantioselective enzymatic hydrolysis using immobilized penicillin acylase (E-PAC).

Results

In our case racemic 4-hydroxyisoleucine lactone was obtained in four steps as described in Scheme 1.



Scheme 1. Synthesis of (3*S*,4*R*,5*S*)-3-amino-4,5-dimethyldihydrofuran-2-one (**6a**) and (3*R*,4*S*,5*R*)-3-amino-4,5-dimethyldihydrofuran-2-one (**6b**): a) 2.2 mmol of dibenzylamine; b) 1) LiHMDS/THF (1 M, 1.5 mmol), 30 min, −30 °C; 2) *cis*-2,3-dimethyloxirane (0.3 mmol/BF_3·Et_2O/TMSCl); 3) NH_4Cl; 4) diastereomers separation by silica gel column chromatography; c) $Pd(OH)_2$/C/MeOH; d) TFA (50% DCM)

tert-Butyl bromoacetate was substituted by dibenzylamine in basic conditions to obtain *tert*-butyl di-*N*-benzylglycinate (**2**) in 90% yield. Direct ring opening of the commercially available *cis*-butylene oxide with the lithium enolate of *tert*-butyl dibenzylglycinate afforded a diastereomeric mixture in 60% yield. The couples of diastereomers **3(a,b)** and **4(a,b)** were separated using silica-gel chromatography and separately identified (*dr* = 60:40) as racemates. Applying the same strategy described in Scheme 1, the final product prepared from the major racemate **3(a,b)** was *inactive* in the biological antidiabetic tests. ^{1}H NMR studies and optical rotation values confirmed the 2*R*,3*S*,4*R* configuration of other isomer.

We describe below the strategy which led to the biologically active compound **1a**.

Starting from the minor racemate **4(a,b)**, catalytic hydrogenolysis and *tert*-butyl ester hydrolysis gave racemate **6(a,b)**, (3*S*,4*R*,5*S*)- and (3*R*,4*S*,5*R*)-3-amino-4,5-dimethyldihydrofuran-2-one in 90% yield.

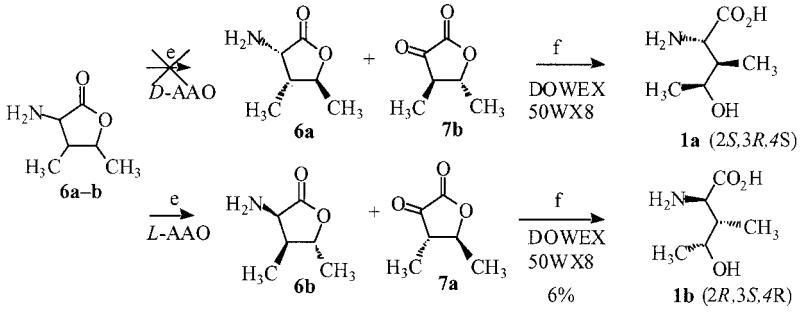
First commercially available α-aminooxidases were tested.[16]

As shown in Scheme 2, D-AAO (EC, 1.4.3.3) from *hog kidney*, a very cheap and easy to use enzyme, could catalyze the enantioselective aminooxidation of the (*D*) α-amino acids leading to the corresponding five-membered ring keto acid (4*R*,5*R*)-4,5-dimethyldihydrofuran-2,3-dione (**7b**) and the natural compound **1a** (2*S*,3*R*,4*S*)-2-amino-4-hydroxy-3-methylpentanoic acid. One **7b** derivative is a very strong smelling furanone called sotolone[17] and in this step the absence of an odour after stirring for three days indicated that aminooxidation had been unsuccessful.

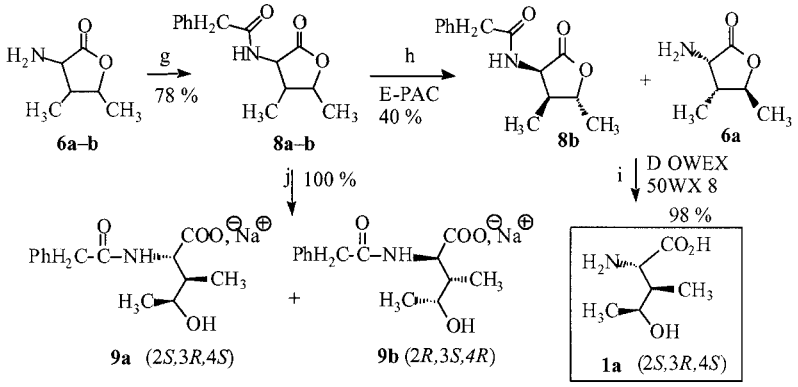
L-AAO (EC, 1.4.3.2) is an expensive and toxic enzyme, isolated from *Crotalus adamanteus* venom and it catalyzed enantioselective aminooxidation of the L-α-amino acids in the corresponding five-membered ring keto acid (4*S*,5*S*)-4,5-dimethyldihydrofuran-2,3-dione (**7a**), also very favourous and (2*R*,3*S*,4*R*)-2-amino-4-hydroxy-3-methylpentanoic acid (**1b**), but in very low yield (6%). (2*R*)-4-hydroxyisoleucine (**1b**) was also a minor product from the fenugreek seeds extraction; after purification on ion-exchange resin DOWEX 50WX8, **1b** was tested for its antidiabetic activity with no effect.

The last chemoenzymatic strategy applied afforded (2*S*,3*R*,4*S*)-2-amino-4-hydroxy-3-methylpentanoic acid (**1a**) as (2*S*,3*R*,4*S*)-4-hydroxyisoleucine (Scheme 3).

The NH_2 group of racemic lactone **6a−b** was protected by a phenylacetic group, the best hydrolytic group for the commercially available metalloenzyme, penicillin acylase G which is immobilized on Eupergit C. E-PAC enantioselec-



Scheme 2. Enzymatic enantioselective aminooxidation of **6a** and **6b** by D-AAO and L-AAO: e) phosphate buffer pH 8.3, O_2 bubbling, 1% H_2O_2, catalase and D-AAO (or L-AAO), 1 day, RT; f) purification of mother solution liquor by DOWEX 50WX8 ion-exchange resin (H^+ form)



Scheme 3. Synthesis of (2*S*,3*R*,4*S*)-2-amino-4-hydroxy-3-methyl-pentanoic acid **1a**; g) phenylacetic chloride/Et_3N; h) phosphate buffer pH 9, penicillin acylase G immobilized on Eupergit C, 1 day, room temp.; i) purification of mother solution liquor by DOWEX 50WX8 ion-exchange resin (H^+ form); j) 1 M NaOH in dioxan

tively hydrolyzed the phenylacetic amide bond of an L-amino acid in the pH range 5−7 when its carboxyl group complexed the metal (Co^{2+}) present inside the active site of this enzyme. At pH 5−7 lactone **8a−b** was formed spontaneously. This resolution was performed in unusual conditions (pH 8.5−9).

The extent of bioconversion was stopped at 40%, before the 2*R* lactone **8b** became substrate by default in these basic conditions. The reaction was followed by HPLC on a C-18 column and by Chiral HPLC on a Chiralcel OJ column (*ee* = 99±1%). Both amino acids **1a** and **8b** were separated on DOWEX 50WX8 and the pure product **1a** was obtained in 38% yield. After recrystallization from 95% ethanol, optically pure (2*S*,3*R*,4*S*)-4-hydroxyisoleucine **1a** was characterized by its ^1H NMR spectrum, m.p., optical activity, and chiral HPLC, which were in complete agreement with the data reported in the literature.[3,5]

As shown in Scheme 3, the ring opening of **8a−b** in 1 M NaOH afforded in quantitative yield *N*-phenylacetyl amino acids **9a−b** as racemate sodium salts easily separated on chiral HPLC [Table 1 column (b)]. The identical absorbance and the different retention time of **9a** and **9b** were determined by chiral-HPLC analysis without any purification or other analysis. This experiment was performed to identify each isomer.

Table 1. HPLC conditions: (a) isocratic conditions on C-18 column inverse phase, λ = 214 nm, 60% H_2O TFA 1‰ and 40% CH_3CN TFA 1‰; flow rate: 1 mL/min; (b) isocratic conditions on Chiralcel OJ column (DAICEL) normal phase, λ = 214 nm, 85% hexane 15% 2-propanol flow rate: 0.8 mL/min

Products	α_D	Retention time (a)	Retention time (b)
1a (2*S*,3*R*,4*S*)	+31 (*c* = 1, D_2O)	8 min	7.3 min
1b (2*R*, 3*S*,*4R*)	−30 (*c* = 1, D_2O)	8 min	5.5 min
phenylacetic acid		6.5 min	/
6a	/	/	22 min
8b	/	10 min	14 min
9a	/	12 min	15 min
9b	/	12 min	17 min

Conclusion

An efficient synthesis of optically pure (2*S*,3*R*,4*S*)-4-hydroxyisoleucine has been performed in only six steps. The optically pure (2*S*,3*R*,4*S*)-4-hydroxyisoleucine has been tested for its antidiabetic activity by Prof. Sauvaire et al. and presented exactly the same activity as the compound extracted from fenugreek seeds.

The other isomers obtained from the compound, **3a−b** (2*S*,3*S*,4*R*)- and (2*R*,3*R*,4*S*)-4-hydroxyisoleucines, have been also characterized and tested for their biological activity with no positive responses.

Using E-PAC to immobilize the enzyme presents the possibility for it to be reused by simple filtration or in continuous flow in a batch reactor. This process will be optimized to decrease the cost of this efficient synthesis.

Experimental Section

General Remarks: Melting points were obtained using a Büchi 510 capillary apparatus and are uncorrected. Infrared spectra were recorded using a Perkin−Elmer Fourier transform spectrometer. ^1H NMR spectra were recorded at 250 MHz or at 400 MHz using a Bruker AC400 instrument. For ^1H NMR or ^{13}C spectra recorded in $CDCl_3$, DCl, D_2O, or MeOD; chemical shifts are quoted in parts per million, referenced to the residual solvent peak. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br, broad. Coupling constants are reported in Hertz (Hz). Diastereoisomeric ratios (*dr*) were determined by ^1H NMR or HPLC analysis on the crude product. Low-resolution mass spectra were recorded on a micromass electrospray instrument with only molecular ion and other major peaks being reported. Optical rotations were determined on a Perkin-Elmer 241 polarimeter at room temperature. Flash chromatography was carried out using E-Merck Silica Gel (Kieselgel 60, 230−400 mesh) as stationary phase. Thin-layer chromatography was carried out on aluminum plates pre-coated with Merck Silica-gel 60F254 and were visualized by quenching of UV fluorescence, by iodine vapor or by ninhydrin spray. THF was distilled from sodium/benzophenone ketyl. Reagents were supplied from commercial sources (Aldrich, Fluka). The immobilized acylase E-PAC (EC, 3.5.1.11), D-AAO (EC, 1.4.3.3) from *hog kidney* and L-AAO (EC, 1.4.3.2) from *Crotalus adamanteus* venom were from Fluka.

***tert*-Butyl *N*,*N*-Dibenzyl Glycinate (2):** Dibenzylamine (2.12 mL, 11 mmol) was added dropwise over 5 min to a stirred solution of *tert*-butyl bromoacetate in ethanol (0.74 mL, 5 mmol) and the mixture was then stirred at room temperature for 4 h. The resulting mixture was filtered, the salt washed with a mixture of diethyl ether/hexane (1:1). The expected compound **2** (1.4 g, 90% yield) was obtained as a colorless solid after purification by chromatography on silica gel (eluent Et_2O/hexane, 10:90). R_f = 0.50 (Et_2O/hexane, 10:90), m.p. 68 °C. ES^+ MS: *m*/*z* = 312 [M + H^+], 334 [M + Na^+]. ^1H NMR (250 MHz, $CDCl_3$): δ = 1.53 (s, 9 H, *t*Bu), 3.24 (s, 2 H, CH_2), 3.87 (s, 4 H, CH_2), 7.26−7.49 (10 H, m, Ph).

***tert*-Butyl (2*S*,3*S*,4*R*)-2-Dibenzylamino-4-hydroxy-3-methylpentanoate (3a), (2*R*,3*R*,4*S*)-... (3b), (2*S*,3*R*,4*S*)-... (4a), (2*R*,3*S*,4*R*)-... (4b):** A solution of 1 M LiHMDS in anhydrous THF (9.6 mL, 9.60 mmol) was added dropwise to a stirred solution of **2** (2 g, 6.42 mmol) in dry THF (12 mL) at 0 °C. After 1 h stirring at −30 °C *cis*-2,3-dimethyloxirane (0.33 mL, 3.8 mmol), $BF_3{\cdot}OEt_2$ (0.8 mL), and TMSCl (0.87 mL) were added. After 15 h stirring at −30 °C the reaction mixture was neutralized with saturated aqueous NH_4Cl. (20 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried with $MgSO_4$ and the solvents were evaporated under reduced pressure. The oxirane opening was afforded in 60% yield (1.4 g). Both racemates (**3a-b** and **4a-b**) were separately identified after chromatography on silica gel, eluting with hexane/diethyl ether/CH_2Cl_2 (50 mL 90:5:5, then 500 mL 85:7.5:7.5, then 500 mL 80:10:10, and 500 mL 75:12.5:12.5). HPLC analysis proved a diastereomeric ratio of 60:40.

3a−b (Major isomers): (2*S*,3*S*,4*R*)-*tert*-Butyl 2-Dibenzylamino-4-hydroxy-3-methylpentanoate (3a); (2*R*,3*R*,4*S*)-... (3b): 885 mg, yield 60%; m.p. 99−100 °C; R_f = 0.22 (hexane/DCM/Et_2O, 3:1:1). ^1H NMR (250 MHz, $CDCl_3$): δ = 1.03 (d, CH_3, *J* = 6.9 Hz, 3 H), 1.05 (d, *J* = 6.3 Hz, 3 H, CH_3), 1.6 (s, 9 H, *t*Bu), 1.80−1.90 (br, 1 H, OH), 3.13 (d, *J* = 9.6 Hz, 1 H, CH), 2.13−2.32 (m, 1 H, CH), 3.56−3.68 (m, 1 H, CH), 3.80 (dd, J_1 = J_2 = 13.9 Hz, 4 H, 2 CH_2), 7.28−7.46 (m, 10 H, 2 Ph) ppm. ^{13}C NMR (300 MHz, $CDCl_3$): δ = 8.5, 20.2, 29.0, 36.7, 56.8, 62.0, 68.0, 73.5, 127.0, 128.2, 128.8, 136.2, 173.7. MS ESI > 0: 384 [M + H^+], 767 [2M + H^+]. $C_{24}H_{33}NO_3$ (383): calcd. C 75.19, H 8.61, N 3.65. found C 75.14, H 8.56, N 3.63.

4a−b (Minor isomers): *tert*-Butyl (2*S*,3*R*,4*S*)-2-Dibenzylamino-4-hydroxy-3-methylpentanoate (4a); (2*R*,3*S*,4*R*)-... (4b): 515 mg, yield 40%; m.p. 96−97 °C; R_f = 0.36 (hexane/DCM/Et_2O, 3:1:1). ^1H NMR (400 MHz, $CDCl_3$): δ = 0.72 (d, *J* = 6.6 Hz, 3 H, CH_3), 1.11 (d, *J* = 6.6 Hz, 3 H, CH_3), 1.63 (s, 9 H, *t*Bu), 2.04−2.17 (m, 1 H, CH), 3.22 (d, *J* = 11 Hz, 1 H), 3.31−3.44 (m, 1 H), 3.73 (dd, J_1 = J_2 = 13.8 Hz, 4 H, 2 CH_2), 6.45 (s, 1 H, OH), 7.29−7.4 (m, 10 H, 2 Ph) ppm. ^{13}C NMR (300 MHz, $CDCl_3$): δ = 9.0, 20.4, 29.0, 36.5, 56.8, 62.2, 68.3, 73.5, 127.0, 128.2, 128.8, 136.2, 173.5. MS ESI > 0: 384 [M + H^+], 767 [2M + H^+]. $C_{24}H_{33}NO_3$ (383): calcd. C 75.19, H 8.61, N 3.65. found C 75.46, H 8.87, N 3.58.

***tert*-Butyl (2*S*,3*R*,4*S*)-2-Amino-4-hydroxy-3-methylpentanoate (5a) and *tert*-Butyl (2*R*,3*S*,4*R*)-2-Amino-4-hydroxy-3-methylpentanoate (5b):** A solution of racemate **4a-b** (524 mg, 1.17 mmol) in methanol (20 mL) was hydrogenated with 10% palladium hydroxide on charcoal (150 mg) and the reaction followed by TLC. When the reaction was finished (4−6 h) (disappearance of TLC spot), the catalyst was removed by filtration through celite and the filtrate was evaporated to dryness to afford the corresponding α-amino ester **5a-b** as a racemic mixture in 95% yield (226 mg). MS ESI > 0: 204 [M + H^+], 407 [2M + H^+], 429 [2M + $Na]^+$. ^1H NMR (250 MHz, $CDCl_3$): δ = 0.74 (d, *J* = 6.8 Hz, 3 H, CH_3), 1.08 (d, *J* = 6.0 Hz, 3 H, CH_3), 1.38 (s, 9 H, *t*Bu), 1.47−1.55 (m, 1 H, CH), 3.17 (d, *J* = 8.2 Hz, 1 H, CH), 3.01−3.26 (br, 2 H, OH), 3.55−3.64 (m, 1 H, CH).

4-Hydroxyisoleucine Lactone: (3*S*,4*R*,5*S*)-3-Amino-4,5-dimethyldihydrofuran-2-one (6a) and (3*R*,4*S*,5*R*)-3-Amino-4,5-dimethyldihydrofuran-2-one (6b): The racemic compound **5a-b** (406 mg, 2 mmol) was dissolved in TFA/DCM (50:50 v/v) (10 mL) and magnetically stirred overnight at room temperature. The solvents were evaporated under reduced pressure and the residue was dissolved in ethyl acetate (15 mL); the organic layer was washed with 10% sodium hydrogencarbonate (10 mL) then with H_2O until neutral pH (2 × 10 mL). The expected compound **6a-b** (220 mg, 80−90% yield) was obtained as a colorless solid after purification by chromatography on silica gel (eluent: diethyl ether/hexane, 20:80 v/v). R_f = 0.58 (diethyl ether/hexane, 1:2 v/v); m.p. 240−245 °C. MS (FAB^+): *m*/*z* = 130 [M + H^+]. ^1H NMR (400 MHz, DCl): δ = 1.22 (d, *J* = 7.3 Hz, 3 H, CH_3), 1.51 (d, *J* = 6.5 Hz, 3 H, CH_3), 2.81 (dqd, *J* = 8.3, 7.3, 2.0 Hz, 1 H, CH), 4.66 (qd, *J* = 6.5, 2.0 Hz, 1 H, CH), 4.67 (d, *J* = 8.3 Hz, 1 H, CH) ppm. ^{13}C NMR (300 MHz, DCl): δ = 12.5, 19.0, 37.6, 51.8, 84.2, 173.8 ppm. IR (KBr): $\tilde{\nu}$ = 3500−2500 (3340, 2900), 1769, 1494, 1384, 1259, 1207, 1133, 1023, 1003, 944.

Resolution by Aminooxidases (D-AAO and L-AAO): Standard Procedure: Phosphate buffer (0.2 M, 25 mL) pH 8.3 was saturated with oxygen by bubbling the gas through the solution for 20 min. Afterwards, 1% H_2O_2 (2 mL), Catalase (10 μL) (260000 U/mL; 30% glycerol; 10% ethanol), 4-hydroxyisoleucine lactone **6a-b** (140 mg, 1.09 mmol) and aminooxidase (2.5 mg) were simultaneously added to the buffer. This mixture was incubated for one day at room temperature. After evaporation the residue was dissolved in 10 mL of 2% acetic acid and applied to the ion-exchange resin DOWEX 50 WX8 previously washed with water and methanol (2 × 20 mL). The amino acid was eluted by 2 N NH_4OH solution (2 × 10 mL), then the solvent was evaporated under reduced pressure and the amino acid was crystallized from water/ethanol.

(2*R*,3*S*,4*R*)-2-Amino-4-hydroxy-3-methylpentanoic Acid (1b): 9.7 mg, yield 6%, m.p. 223−226 °C. MS (FAB+): *m*/*z* = 148 [M + H^+], $[\alpha_D]$ = −30 (*c* = 1, H_2O). ^1H NMR (400 MHz, D_2O): δ = 1.0 (d, *J* = 7.1 Hz, 3 H, CH_3), 1.30 (d, *J* = 6.4 Hz, 3 H, CH_3), 2.00 (qdd, CH, *J* = 7, 4, 8 Hz, 1 H), 3.85 (dq, *J* = 6.3, 8.1 Hz, 1 H, CH), 3.93 (d, *J* = 4 Hz, 1 H, CH) ppm. ^{13}C NMR (300 MHz, D_2O): δ = 12.6, 21.1, 41.6, 57.2, 70.1, 174.0. $C_6H_{13}NO_3$ (147.17): calcd. C 49.9, H 8.9, N9.5; found C 49.5, H 9.0, N 9.4.

Phenylacetic Protection: *N*-[(3*S*,4*R*,5*S*)-4,5-Dimethyltetrahydro-2-oxofuran-3-yl]-2-phenylacetamide (8a) and *N*-[(3*R*,4*S*,5*R*)-4,5-Dimethyltetrahydro-2-oxofuran-3-yl]-2-phenylacetamide (8b): Racemic lactone **6a-b** (323 mg, 2.5 mmol) was dissolved in anhydrous dichloromethane (15 mL) and the solution was cooled to 0 °C in an ice bath. Then triethylamine (0.452 mL, 3.25 mmol) was added and the mixture was magnetically stirred at 0 °C for 15 min. Phenylacetyl chloride (0.43 mL, 3.25 mmol) diluted in anhydrous DCM (3 mL) was added dropwise. The mixture was stirred for 1 h at 0 °C and 16 h at room temperature. The mixture was washed with 15% citric acid solution (10 mL) and $NaHCO_3$ saturated solution (10 mL). The organic layer was dried with magnesium sulfate, concentrated under reduced pressure, and the residue was purified by silica-gel column chromatography (eluent: Et_2O/hexane, 1:1 v/v; then 2:1, 3:1, and 4:1 gradients). The compound **8a-b** was obtained as white crystals in 78% yield (482 mg). R_f = 0.37 (Et_2O). m.p. 131−132 °C. MS ESI > 0: 248 [M + H^+], 270 [M + $Na]^+$, 517

[(2M + Na)$^+$]. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.81 (d, *J* = 7.2 Hz, 3 H, CH_3), 1.40 (d, *J* = 6.7 Hz, 3 H, CH_3), 2.53−2.65 (m, 1 H, CH), 3.62 (2 H, s, CH_2), 4.3−4.4 (m, 1 H, CH), 4.7 (dd, *J* = 6.0, *J* = 8 Hz, 1 H, CH), 5.8 (br, 1 H, OH), 7.25−7.4 (m, 5 H, Ph).

9a-b: Sodium (2*S*,3*R*,4*S*)- and (2*R*,3*S*,4*R*)-2-[(*N*-Phenylacetyl)-amino]-4-hydroxy-3-methylpentanoate: Racemate **8a-b** (350 mg, 1.42 mmol) was stirred in a dioxan/H_2O mixture (9:1 v/v, 15 mL). After solubilization 1 M NaOH was added (3.25 mmol). The ring opening was followed by TLC. The solvent was concentrated under reduced pressure and the racemate **9a-b** was obtained as an oil in quantitative yield (375 mg). Both isomers were eluted on HPLC on a Chiralcell OJ column (eluent: 85% hexane, 15% 2-propanol, flow rate: 0.8 mL/min). The elution results are given in Table 1. MS ESI > 0: 265 [M + H$^+$], 287 [M + Na$^+$]. ^{1}H NMR (400 MHz, D_2O): δ = 1.05 (d, *J* = 7.0 Hz, 3 H, CH_3), 1.35 (d, *J* = 6.3 Hz, 3 H, CH_3), 2.10 (qdd, CH, *J* = 7, 4, 8 Hz, 1 H), 3.58 (s, 2 H, CH_2), 3.86 (dq, *J* = 6.3, 8.0 Hz, 1 H, CH), 3.94 (d, *J* = 4.0 Hz, 1 H, CH), 7.20−7.4 (m, 5 H, Ph).

(2*S*,3*R*,4*S*)-4-Hydroxyisoleucine (1a): Resolution by E-PAC (Eupergit Penicillin Acylase): Racemate **8a-b** (1.4 mmol, 400 mg) was dissolved in 0.2 M NaOH solution (75 mL) at pH 9. Then the immobilized amidase was added and left in suspension. The mixture was moderately stirred at room temperature. The pH decreased during the bioconversion and had to be maintained at pH 9 by addition of concentrated NaOH solution. The reaction was followed by HPLC and stopped at 40% bioconversion (after around 2 h). The immobilized enzyme was filtered and washed with water (2 × 10 mL). The filtrate was acidified to pH 3 and the phenylacetic acid was eliminated by extraction with dichloromethane (3 × 30 mL). The aqueous layer was concentrated under reduced pressure to 20 mL and the solution was eluted on DOWEX 50WX8 previously washed by three cycles of water/MeOH over 30 min. The adsorption was followed by observing the disappearance of the free amino acid from the solution using TLC with EtOH/NH_4OH (4:1, v/v) as eluent and ninhydrin for visualization. (2*S*,3*R*,4*S*)-4-Hydroxyisoleucine (**1a**) was eluted from the DOWEX resin by 2 M NH_4OH solution. Then after evaporation and recrystallization from 95% ethanol, **1a** was obtained in 38% yield (78 mg) (*ee* = 99±1%). R_f = 0.68 (NH_4OH/EtOH, 1:4, v/v). m.p. 220−225 °C (224−225 °C[5]). MS (FAB+): *m*/*z* = 148 [M + H$^+$], [α_D] = +31 (*c* = 1,H_2O). [α_D] =+31.5 (*c* = 1, H_2O[14]). ^{1}H NMR (400 MHz, D_2O): δ = 1.0 (d, *J* = 7.0 Hz, 3 H), 1.3 (d, *J* = 6.3 Hz, 3 H), 2.0 (qdd, *J* = 7, 4, 8 Hz, 1 H), 3.88 (dq, *J* = 6.3, 8.0 Hz, 1 H), 3.94 (d, *J* = 4 Hz, 1 H) ppm. ^{13}C NMR (300 MHz, D_2O): δ = 12.5, 21.0, 41.7, 57.3, 70.2, 174.1 ppm. IR (KBr): ṽ = 3500−2500, 1634, 1490, 1415, 1259, 1356, 1312, 1270, 1180, 1104, 1053, 1017, 962, 931, 899, 858, 817, 794. $C_6H_{13}NO_3$ (147.17): calcd. C 49.9, H 8.9, N 9.5; found C 49.3, H 9.1, N 9.4.

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