Action of L-Aminoacylase and L-Amino Acid Oxidase on 2-Amino-3-methylpent-4-enoic acid [Δ(4)-Dehydroisoleucine and alloisoleucine] Stereoisomers: An Alternative Route to a Stereochemically Pure Compound and the Application to the Synthesis of *(R)*-2-Methylbutan-1-ol

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Abstract: The action of L-aminoacylase and L-amino acid oxidase on the stereoisomers of 2-amino-3-methylpent-4-enoic acid was examined, to secure stereochemically pure compounds related to L-alloisoleucine. A scalemic mixture [(2*S*,3*R*)- (90.0%), (2*S*,3*S*)- (3.2%), (2*R*,3*S*)- (5.9%), (2*R*,3*R*)- (0.9%)] of the *N*-trifluoroacetyl derivatives of the title compound, which was prepared by an asymmetric Claisen rearrangement, was submitted to *Aspergillus* L-aminoacylase-catalyzed hydrolysis. Only the (2*S*,3*R*)- and (2*S*,3*S*)-isomers were hydrolyzed to give the corresponding amino acids. The subsequent treatment of these isomers with *Crotalus adamanteus* L-amino acid oxidase afforded the pure (2*S*,3*R*)-isomer, related to L-alloisoleucine, in more than 99% stereochemically pure form. Moreover, the product was converted to L-alloisoleucine and *(R)*-2-methylbutan-1-ol via a chemoenzymatic procedure.

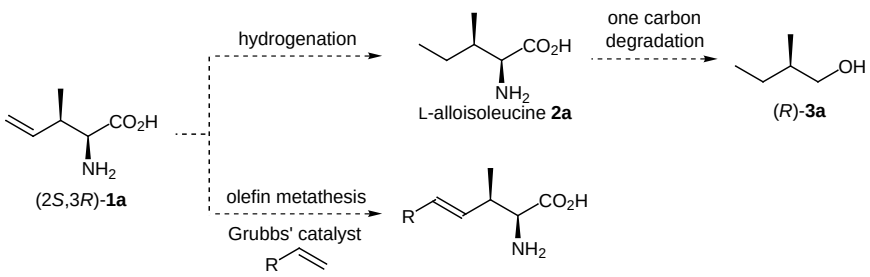
Key words: L-aminoacylase, L-amino acids oxidase, chelation-controlled enolate Claisen rearrangement, L-alloisoleucine, *(R)*-2-methylbutan-1-ol

Among the amino acids with branched side chains, isoleucine and alloisoleucine play important roles in biologically active oligopeptides and depsipeptides[1] such as antibiotics. From the view of synthetic organic chemistry, it has been well recognized that these amino acids bearing the nature-made chirality which consists of methyl and ethyl substituents [$CH_3CH_2(CH_3)CH$] serve as enantiomerically enriched building blocks for natural product syntheses. In this context, the potentiality of 2-amino-3-methylpent-4-enoic acid (**1a**), a γ, δ-unsaturated amino acid, closely related to alloisoleucine in terms of its structural backbone and stereochemistry, has been gaining interest. Indeed, it has recently been utilized as a starting material for isostatine.[2] Moreover, by taking advantage of the terminal double bond, **1a** is expected to be a potential precursor of homologous dehydroamino acids by means of a recently developed alkene metathesis reaction (Scheme 1).[3]

For a long time, enantiomers of amino acids whose stereochemistry was related to alloisoleucine had been prepared as follows: a combination of Strecker amino acid synthesis and the subsequent L-aminoacylase-catalyzed enantiomeric resolution of the corresponding *N*-acyl derivatives.[4] The first step of this process, however, yields all four possible stereoisomers in nearly the same quantities, and a separation of the diastereomers requires a laborious procedure.

Among several reports on the efforts for stereoselective synthesis,[5] a decisive clue to a solution of this issue has recently been presented by one of these authors (Kazmaier).[6] A metal-chelated asymmetric Claisen rearrangement of *N*-trifluoroacetylglycine *(E)*-crotyl ester **4**[7] provides a highly scalemic stereoisomeric mixture in which (2*S*,3*R*)-**1b** comprises ca. 90% of the compounds. Any desired enantiomer of **1b** is obtained at will, by changing the absolute configuration of amine base as the additive. The stereoisomers as impurities could be removed by crystallization of the corresponding diastereomeric salt with α-phenethylamine.

Current studies of one of the authors (Sugai)[8] prompted us to study enzyme actions and substrate specificities on the stereoisomers of **1**, hopefully to establish an alternative, facile enzyme-mediated way for the purification of the



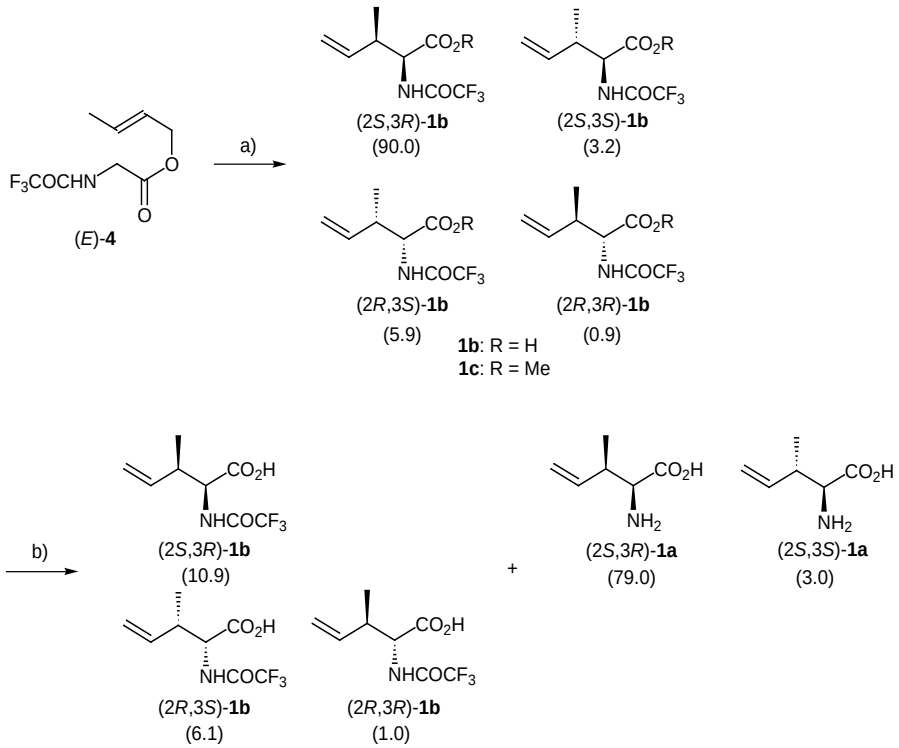
Scheme 1

(2*S*,3*R*)-isomer. At first, we focused on the beneficial character of the substrate, the *N*-trifluoroacetamide group, which is essentially required as the chelation-controlled Claisen rearrangement to give the (2*S**,3*R**)-isomer through the most thermodynamically stable transition state. During the present study, we became aware that the *N*-trifluoroacetamide group indeed worked very well under *Aspergillus* aminoacylase-catalyzed hydrolysis conditions for the enantiomeric kinetic resolution of phenylglycine derivatives.[9] So far, in some kinetic studies, porcine kidney aminoacylase-catalyzed hydrolysis of the *N*-trifluoroacetamide group has been reported to be very fast,[10] compared with those of the conventional *N*-acetyl and/or *N*-chloroacetyl groups.[11] The high rate of hydrolysis especially observed in the case of amino acids with a branched chain[10a] suggested to us a more efficient resolution of enantiomers due to an exhaustive hydrolysis of the highly reactive enantiomer.

Based on the foregoing facts, a scalemic substrate mixture [(2*S*,3*R*)-**1b** (90.0%), (2*S*,3*S*)-**1b** (3.2%), (2*R*,3*S*)-**1b** (5.9%), (2*R*,3*R*)-**1b** (0.9%)] was submitted to *Aspergillus* aminoacylase-catalyzed hydrolysis. The reaction proceeded smoothly, and the conversion reached 83% within 1 day. The desired (2*S*,3*R*)-**1b** and another L-isomer, (2*S*,3*S*)-**1b** were preferentially hydrolyzed while two D-isomers, (2*R*,3*R*)-**1b** and (2*R*,3*S*)-**1b** remained intact. In this case, the major (2*S*,3*R*)-**1b** isomer was recovered to some extent in the fractions of *N*-trifluoroacetamides **1b**, while (2*S*,3*S*)-**1b** was completely hydrolyzed, and these results indicated that the hydrolysis of the (2*S*,3*R*)-isomer was substantially slower than that of the (2*S*,3*S*)-isomer. This conclusion was supported by an earlier observation by Chibata that the hydrolysis of *N*-acetyl-L-alloisoleucine was slower (ca. 1:15) than that of *N*-acetyl-L-isoleucine (Scheme 2).[11a,b]

The second task was the removal of the obstinate (2*S*,3*S*)-isomer. As the conventional recrystallization proved ineffective, we attempted the kinetic resolution of the diastereomers based on the difference in the rates of aminoacylase-catalyzed hydrolysis between (2*S*,3*R*)-**1b** and (2*S*,3*S*)-**1b**. The hydrolysis was stopped at a lower (16%) conversion, expecting the most highly reactive (2*S*,3*S*)-isomer would be almost hydrolyzed, as mentioned above. In the case of the present experiment, the ratio of the reactivity between (2*S*,3*R*)-**1b** and (2*S*,3*S*)-**1b** was estimated to be 1:7. To our disappointment, the hydrolysis of (2*S*,3*S*)-isomer did not go to completion and a small portion was still observed in the fraction of **1b**; 90.6% of (2*S*,3*R*), 1.3% of (2*S*,3*S*) and 8.1% of the D-isomers.

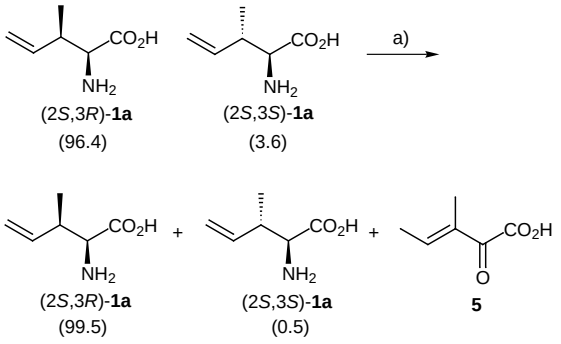
At this stage, we turned our attention to another enzyme, L-amino acid oxidase from snake venom.[12] Through extensive studies on the substrate specificity, the relative rate of oxidation on isoleucine and alloisoleucine stereoisomers has been estimated, and L-isoleucine reacts ten to



a) LHMDS, quinidine, $Al(OiPr)_3$ / THF, –80 °C → r.t., 1 day, 90% yield. b) *Aspergillus* L-aminoacylase, $CoCl_2$, pH 7.0, 30 °C, 1 day, 18% conv., 16% yield. The values in parentheses show the distribution of stereoisomers (total = 100).

Scheme 2

one hundred times faster than L-alloisoleucine.[12a] The mixture of (2*S*,3*R*)-**1a** and (2*S*,3*S*)-**1a** [96.4:3.6] was subjected to this enzyme-catalyzed oxidation. The reaction worked well to give highly pure (2*S*,3*R*)-**1a** (82%) as the unchanged recovered product. Initially formed 3-methyl-2-oxopent-4-enoic acid was isomerized in situ to the corresponding conjugated 2-oxo acid **5**, and this could easily be separated from (2*S*,3*R*)-**1a** by anion exchange [Dowex 1x8–50 (OH^-)] chromatography (Scheme 3).

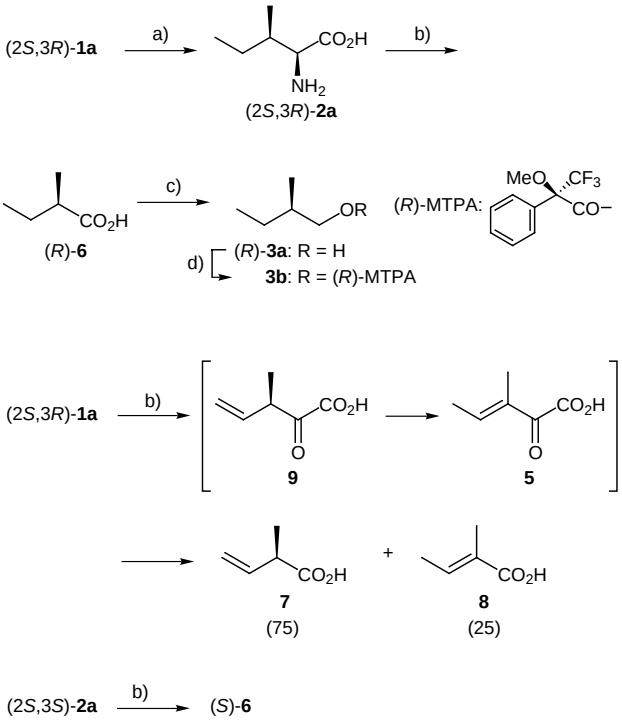


a) *Crotalus* L-amino acid oxidase, catalase, tris-maleate buffer (pH 7.8), KCl, 30 °C, 3 h, 8% conv., 82% yield. The values in parentheses show the distribution of stereoisomers (total = 100).

Scheme 3

With the highly pure (2*S*,3*R*)-**1a** in hand, we further embarked upon the conversion to *(R)*-**3a**. The alcohol *(R)*-**3a** and its precursor, *(R)*-2-methylbutyric acid (**6**) are important starting materials for natural products[13,14a–d] and liquid crystals[14e] syntheses, and methods for preparation of *(R)*-**3a**[14] or *(R)*-**6**[15] were extensively studied. Toward this end, the double bond of **1a** was saturated; the hydrogenation smoothly proceeded to give L-alloisoleucine [(2*S*,3*R*)-**2**] without any epimerization of the chiral center adjacent to the double bond. Further one-carbon degradation of L-alloisoleucine ultimately might lead to *(R)*-2-methylbutan-1-ol (**3a**), which is a mirror image of naturally occurring *(S)*-**3a**, isolated from fusel oil as the yeast-mediated metabolite of L-isoleucine.

Although the oxidative one-carbon degradation of a closely related substrate mediated by AgO was reported,[16] the yield was recorded to be as low as 50%. The moderate yield could be raised by an elaborated enzymatic reaction. When an L-amino acid oxidase-catalyzed reaction was performed, concomitantly formed hydrogen peroxide attacked the α-oxo group, under the special conditions that no catalase was added to the reaction. Subsequently, the C–C bond cleavage and the loss of carbon dioxide ultimately led to the lower homologous carboxylic acid.[12b] In this case, *Crotalus adamanteus* (rattlesnake) venom oxidase[12a,c] was the best choice among some commercially available candidates, such as those from *Crotalus atrox* and *Bothrops atrox*, in terms of the the reaction rate of oxidation and the availability. L-Alloisoleucine (**2a**), as obtained before was incubated with this enzyme for 2 days at pH 7.2, expecting to avoid the epimerization of the chiral center adjacent to the carbonyl group present in the α-oxo acid intermediate. Acid *(R)*-**6** was obtained in 82% yield and was immediately reduced with $LiAlH_4$[14d] to give the desired alcohol *(R)*-**3a** in 73% yield. The enantiomeric excess (ee) was determined to be 96% by an ^1H NMR measurement of the corresponding *(R)*-MTPA ester **3b**.[14b,d] Judged from the ee of the starting material (**2a**), racemization as low as 1% was observed even after prolonged incubation (Scheme 4).



a) PtO_2, H_2O, H_2, r.t., 15 h, 98% yield. b) Crotalus L-amino acid oxidase, tris-HCl buffer (pH 7.2), O_2, 33 °C, 1–2 days, 82% yield. for (*R*)-6; 91% yield. for (*S*)-6. c) LAH, Et_2O, r.t., 4 h, 73% yield. d) MTPACl, pyridine, r.t., 20 h. The values in parentheses show the distribution of stereoisomers (total = 100).

Scheme 4

So far, *Crotalus* amino acid oxidase-catalyzed oxidation of branched chain α-amino acids have been performed on a small scale, only for the kinetic resolution of racemate. It is noteworthy that some results on the related substrates are described here. In the case of unsaturated substrate (2*S*,3*R*)-**1a**, the desired one-carbon degraded product **7**[17] was contaminated with an α, β-unsaturated acid **8** (ca. 25%). This was probably due to the migration of the double bond to a stable conjugated position at the stage of intermediates (from **9** to **5**), even under mild reaction conditions. In contrast, the oxidation was proved to be an excellent tool for the preparation of *(S)*-**6**. Oxidation of L-isoleucine afforded *(S)*-**6** (>99% *e.e.*, determined at the stage of *(S)*-**3b**] in 91% yield. In this case, the enzyme/

substrate ratio could certainly be suppressed, as the oxidation of L-isoleucine was much faster than that of L-alloisoleucine, as mentioned earlier.

In conclusion, the action and rate of L-aminoacylase and L-amino acid oxidase to the stereoisomers of 2-amino-3-methylpent-4-enoic acid derivatives were clarified in a preparative scale, and a new alternative way to secure highly isomerically pure **1a** was established.

All mps were uncorrected. IR spectra were measured on a FT/IR-410 spectrometer. ^{1}H and ^{13}C NMR spectra were measured at 270 MHz on a JEOL JNM EX-270 or 400 MHz on a JEOL JNM α-400 spectrometer unless otherwise stated. Mass spectra were recorded on a Hitachi M-80B spectrometer at 70 eV. Optical rotations were recorded on a Jasco DIP 360 polarimeter. Silica gel 60 K070-WH (70–230 mesh) of Katayama Chemical Co. was used for column chromatography. Amicon Stirred Ultra-filtration Cell 8200 was used for ultra-filtration.

(2*S*,3*R*)-(+)-3-Methyl-2-(trifluoroacetylamino)pent-4-enoic Acid (1b)

This was prepared according to the published procedure[6a] with a slight modification. LHMDS solution was prepared by adding BuLi (39.8 mL of a 1.71 M solution in hexane, 68.1 mmol, 5.1 eq.) at –20 °C under Ar to hexamethyldisilazane (16.1 mL, 79.1 mmol, 5.9 eq.) in THF (38 mL) and the mixture was stirred for 1 h. A homogeneous solution of the *N*-TFA glycine crotyl ester *(E)*-**4**[7,18] (*E*/*Z* = 10/1,[18c] 3.00 g, 13.3 mmol), Al(*O*-*i*-Pr)$_3$ (3.27 g, 16.0 mmol, 1.2 eq.) and quinidine (10.9 g, 33.6 mmol, 2.5 eq.) in THF (135 mL) was stirred for 10 min under Ar at r.t., and cooled to –78 °C. To the mixture, the freshly prepared LHMDS solution was added slowly, and the mixture was stirred overnight under Ar at r.t. The progress of the hydrolysis was confirmed by a TLC analysis [silica gel, developed with hexane–EtOAc (3:1) and $CHCl_3$–MeOH–HOAc (60:6:4), detected with I_2 or $KMnO_4$ solution]. The mixture was cooled at 0 °C again, successively the reaction was quenched slowly by sat. aq potassium hydrogensulfate solution, and the mixture was stirred for 10 min at r.t. The organic layer was diluted with EtOAc, washed with sat. aq potassium hydrogensulfate solution. The product was extracted with sat. aq $NaHCO_3$. The basic solution was subsequently acidified with solid potassium hydrogensulfate to pH 1 and extracted with EtOAc (3 x 20 mL). The combined extracts were dried (Na_2SO_4) and concentrated in vacuo. The residue was chromatographed [silica gel, 40 g, hexane–EtOAc (20:1 to 10:1)] to give **1b** (2.71 g, 90%) as brownish oil; $[\alpha]^{24}_D$ = +70.3 (*c* = 0.98, $CHCl_3$).

IR (film): ν = 3301, 3091, 2981, 1714, 1552, 1456, 1419, 1382, 1171, 996, 927, 771, 729, 698, 607, 520, 444, 424 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.15 (d, 3 H, *J* = 7.3 Hz, CHC*H*$_3$), 2.82 (m, 1 H, C*H*CH=CH$_2$), 4.69 (dd, 1 H, *J* = 4.8, 8.8 Hz, C*H*NHCO), 5.15 (dd, 1 H, *J* = 1.1, 16.9 Hz, CH=C*H*$_2$), 5.17 (dd, 1 H, *J* = 1.1, 10.3 Hz, CH=C*H*$_2$), 5.71 (ddd, 1 H, *J* = 7.0, 10.3, 16.9 Hz, C*H*=CH$_2$), 6.89 (d, 1 H, *J* = 8.8 Hz, NHCO), 10.30 (br s, 1 H, CO_2H).

^{1}H NMR (400 MHz, D_2O): δ = 1.05 (d, 3 H, *J* = 6.8 Hz, CHC*H*$_3$), 2.78 (m, 1 H, C*H*CH=CH$_2$), 4.28 (d, 1 H, *J* = 5.9 Hz, C*H*NHCO), 5.07 (dd, 1 H, *J* = 1.5, 10.5 Hz, CH=C*H*$_2$), 5.13 (dd, 1 H, *J* = 1.5, 16.3 Hz, CH=C*H*$_2$), 5.82 (ddd, 1 H, *J* = 7.3, 10.5, 16.3 Hz, C*H*=CH$_2$).

^{13}C NMR ($CDCl_3$): δ = 15.0, 40.1, 55.9, 115.5 (q, *J* = 288 Hz), 117.7, 136.8, 157.0 (q, *J* = 38.0 Hz), 174.4.

A small portion of this was converted to the corresponding methyl ester **1c** in the following manner. To the mixture of **1b** (27.0 mg, 120 μmol) and MeOH, TMS-diazomethane solution was added and concentrated in vacuo. The residue was chromatographed [silica gel, 1.5 g, hexane–EtOAc (1:0 to 30:1)] to afford **1c** (24.7 mg, 87%) as colorless oil, $[\alpha]^{23}_D$ = +56.2 (*c* = 1.13, $CHCl_3$).

IR (film): ν = 3329, 3086, 2977, 1721, 1643, 1548, 1440, 1165, 996, 927, 861, 771, 726, 670, 519, 444, 423 cm^{-1}.

^{1}H NMR (270 MHz, $CDCl_3$): δ = 1.10 (d, 3 H, *J* = 6.9 Hz, CHC*H*$_3$), 2.75 (m, 1 H, C*H*CH=CH$_2$), 3.79 (s, 3 H, CO_2CH_3), 4.65 (dd, 1 H, *J* = 5.0, 8.6 Hz, C*H*NHCO), 5.10 (dd, 1 H, *J* = 1.3, 16.8 Hz, CH=C*H*$_2$), 5.14 (dd, 1 H, *J* = 1.3, 10.2 Hz, CH=C*H*$_2$), 5.67 (ddd, 1 H, *J* = 7.6, 10.2, 16.8 Hz, C*H*=CH$_2$), 6.80 (br s, 1 H, NHCO).

^{13}C NMR ($CDCl_3$): δ = 15.3, 40.6, 52.6, 56.2, 115.6 (q, *J* = 288 Hz), 117.3, 137.2, 156.6 (q, *J* = 38.1 Hz), 170.2.

HRMS for $C_9H_{12}F_3NO_3$ calc. 239.0768, found 239.0756.

The ee and de of **1b** was estimated by a GC analysis of **1c**.[19] GC [column, CHROMPACK CHIRASIL-L-VAL (0.25 mm x 25 m); Oven, 80 °C; Inj, 250 °C; Det, 250 °C]: Rt = 7.3 min [(2*R*,3*R*)-**1c**, 0.9%], 7.9 min [(2*R*,3*S*)-**1c**, 5.9%], 8.8 min [(2*S*,3*S*)-**1c**, 3.2%], 9.9 min [(2*S*,3*R*)-**1c**, 90.0%].

L-Amino Acylase-Catalyzed Hydrolysis of 1b. Preparation of the (2*S*,3*R*)-(+)-2-Amino-3-methylpent-4-enoic Acid (1a)

N-TFA-Amino acid **1b** (90% isomerically pure, 3.50 g, 15.5 mmol) was dissolved in H_2O by adding aq KOH (ca. 870 mg, 15.5 mmol in H_2O) until the pH of the mixture exactly became 7.0, and then total volume was adjusted to 145 mL by the addition of H_2O. To the mixture was added $CoCl_2 \cdot 6H_2O$ (1.5 mg, 6.3 μmol, 3.9 x 10^{-4} eq) and L-aminoacylase (L-acylase ACU06514, *Aspergillus*, Amano pharmaceutical Co., 1.08 g, 70 mg/mmol of the substrate), and the mixture was stirred for 1 day at 30 °C. The progress of the hydrolysis was confirmed by a TLC analysis [silica gel, developed with BuOH–CH_3CO_2H–H_2O (4:1:1) and detected with I_2, R_f: product, 0.4; **1b**, 0.9]. The enzyme was removed from the mixture (pH 6.7) by using ultra-filtration (Amicon YM10 membrane, 4.5 kg/m^2), and the filtrate was concentrated in vacuo. The ^{1}H NMR spectrum (400 MHz, D_2O) of the crude product indicated that it was a mixture of *N*-acylamino acid **1b** (δ 4.32, d, 18%) and free amino acid **1a** (δ 3.79, d, 82%) by judging from the signals of α-proton. The mixture was charged on a cation-exchange resin column [Amberlite IR-120B (H^+), volume: 280 mL], and the column was washed with H_2O. The washings were acidified with solid potassium hydrogensulfate to pH 1, and extracted with EtOAc (3 × 20 mL). The combined extracts were dried (Na_2SO_4) and concentrated in vacuo. The residue was chromatographed [silica gel, 15 g, hexane–EtOAc (20:1 to 10:1)] to give the recovery of **1b** (570 mg, 16%) as yellow solid. A small portion of this was converted to the methyl ester and analyzed by GC as above: the recovery consisted of 60.5% of (2*S*,3*R*)-**1b**, 34.1% of (2*R*,3*S*)-**1b** and 5.4% of (2*R*,3*R*)-**1b**.

Then the ion-exchange resin was eluted with 1 M aq NH_3 and the eluent was concentrated in vacuo to give a light brownish solution. Decoloration with Norit, and concentration in vacuo afforded **1a** in a colorless aqueous solution.

A small portion of this was *N*-trifluoroacetylated[20] and converted to the corresponding methyl ester **1c** in the following manner. A mixture of dried **1a** (8.7 mg, 67.4 μmol), small portions of anhyd MeOH, Et_3N, and ethyl trifluoroacetate was stirred under Ar at r.t. until the amino acid had completely dissolved. The mixture was concentrated in vacuo, diluted with EtOAc and washed with sat. aq $KHSO_4$. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was chromatographed [silica gel, 1.5 g, hexane–EtOAc (20:1 to 10:1)] to give **1b** (12.6 mg, 78%). Additions of MeOH and TMS-diazomethane solution, and concentration in vacuo afforded methyl ester **1c**. Based on the GC analysis of this as above, the ee and de of (2*S*,3*R*)-**1a** was estimated >99.8% and 92.8% respectively.

Lyophilization of (2*S*,3*R*)-**1a** in aqueous solution afforded a colorless solid (1.47 g, 73%).

^{1}H NMR (400 MHz, D_2O): δ = 1.12 (d, 3 H, *J* = 7.3 Hz, CH_3), 2.89 (m, 1 H, C*H*CH=CH_2), 3.77 (d, 1 H, *J* = 4.0 Hz, C*H*NH_2), 5.26 (dd, 1 H, *J* = 1.0, 16.2 Hz, CH=C*H*$_2$), 5.27 (dd, 1 H, *J* = 1.0, 10.6 Hz, CH=C*H*$_2$), 5.86 (ddd, 1 H, *J* = 6.6, 10.6, 16.2 Hz, C*H*=CH_2).

This was employed for the next step without further purification.

L-Amino Acid Oxidase-Catalyzed Oxidation of 1a. Preparation of the (2*S*,3*R*)-(+)-2-Amino-3-methylpent-4-enoic Acid (1a)

This experiment was carried out according to the published procedure.[12d] Amino acid **1a** (>99.8% *e.e.*, 92.8% *d.e.*, 1.45 g, 11.2 mmol) was dissolved in tris-maleate buffer (185 mL, 0.05 M, pH 7.8). To this mixture was added KCl (1.40 g, 18.8 mmol) followed by L-amino acid oxidase (*Crotalus adamanteus* venom, Sigma A9253; type I, 0.31 units/mg, 36 mg) and catalase (Tokyo Chemical Industry C0052, 10 mg). The mixture was vigorously stirred for 3 h at 30 °C. The progress of the oxidation was confirmed by a TLC analysis [silica gel, developed with BuOH–CH_3CO_2H–H_2O (4:1:1) and detected with phosphomolybdic acid–EtOH, R_f: **1a**, 0.4; product, 0.5]. Oxidase and catalase were removed from the mixture by using ultra-filtration (Amicon YM10 membrane, 4.5 kg/m^2), and the filtrate was concentrated in vacuo. The ^{1}H NMR spectrum of the crude product indicated that it was a mixture of amino acid **1a** (92%) and 2-oxo acid **5** (8%).

^{1}H NMR (400 MHz, D_2O): δ = 1.11 (d, 3 H, *J* = 7.0 Hz, **1a**, CH_3), 1.77 (s, 3 H, **5**, CH_3CO), 1.95 (d, 3 H, *J* = 6.6 Hz, **5**, C*H*$_3$CH=C), 2.89 (m, 1 H, **1a**, C*H*CH=CH_2), 3.77 (d, 1 H, *J* = 4.0 Hz, **1a**, C*H*NH_2), 5.26 (d, 1 H, *J* = 17.0 Hz, **1a**, CH=C*H*$_2$), 5.27 (d, 1 H, *J* = 11.7 Hz, **1a**, CH=C*H*$_2$), 5.85 (ddd, 1 H, *J* = 6.8, 11.7, 17.0 Hz, **1a**, C*H*=CH_2), 6.86 (q, 1 H, *J* = 6.6 Hz, **5**, CH_3C*H*=C).

The mixture was charged on an anion-exchange resin column [Dowex 1X8–50 (OH^-), volume:420 mL], and the column was washed with H_2O. The subsequent elution with 1 M HOAc and concentration in vacuo gave a mixture of amino acid with some impurities. The mixture was charged on a cation-exchange resin column [Amberlite IR-120B (H^+), volume:280 mL], and the column was washed with H_2O. The subsequent elution with 1 M aq NH_3 and concentration in vacuo gave an amino acid solution. The solution was decolorised with Norit and concentrated in vacuo. Lyophilization afforded L-(2*S*,3*R*)-**1a** (1.19 g, 82%) as a colorless solid.

A small portion of this was *N*-trifluoroacetylated and converted to the corresponding methyl ester **1c** in the same manner as described above. Based on the GC analysis of this, the ee and de of (2*S*,3*R*)-**1a** was estimated >99.8% and 99.0% respectively.

Recrystallization of (2*S*,3*R*)-**1a** from H_2O–EtOH gave an analytical sample (181 mg, 13%) as needles, mp 214–216 °C, $[\alpha]_D^{24}$+16.0 (*c* = 1.02, H_2O). Its de was not changed by recrystallization.

IR (KBr disc): ν = 3422, 3082, 2983, 2944, 2621, 2117, 1611, 1585, 1511, 1418, 1392, 1353, 1322, 1265, 1244, 1187, 1131, 1075, 1057, 1023, 1001, 936, 915, 881, 854, 807, 795, 766, 715, 669, 573, 546, 504, 465, 402 cm^{-1}.

^{13}C NMR (D_2O): δ = 13.8, 38.5, 59.1, 118.3, 138.3, 174.2.

Anal. calcd. for $C_6H_{11}NO_2$: C, 55.80; H, 8.58; N, 10.85 (129.16). Found: C, 55.79; H, 8.48; N, 10.92.

(2*S*,3*R*)-(+)-2-Amino-3-methylpentanoic Acid (2a) (L-Alloisoleucine)

Unsaturated L-amino acid (2*S*,3*R*)-**1a** (807 mg, 6.25 mmol) was dissolved in H_2O (10 mL) and catalytic amount of platinum oxide was added. After stirring for 15 h under H_2 at r.t., the disappearance of the starting material was confirmed by a TLC analysis [silica gel, detected with I_2]. The mixture was filtered by a suction and the residue was washed with H_2O. The combined filtrate and washings were decolorated with Norit and lyophilized to give **2a** (802 mg, 98%).

A small portion of this was *N*-trifluoroacetylated and converted to the corresponding methyl ester **2b** in the same manner as described above.

^{1}H NMR (270 MHz, $CDCl_3$): δ = 0.88 (d, 3 H, *J* = 6.9 Hz, CHC*H*$_3$), 0.93 (t, 3 H, *J* = 7.4 Hz, CH_2C*H*$_3$), 1.16 (m, 1 H, C*H*$_2$$CH_3$), 1.40 (m, 1 H, C*H*$_2$$CH_3$), 2.01 (m, 1 H, C*H*$CH_3$), 3.76 (s, 3 H, CO_2CH_3), 4.69 (dd, 1 H, *J* = 4.0, 8.9 Hz, C*H*NHCO), 6.89 (br s, 1 H, NHCO). The NMR spectrum was identical with an authentic sample derived from commercially available D-alloisoleucine (Tokyo Chemical Industry A0212).

GC analysis of the **2b** was compared with an authentic sample derived from DL-alloisoleucine,[21] D-alloisoleucine, DL-isoleucine, and L-isoleucine,: Rt = 10.1 min [(2*S*,3*R*)-**2b**, 99.5%], 10.8 min [(2*S*,3*S*)-**2b**, 0.5%]. No peak on 8.2 min [(2*R*,3*S*)-**2b**] or 9.2 min [(2*R*,3*R*)-**2b**] were observed.

Recrystallization of (2*S*,3*R*)-**2a** from H_2O–EtOH gave an analytical sample (217 mg, 26.4%) as needles, mp 219–221 °C, $[\alpha]_D^{24}$+14.7 (*c* = 0.99, H_2O); lit.[22]: $[\alpha]_D$+15.7 (*c* = 2, H_2O), authentic sample of D-alloisoleucine: $[\alpha]_D^{24}$ –14.1 (*c* = 1.00, H_2O).

^{1}H NMR (270 MHz, $CDCl_3$): δ = 0.97 (d, 3 H, *J* = 6.9 Hz, CHC*H*$_3$), 0.99 (t, 3 H, *J* = 7.6 Hz, CH_2C*H*$_3$), 1.42 (m, 2 H, C*H*$_2$$CH_3$), 2.11 (m, 1 H, C*H*$CH_3$), 3.76 (d, 1 H, *J* = 3.3 Hz, C*H*NH_2). This NMR spectrum was identical with authentic sample of D-alloisoleucine.

Oxidase-Catalyzed Oxidation of (2*S*,3*R*)-2a: Preparation of *(R)*-(–)-2-Methylbutyric Acid (6)

A solution of L-alloisoleucine **2a** (>99.8% *e.e.*, 99.0% *d.e.*, 100 mg, 0.76 mmol) in tris-hydrochloride buffer (0.1 M, pH 7.2, 30 mL) was filtered through a cellulose acetate filter (Advantec TOYO, DISMIC-25CS020AS). To the mixture was added L-amino acid oxidase (*Crotalus adamanteus* venom, as descibed above, 0.53 units/mg, 200 mg), sodium azide (3 mg, 46 μmol) and a small portion of antifoam, and the mixture was vigorously stirred for 1 day at 30 °C. A stream of oxygen through a cellulose acetate filter (Advantec TOYO, DISMIC-25JP020) was continuously bubbled into the solution (7 cm^3/min). To the mixture, L-amino acid oxidase (200 mg) and sterilized H_2O (3 mL) were added and the mixture was further stirred for 1 day at 30 °C with oxygen bubbling. The consumption of the starting material was confirmed by a TLC analysis [silica gel, developed with BuOH–CH_3CO_2H–H_2O (4:1:1) and detected with ninhydrin reagent]. Oxidase was removed from the mixture by using ultra filtration (Amicon YM10 membrane, 4.5 kg/m^2), and the filtrate was concentrated in vacuo. The ^{1}H NMR spectrum of the crude product indicated that it was a mixture of amino acid (2*S*,3*R*)-**2a** (ca. 10%) and product **6** (ca. 90%). In the present experiment, 2-oxo-3-methylpentanoic acid (**9**) was not detected.

^{1}H NMR (400 MHz, D_2O): δ = 0.79 (t, 3 H, *J* = 7.5 Hz, **6**, C*H*$_3$$CH_2$), 0.88 [d, 3 H, *J* = 7.0 Hz, (2*S*,3*R*)-**2a**, C*H*$_3$CH], 0.91 [t, 3 H, *J* = 7.0 Hz, (2*S*,3*R*)-**2a**, C*H*$_3$$CH_2$], 0.98 (d, 3 H, *J* = 7.0 Hz, **6**, C*H*$_3$CH), 1.31 [m, 1 H, (2*S*,3*R*)-**2a**, CH_3C*H*$_2$], 1.32 (m, 1 H, **6**, CH_3C*H*$_2$), 1.37 [m, 1 H, (2*S*,3*R*)-**2a**, CH_3C*H*$_2$], 1.41 (m, 1 H, **6**, CH_3C*H*$_2$), 2.02 [m, 1 H, (2*S*,3*R*)-**2a**, CH_3C*H*], 2.14 (m, 1 H, **6**, CH_3C*H*), 3.69 [d, 1 H, *J* = 3.3 Hz, (2*S*,3*R*)-**2a**, C*H*NH_2]. An authentic sample of **9** showed δ = 2.90 (m, 1 H, CH_3C*H*).

The crude mixture was cooled at 0 °C, successively the mixture was acidified to pH 1 with concentrated hydrochloric acid. Extraction with Et_2O (7 x 10 mL) and the combined ethereal extracts were dried (Na_2SO_4). Ethereal solution was concentrated under atmospheric pressure. The residue was distilled in vacuo to give **6** as colorless oil (63.8 mg, 82%), bp 120 °C / 12 mmHg, $[\alpha]_D^{28}$ –18.7 (*c* = 0.76, EtOH) [lit.[15h]: $[\alpha]_D$ –20.7 (*c* = 0.77, EtOH)].

^{1}H NMR (270 MHz, $CDCl_3$): δ = 0.95 (t, 3 H, *J* = 7.4 Hz, CH_3CH$_2$), 1.18 (d, 3 H, *J* = 7.3 Hz, CH_3CH), 1.50 (m, 1 H, CH$_3$CH_2), 1.71 (m, 1 H, CH$_3$CH_2), 2.40 (m, 1 H, CH$_3$C*H*). Its NMR spectrum was identical with that reported previously.[15c]

Although *Bothrops atrox* (Sigma A4257; type II, 0.64 units/mg) and *Clotalus atrox* (Sigma A5147; type VI, 0.2 units/mg) oxidases gave the same product under the same condition, the reactions were very slow compared with that of *Crotalus adamanteus*.

(*R*)-(+)-2-Methylbutan-1-ol (3a)

Alcohol (**3a**) was prepared according to the published procedure[14d] in 73% yield as colorless oil, bp 80 °C / 130 mm Hg, $[\alpha]_D^{21}$+6.48 (*c* = 0.92, $CHCl_3$); [lit.[14a]:82% ee, $[\alpha]_D^{22}$+5.89 (*c* = 1.08, $CHCl_3$)].

^{1}H NMR (270 MHz, $CDCl_3$): δ = 0.91 (t, 3 H, *J* = 7.3 Hz, CH_3CH$_2$), 0.91 (d, 3 H, *J* = 6.6 Hz, CH_3CH), 1.13 (m, 1 H), 1.43 (m, 1 H), 1.56 (m, 1 H), 1.70 (brs, 1 H, OH), 3.43 (dd, 1 H, *J* = 6.4, 10.4 Hz, CH_2OH), 3.52 (dd, 1 H, *J* = 5.8, 10.4 Hz, CH_2OH). Its NMR spectrum was almost identical with that reported previously with an exception of the chemical shift of OH signal.[14b]

A small portion of this was converted to the corresponding *(R)*-MTPA ester **3b**. The ee of *(R)*-**3a** was estimated by ^{1}H NMR spectrum (270 MHz, $CDCl_3$) of **3b**.[14b, d] Judging from the protons at δ = 4.08 (dd, 1H, *J* = 6.6, 10.6 Hz, CH_2O–MTPA) and 4.25 (dd, 1 H, *J* = 5.6, 10.6 Hz, CH_2O–MTPA) for *(R)*-**3a** (98%) and δ = 4.17 (d, 2 H, *J* = 6.3 Hz, CH_2O–MTPA) for *(S)*-**3a** (2%), the ee of *(R)*-**3a** was estimated to be 96%.

Oxidase-Catalyzed Oxidation of (2*S*,3*R*)-1a

The reaction was carried out in the same manner as described for (2*S*,3*R*)-**2a**. A mixture of **7** and **8** was obtained as colorless oil (70%).

^{1}H NMR (270 MHz, $CDCl_3$): δ = 1.30 (t, 3 H, *J* = 6.9 Hz, **7**, CH_3CH), 1.82 (d, 3 H, **8**, *J* = 7.9 Hz, CH_3CCO$_2$H), 1.83 (s, 3 H, **8,** CH_3C=CCO$_2$H), 3.19 (m, 1 H, **7**, CH$_3$C*H*), 5.15 (d, 1 H, *J* = 10.2 Hz, **7**, CH_2=CH), 5.18 (d, 1 H, *J* = 17.2 Hz, **7**, CH_2=CH), 5.94 (ddd, 1 H, *J* = 7.3, 10.2, 17.2 Hz, **7**, CH$_2$=C*H*), 7.01 (m, 1 H, **8**). The spectrum of **7** (ca. 75%) was identical with that reported previously,[17d] and that of tiglic acid **8** (ca. 25%) was in agreement with the authentic sample.

Oxidase-Catalyzed Oxidation of (2*S*,3*S*)-2a: Preparation of *(S)*-6

In a similar manner as described for (2*S*,3*R*)-**2a**, the oxidation of (2*S*,3*S*)-**2a** (L-isoleucine) was carried out. The reaction completed within 2 days with a use of less amount of enzyme (1 mg of enzyme / 12.5 mg of substrate), and *(S)*-**6** was isolated in 91% yield. This was converted to *(S)*-**3b**, and no signals due to *(R)*-**3b** were detected in ^{1}H NMR analysis.

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