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Supporting Information for:

In vitro biocatalytic pathway design: orthogonal network for the quantitative and stereospecific amination of alcohols

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1. Abbreviations

AmDH amine dehydrogenase (variant)

Bb-PhAmDH amine dehydrogenase variant originated from the phenylalanine dehydrogenase from *Bacillus badius*.

Rs-PhAmDH amine dehydrogenase variant originated from the phenylalanine dehydrogenase from *Rhodoccoccus species*.

Ch1-AmDH chimeric amine dehydrogenase generated through domain shuffling of Bb-PhAmDH variant and L-AmDH variant, the latter originated from the leucine dehydrogenase from *Bacillus stearothermophilus*.

YcnD NADPH oxidase from *Bacillus subtilis*

Cb-FDH formate dehydrogenase from *Candida boidinii*

TeS-ADH wild-type alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (also known as ADH-T)

TeS-ADH W110A variant of the alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*

TeS-ADH I86A variant of the alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*

TeS-ADH I86A W110A variant of the alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*

LB-ADH wild-type alcohol dehydrogenase from *Lactobacillus brevis*

ee enantiomeric excess

n.a. not applicable

n.d. not determined

n.m. not measureable

2. List of substrates

Figure S1. Secondary alcohols tested in this study.

Figure S2. Primary alcohols tested in this study.

Figure S2. Primary alcohols tested in this study.

Figure S3. Additional ketones tested with TeS-ADH W110A, I86A and I86A W110A.

3. General information

Ketones **2a**, **b**, **e**-**i**, **l**-**p** were purchased from Sigma-Aldrich (Steinheim, Germany). Ketones **2c**, **d**, **j**, **k**, were purchased from Alfa Aesar (Shore Road, Heysham, UK).

Aldehydes **2q**-**t** were purchased from Sigma-Aldrich (Steinheim, Germany).

Racemic secondary alcohols **1a**, **f**, **i**-**p** primary alcohols **1q**-**t** were purchased from Sigma-Aldrich (Steinheim, Germany). Racemic secondary alcohols **1b**-**e**, **g**, **h** were chemically synthesised by reduction with $LiAlH_4$ from the related ketones. Enantiopure secondary alcohols (*S*)-**1a**, **i**, **l**-**o** and enantiopure secondary alcohols (*R*)-**1a**, **i**, were purchased from Sigma-Aldrich (Steinheim, Germany). Enantiopure secondary alcohols (*S*)-**1b**-**h**, **j**, **k**, **p** and (*R*) **1b**-**h**, **j**-**p** were synthesised by enzymatic reduction of the related ketones with stereocomplementary ADHs according to procedures reported in literature.[1]

Enantiopure (*R*), (*S*) or racemic amines **3f**, **i**, **o**, **p**, and non-chiral amines **3q**-**t** were purchased from Sigma-Aldrich (Steinheim, Germany). Enantiopure (*S*) and (*R*) amines **3a**-**e**, **g**, **h**, **j**-**n** were synthesised by stereoselective amination using established enzymatic methods (commercially available stereocomplementary ω-transaminases).[2]

Nicotinamide cofactors (NADH and NAD^+) were purchased from Melford Biolaboratories (Chelsworth, Ipswich, UK).

Catalase from bovine liver was purchased by Sigma-Aldrich (lyophilized powder, >10000 U mg^{-1} of protein). Catalase (50 U) was added to the reactions from a stock solution previously prepared in aqueous buffer (5 µL of stock solution 1 mg mL^{-1}). The concentration of the catalase in the reaction mixture (ca. 0.2 µM) was calculated considering the MM of the monomer (60 kDa) since each monomer contains a catalytic iron site.

Ni^{2+} affinity columns (HisTrap FF, 5 mL) and Strep-tag columns (StrepTrap HP, 5 mL) were purchased from GE Healthcare Bio-Sciences (Munich, Germany).

Biotinylated thrombin was purchased from Merck.

4. Site directed Mutagenesis

The three TeS-ADH variants were obtained by site directed mutagenesis using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the protocol of the supplier and by using following template DNA (already subcloned into pET21a):

```
>TeS-ADH
CATATGAAAGGTTTTGCAATGCTGAGCATTGGTAAAGTTGGCTGGATTGAAAAAGAAAAACCGGCACCGGGTCCG
TTTGATGCAATTGTTCGTCCGCTGGCAGTTGCACCGTGTACCAGCGATATTCATACCGTTTTTGAAGGTGCAATT
GGCGAACGCCATAATATGATTCTGGGTCATGAAGCAGTTGGTGAAGTTGTTGAAGTTGGTAGCGAAGTGAAAGAT
TTTAAACCGGGTGATCGTGTTGTTGTTCCGGCAATTACACCGGATTGGTGGACCAGCGAAGTTCAGCGTGGTTAT
CATCAGCATAGCGGTGGTATGCTGGCAGGTTGGAAATTTAGCAATGTTAAAGATGGCGTGTTTGGCGAATTTTTT
CATGTTAATGATGCCGATATGAATCTGGCACATCTGCCGAAAGAAATTCCGCTGGAAGCAGCAGTTATGATTCCG
GATATGATGACCACCGGTTTTCATGGTGCAGAACTGGCAGATATTGAACTGGGTGCAACCGTTGCAGTTCTGGGT
ATTGGTCCGGTTGGTCTGATGGCAGTTGCCGGTGCAAAACTGCGTGGTGCAGGTCGTATTATTGCAGTTGGTAGC
CGTCCGGTTTGTGTTGATGCAGCAAAATACTATGGTGCCACCGATATTGTGAATTATAAAGATGGTCCGATTGAA
AGCCAGATTATGAATCTGACCGAAGGTAAAGGTGTTGATGCCGCAATTATTGCCGGTGGTAATGCAGATATCATG
GCAACCGCAGTGAAAATTGTGAAACCGGGTGGCACCATTGCCAATGTTAATTATTTTGGTGAAGGTGAAGTTCTG
CCGGTTCCGCGTCTGGAATGGGGTTGTGGTATGGCACATAAAACCATTAAAGGTGGTCTGTGTCCGGGTGGTCGT
CTGCGTATGGAACGTCTGATTGATCTGGTTTTTTATAAACCGGTGGATCCGAGCAAACTGGTTACCCATGTTTTT
CAGGGCTTTGATAATATTGAAAAAGCCTTTATGCTGATGAAAGATAAACCGAAAGATCTGATTAAACCGGTTGTT
ATTCTGGCAAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAACTAGAG
```

Following primers have been used:

```
ADH-TE I86A_forw      GTTGTTGTTCCGGCAGCTACACCGGATTGGTGG
ADH-TE I86A_rev       CCACCAATCCGGTGTAGCTGCCGGAACAACAAC
ADH-TE W110A_forw     GGTGGTATGCTGGCAGGTGCGAAATTTAGCAATGTTAAAG
ADH-TE W110A_rev      CTTTAACATTGCTAAATTTCGCACCTGCCAGCATACCACC
```

For the double variant, both primer pairs were added to the PCR reaction mixture.

After verifying positive clones by sequencing, *E. coli* C43 cells were transformed with the DNA and used as host for expression of the proteins.

5. Expression and purification of recombinant proteins in *E. coli* host cells

5.1 *Strep-II-tagged ADH variants from Thermoanaerobacter ethanolicus (Strep TeS-ADH W110A and I86A W110A)*

The C-terminal Strep-II-tagged TeS-ADH variants W110A and I86A W110A - subcloned in pET21a - were expressed in *E. coli* C43 cells. Cells were grown at 37 °C until an OD_{600} of ca. 0.7 was reached prior to the addition of IPTG (final concentration 0.5 mM) and expression was carried out at 20 °C over night. Cultures were harvested, centrifuged and the pellet was washed with an aqueous solution of NaCl (0.9% w w^{-1}). Cells were then resuspended in binding buffer (142 mL, pH 8.0, Tris-HCl 100 mM, NaCl 150 mM, $NADP^+$ 0.1 mM, dithiothreitol (DTT) 0.5 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, DNase 1.32 mg from aqueous stock solution) and disrupted by sonication. Protein purification was performed by Strep-tactin affinity chromatography using pre-packed StrepTrap HP columns (GE Healthcare). After loading of the

filtered lysate on the equilibrated column, the column was washed with sufficient amounts of binding buffer and bound protein was recovered with elution buffer (pH 8.0, Tris-HCl 100 mM, NaCl 150 mM, d-desthiobiotine 2.5 mM, $NADP^+$ 0.1 mM). After SDS-PAGE, fractions containing the desired proteins in a sufficient purity were pooled and buffer exchange was carried out with PD10 columns in Tris-HCl buffer (100 mM, pH 8.0, $NADP^+$ 0.1 mM). Proteins were concentrated using Centripreps (Millipore) and their final concentrations determined at 280 nm (ε_{280} = 36,600 M^{-1} cm^{-1}, MW = 38.6 kDa). 1.4 mg and 1.7 mg purified protein per g wet cells were obtained for TeS-ADH W110A and I86A W110A, respectively, which were frozen in liquid nitrogen and stored at -80 °C.

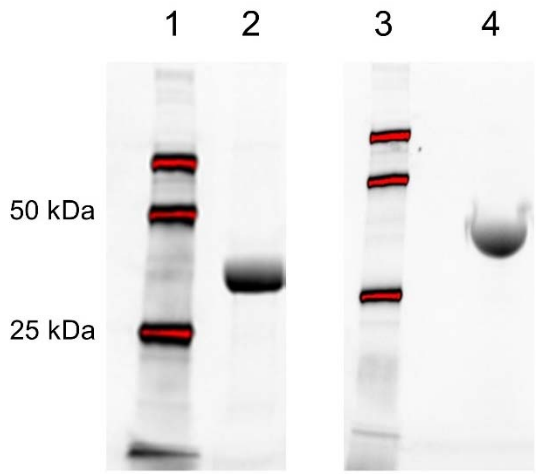


Figure S4. SDS-PAGE of purified Strep-II-tagged TeS-ADHs. Lane 1 and 3: Biorad Precision Plus Protein™ Unstained Standard, lane 2: Strep TeS-ADH W110A, lane 4: Strep TeS-ADH I86A W110A.

5.2 NADP oxidase (YcnD) from Bacillus subtilis and Formate dehydrogenase from Candida boidinii (Cb-FDH)

BL21 DE3 cells containing the plasmid for the expression of YcnD and Cb-FDH (pET21a, no Tag) were grown at 37 °C until an OD_{600} of 0.6 to 0.8 was reached. The expression of the protein was initiated by the addition of IPTG (final concentration 0.5 mM) and carried out at 25 °C overnight. Cells were harvested by centrifugation and washed with an aqueous solution of NaCl (0.9% w w^{-1}).

The non-tagged proteins were purified by anion exchange chromatography using a HiPrepQ HP 16/10 column (GE Healthcare). The pellets were dissolved in start buffer (20 mM Tris/HCl, pH 8.0 buffer), a tiny spatula of FMN was added to the YcnD protein solution and after cell disruption and centrifugation the lysates were loaded onto the column and washed with start buffer until the absorbance went down to zero. The elution of the proteins was performed with a gradient between start buffer and elution buffer (20 mM Tris/HCl, 1M NaCl, pH 8.0 buffer).

After SDS-PAGE, fractions containing the desired protein in a sufficient purity were pooled and dialyzed against 50 mM phosphate buffer (pH 8.0) overnight, and concentrated using Centripreps (Millipore). The final concentration was determined spectrophotometrically at 280 nm for Cb-FDH (ε_{280} = 51,400 M^{-1} cm^{-1}, MW = 40.3 kDa) or for YcnD using the wavelength/extinction coefficient for the protein bound flavin (approx. 450 nm/12,190 M^{-1} cm^{-1}, MW = 28.9 kDa) as determined by Morokutti et al. [3] Approximately 4 mg and 34 mg pure protein per g wet cells were obtained for YcnD and Cb-FDH, respectively, which were frozen in liquid nitrogen and stored at -80 °C.

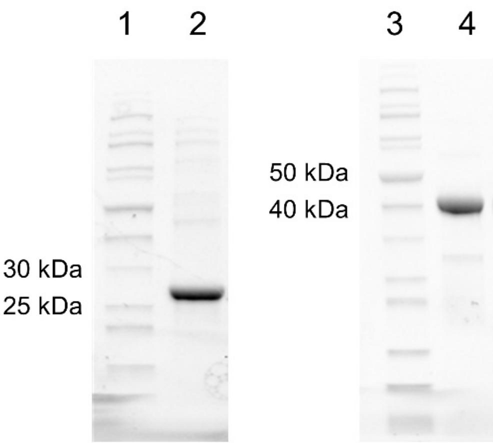


Figure S5. SDS-PAGE of purified YcnD and Cb-FDH. Lane 1 and 3: PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific), lane 2: YcnD, lane 4: Cb-FDH.

5.3 Amine dehydrogenases

The amine dehydrogenases Bb-PhAmDH and Ch1-AmDH were expressed and purified as His_6-tagged recombinant enzymes as described previously.[4] When required, the His_6-tag was cleaved following the previous protocol.[4] Rs-PhAmDH was expressed and purified as His_6-tagged recombinant enzyme as described previously. [5] When required, the His_6-tag was cleaved as follows.

A solution of His_6-tagged Rs-PhAmDH (70 mg of enzyme in ca. 1.4 mL of potassium phosphate buffer pH 8.0, 50 mM) was added to the cleavage buffer (20 mL, 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). A solution of biotinylated thrombin was then added (2.8 U). The mixture was incubated at 20 °C for 2 h. Partial precipitation was observed. SDS electrophoresis showed that the cleavage of the His_6-tag was not complete. Thus, a second aliquot of biotinylated thrombin was added (2.8 U) and incubation was prolonged for other 2 h.

The residual thrombin present in the solution was removed by the addition of streptavidin agarose beads (192 µL of 50% slurry). The beads were then removed by initial centrifugation (4,000 rpm, 10 min, 4 °C) and subsequent further filtration of the supernatant. The final solution contained a mixture of His_6-tagged Rs-AmDH, Rs-AmDH devoid of His_6-tag and free His_6-tag. The fraction of His_6-tagged Rs-AmDH and the free His_6-tag were removed through Ni^{2+} affinity chromatography. The final solution of highly pure Rs-AmDH devoid of the His-tag was dialyzed overnight in phosphate buffer (pH 8.0, 50 mM), concentrated, frozen in liquid nitrogen and stored at -80 °C. The enzyme concentration was measured spectrophotometrically (ε_{280} = 26,500 M^{-1} cm^{-1}, MW = 36.7 kDa). The amount of recovered Rs-AmDH devoid of His_6-tag was 29 mg.

6. Calculation of the $\Delta_rG'°$ for (A) biocatalytic hydrogen-borrowing amination (i.e. redox-interconnected steps) (B) orthogonal biocatalytic network (i.e. concurrent redox separated steps)

$\Delta_rG°$ is the change in Gibbs free energy due to a chemical reaction in standard conditions (T 298.15 K, P 1 bar) and without accounting for pH and ionic strength. $\Delta_rG'°$ represents the change in Gibbs free energy due to a chemical reaction in standard conditions considering a particular pH and ionic strength. In this, case we set pH 8.5 and ionic strength 1 M in water as solvent. We chose these conditions as they are analogous to the experimental conditions applied for cascade A (previous work)[4] and cascade B (the present work).

The calculations were performed with the aid of eQuilibrator, a tool made available by Milo Lab at the Weizmann Institute in Rehovot, Israel. [6] eQuilibrator uses a well-studied approximation of Δ_fG (the ΔG related to the formation of a given molecule from its components) called group contribution, enabling thermodynamic analysis of many biochemical reactions and pathways.[7] eQuilibrator also allows for manipulation of the conditions of a reaction - pH, ionic strength, and reactant and product concentrations - to help exploring the thermodynamic landscape of a biochemical reaction. Currently, eQuilibrator can provide estimates for many compounds in the KEGG database (about 4500).[8]

For simplicity, the calculations were performed using 2-propanol, acetone and 2-propylamine for a representative amination of a secondary alcohol. 1-Butanol, butanal and 1-butylamine were considered in the calculation of a representative amination of primary alcohols.

The calculations of the $\Delta_rG'°$ values are reported in the tables below

The equilibrium constant for the overall reactions was also calculated as follows:

$$\Delta rG'° = -RT \ln K'_{eq}$$

where

$\Delta_rG'°$ is the calculated free Gibbs energy of the reaction in standard conditions and in aqueous buffer at pH 8.5, ionic strength 1 M

R = 0.00831447 KJ mol K^{-1}

T = 298.15 K

K'_{eq} is the equilibrium constant for the reaction in aqueous buffer at pH 8.5, ionic strength 1 M

Cascade A

Scheme S1 Biocatalytic hydrogen-borrowing amination (i.e. redox-interconnected steps) for the one-pot conversion of alcohols to enantiopure amines employing alcohol dehydrogenases (ADHs) and amine dehydrogenases (AmDHs); previous work.[4]

Table S1. Thermodynamic calculation for the hydrogen-borrowing amination of secondary alcohols

Equation	$\Delta_rG'^{\circ}$ [KJ mol^{-1}] [(a)]
2-propanol + NAD^+ => acetone + NADH	- 3.1 ± 0.7
acetone + NADH + $NH_{3(aq)}$ => 2-propylamine + NAD^+ + H_2O	2.5 ± 5.7
Combination: hydrogen-borrowing amination	**- 0.6 ± 6.4**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

K'_{eq} = 1.27

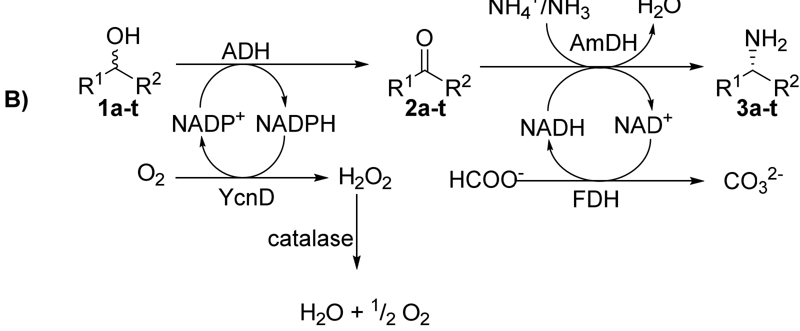
Table S2. Thermodynamic calculation for the hydrogen-borrowing amination of primary alcohols

Equation	$\Delta_rG'^{\circ}$ [KJ mol^{-1}] [(a)]
1-butanol + NAD^+ => butanal + NADH	13.7 ± 4.8
butanal + NADH + $NH_{3(aq)}$ => 1-butylamine + NAD^+ + H_2O	- 20.5 ± 6.2
Combination: hydrogen-borrowing amination	**- 6.8 ± 11**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

K'_{eq} = 15.53

Cascade B



Scheme S2. Orthogonal biocatalytic network (i.e. concurrent redox separated steps) for the one-pot conversion of alcohols to enantiopure amines employing alcohol dehydrogenases (ADHs) and amine dehydrogenases (AmDHs).

Table S3. Thermodynamic calculation for the orthogonal, oxidation-reduction amination of secondary alcohol: the oxidative step

Equation	$\Delta_rG'^{\circ}$ [KJ mol^{-1}] [(a)]
2-propanol + $NADP^+$ => acetone + NADPH	- 4.1 ± 0.6
$O_{2(aq)}$ + NADPH => H_2O_2 + $NADP^+$	- 121.8 ± 8.3
H_2O_2 => ½ $O_{2(aq)}$ + H_2O	- 96.5 ± 6.7
Combination: oxidative step, orthogonal cascade	**- 222.4 ± 15.6**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

Table S4. Thermodynamic calculation for the orthogonal, oxidation-reduction amination of secondary alcohol: the reductive amination step

Equation	$\Delta_rG'°$ [KJ mol^{-1}] [(a)]
acetone + NADH + $NH_{3(aq)}$ => 2-propylamine + NAD^+ + H_2O	2.5 ± 5.7
formate + NAD+ => $CO_{2(aq)}$ + NADH	- 15.1 ± 6.5
Combination: reductive amination step, orthogonal cascade	**- 12.6 ± 12.2**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

Table S5. Thermodynamic calculation for the orthogonal, oxidation-reduction amination of secondary alcohol: the overall cascade

Steps	$\Delta_rG'°$ [KJ mol^{-1}] [(a)]
Combination: oxidative step, orthogonal cascade	- 222.4 ± 15.6
Combination: reductive step, orthogonal cascade	- 12.6 ± 12.2
Combination: overall orthogonal cascade	**- 235.0 ± 27.8**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

For the overall orthogonal cascade: **K'eq = 1.48 x 10^{41}**

Table S6. Thermodynamic calculation for the orthogonal, oxidation-reduction amination of primary alcohol: the oxidative step

Equation	$\Delta_rG'°$ [KJ mol^{-1}] [(a)]
1-butanol + $NADP^+$ => butanal + NADPH	12.6 ± 4.8
$O_{2(aq)}$ + NADPH => H_2O_2 + $NADP^+$	- 121.8 ± 8.3
H_2O_2 => ½ $O_{2(aq)}$ + H_2O	- 96.5 ± 6.7
Combination: oxidative step, orthogonal cascade	**- 205.7 ± 19.8**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

Table S7. Thermodynamic calculation for the orthogonal, oxidation-reduction amination of primary alcohol: the reductive amination step

Equation	$\Delta_rG'°$ [KJ mol^{-1}] [(a)]
butanal + NADH + $NH_{3(aq)}$ => 1-butylamine + NAD^+ + H_2O	-20.5 ± 6.2
formate + NAD+ => $CO_{2(aq)}$ + NADH	- 15.1 ± 6.5
Combination: reductive amination step, orthogonal cascade	**- 35.6 ± 12.7**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

Table S8. Thermodynamic calculation for the orthogonal, oxidation-reduction amination of primary alcohol: the overall cascade

Steps	$\Delta_rG'°$ [KJ mol^{-1}] [(a)]
Combination: oxidative step, orthogonal cascade	- 205.7 ± 19.8
Combination: reductive step, orthogonal cascade	- 35.6 ± 12.7
Combination: overall orthogonal cascade	**- 241.3 ± 32.5**

[(a)] calculated in aqueous buffer at pH 8.5 and ionic strength 1 M

For the overall orthogonal cascade: **K'eq = 1.88 x 10^{42}**

7. Determination of the stereoselectivity for TeS-ADH W110A and TeS-ADH I86A.

The determination of the stereoselective outcome for the reduction of selected prochiral ketones was carried out using *E. coli* lyophilized cells overexpressing the TeS-ADH variants. 2-propanol was used as sacrificial cosubstrate for recycling of NADPH.

Scheme S3. Reduction of prochiral ketones with lyophilized cells of *E. coli* overexpressing TeS-ADH variants.

E. coli/TeS-ADH W110A or *E. coli*/TeS-ADH I86A (20 mg) were resuspended in Tris-HCl buffer (1 mL, pH 7, 50 mM) containing $NADP^+$ (1 mM). The suspension was incubated for 20 min, at 30 °C with orbital agitation (150 rpm). Then, the ketone substrate (50 mM) and isopropanol (50 µl, equal to 5% v v^{-1}) were added. The reactions were run for 16 h at 30°C and orbital agitation (150 rpm). The reaction mixtures were extracted with EtOAc (2 x 500 µL) and conversions were measured by GC-FID. The enantiomeric excess of the alcohol was analyzed by GC on a chiral phase after derivatization. Derivatization was performed by adding 4-(N,N-dimethylamino)pyridine (5 mg) dissolved in acetic anhydride (100 µL). After washing with water and drying ($MgSO_4$) the *ee* value of the derivatized compound was measured. For details on analytical methods and retention times, refer to section “Analytics”.

Table S9. Reduction of selected prochiral ketones with TeS-ADH W110A

Entry	Ketone	Conversion [%]	*ee* [%]
1	**2a**	69	73 (*S*)
2	**2b**	97	92 (*S*)
3	**2c**	84	>99 (*S*)
4	H_3CO **2d**	40	82 (*S*)
5	**2e**	94	93 (*S*)
6	**2g**	21	99 (*S*)
7	**2m**	88	39 (*S*)
8	**2n**	89	5 (*S*)
9	**2p**	76	95 (*S*)
10	**2u**	14	>99 (*S*)
11	H_3CO **2v**	96	>99 (*S*)
12	OCH_3 **2w**	4	19 (*S*)
13	F_3C **2x**	97	87 (*S*)

Table S10. Reduction of selected prochiral ketones with TeS-ADH I86A

Entry	Ketone	Conversion [%]	*ee* [%]
1	**2b**	70	97 (*R*)
2	**2c**	11	89 (*S*)
3	**2d**	2	49 (*S*)
4	**2e**	60	97 (*S*)
5	**2g**	62	>99 (*S*)
6	**2m**	85	27 (*S*)
7	**2n**	85	56 (*R*)
8	**2p**	80	78 (*R*)
9	**2u**	83	>99 (S)
10	**2v**	9	>99 (S)
11	**2x**	4	>99 (S)

8. Studies on the first oxidative cycle of the orthogonal biocatalytic network for the amination of alcohols

Table S11. Oxidation of (*S*)-phenyl 2-propanol ((*S*)-**1a**) to phenylacetone (**2a**). Reaction conditions: (*S*)-**1a** (20 mM), TeS-ADH W110A (13 µM), His_6-tagged YcnD (5 µM), catalase (0.2 µM) and $NADP^+$ (0.5 mM) in Tris-HCl buffer at varied pH (7 - 8.5), 30 °C, 24 h, orbital agitation 170 rpm.

Entry	pH	Conversion [%]
1	7	82
2	7.5	91
3	8	86
4	8.5	85

Table S12. Oxidation of (*S*)-phenyl 2-propanol ((*S*)-**1a**) to phenylacetone (**2a**). Reaction conditions: (*S*)-**1a** (20 mM), varied concentration of TeS-ADH W110A (13 – 26 µM), His_6-tagged YcnD (5 – 10 µM), catalase (0.2 µM) and $NADP^+$ (0.5 mM) in Tris-HCl buffer at pH 8.5, 30 °C, 24 h or 48 h, orbital agitation 170 rpm.

Entry	TeS-ADH W110A [µM]	YcnD [µM]	Reaction Time [h]	Conversion [%]
1	13	5	24	73
2	13	10	24	85
3	26	5	24	93
4	26	10	24	95
5	26	10	48	98

9. Initial studies on the orthogonal biocatalytic network for the asymmetric amination of alcohols

Table S13. Initial studies on the orthogonal biocatalytic network for the amination of (*S*)-**1b** (20 mM). Different variants possessing His_6-tags and devoid of His_6-tags (YcnD and Ch1-AmDH) were applied at varied concentrations. The other enzymes were Strep-tagged TeS-ADH W110A (varied concentration), Cb-FDH (20 µM), catalase (0.2 µM), NAD^+ (0.5 mM) and $NADP^+$ (0.5 mM). The reaction was carried out in $HCOONH_4$ buffer (1 M, pH 8.5) at 30 °C with orbital agitation (170 rpm).

Entry	Type of TeS-ADH W110A	Type of YcnD	Type of Ch1-AmDH	Time [h]	Conversion [%] (*)		
					Amine	Ketone	Alcohol
1	Strep-tag 26 µM	His_6-tag 5 µM	His_6-tag 126 µM	48	64	1	35
2	Strep-tag 26 µM	His_6-tag 10 µM	His_6-tag 126 µM	48	51	<1	49
3	Strep-tag 52 µM	His_6-tag 5 µM	His_6-tag 126 µM	48	78	1	21
4	Strep-tag 52 µM	His_6-tag 10 µM	His_6-tag 126 µM	48	73	1	26
5	Strep-tag 26 µM	His_6-tag 5 µM	No tag 100 µM	48	78	1	21

(*) Every data is the average of three independent experiments showing deviation of ± 1 – 2%

10. Time study for the amination of (*S*)-1b with inversion of configuration

Table S14. Time study for the amination of (*S*)-**1b** (20 mM) with inversion of configuration. Figure 2 in main paper.

time [h]	Amine [%]	Ketone [%]	Alcohol [%]
0.5	14.09	63.36	22.55
1	45.33	41.99	12.68
2	74.44	23.97	1.58
3	89.02	9.16	1.82
6	97.77	0.79	1.44
10	98.78	0.12	1.09
12	98.73	0.24	1.04
16	99.03	0.18	0.79
24	99.14	0.11	0.74

11. Determination of the stereoselectivity for TeS-ADH I86A W110A

Table S15. Reduction of selected prochiral ketones with TeS-ADH I86A W110A.

Entry	Ketone	Conversion [%]	*ee* [%]
1	**2a**	30	8 (*R*)
1	F **2b**	66	15 (*S*)
2	**2c**	17	89 (*S*)
3	H_3CO **2d**	59	20 (*S*)
4	**2e**	75	72 (*S*)
5	**2g**	27	96 (*S*)
6	**2m**	88	24 (*S*)
7	**2n**	89	0

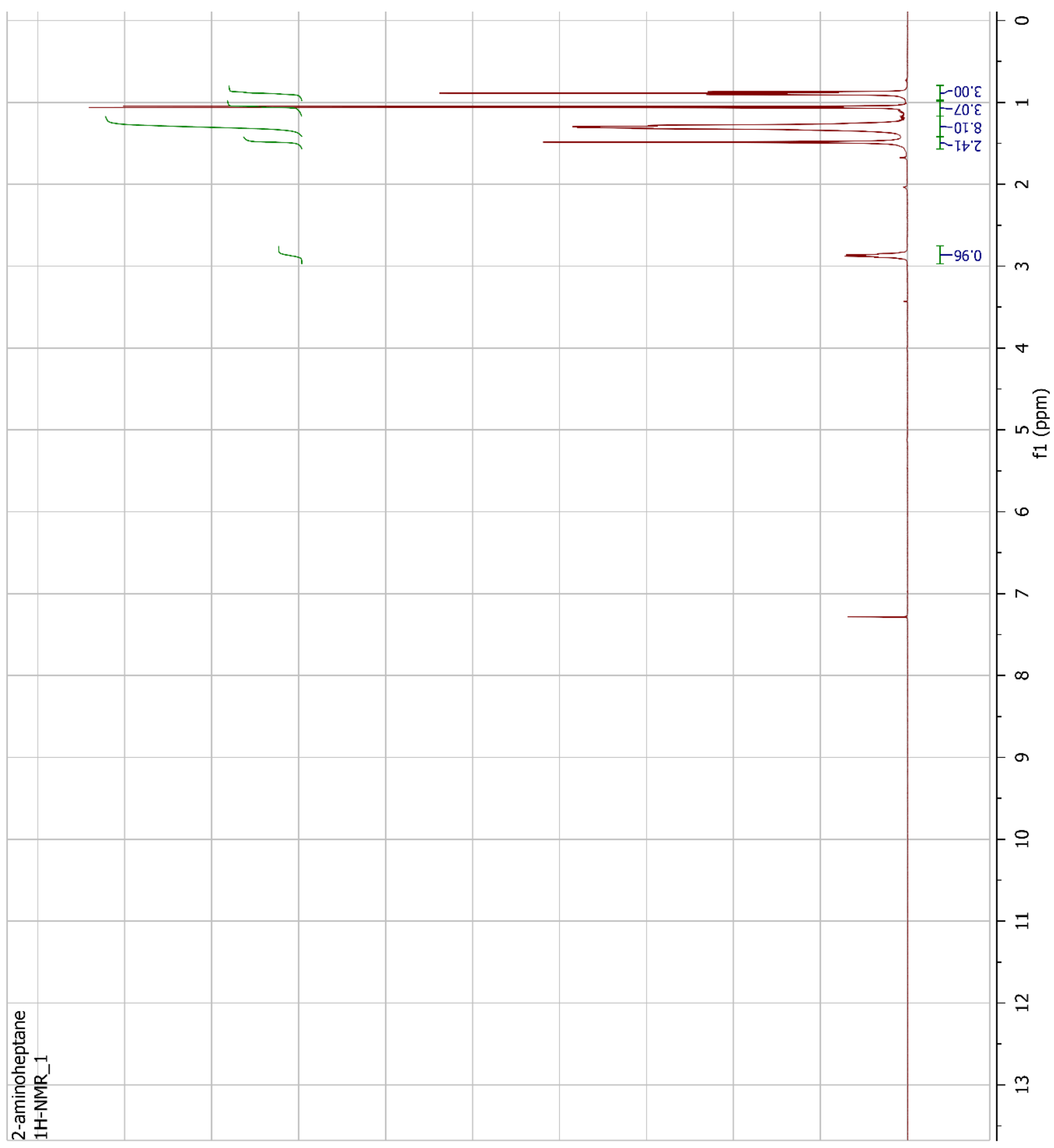
8	**2p**	83	10 (*S*)
9	**2u**	22	>99 (S)
10	H_3CO **2v**	52	>99 (S)
11	F_3C **2x**	90	63 (S)

12. Sequence alignment between TeS-ADH and Tb-ADH

Sequence alignment between the ADH from *Thermoanaerobacter ethanolicus* (TeS-ADH) and the ADH from *Thermoanaerobacter brockii* (Tb-ADH)

```
                    ************************************************************
TeS-ADH   (     1)  MKGFAMLSIGKVGWIEKEKPAPGPFDAIVRPLAVAPCTSDIHTVFEGAIGERHNMILGHE
Tb-ADH    (     1)  MKGFAMLSIGKVGWIEKEKPAPGPFDAIVRPLAVAPCTSDIHTVFEGAIGERHNMILGHE

                    ****************************** *****************************
TeS-ADH   (    61)  AVGEVVEVGSEVKDFKPGDRVVVPAITPDWWTSEVQRGYHQHSGGMLAGWKFSNVKDGVF
Tb-ADH    (    61)  AVGEVVEVGSEVKDFKPGDRVVVPAITPDWRTSEVQRGYHQHSGGMLAGWKFSNVKDGVF

                    ************************************************************
TeS-ADH   (   121)  GEFFHVNDADMNLAHLPKEIPLEAAVMIPDMMTTGFHGAELADIELGATVAVLGIGPVGL
Tb-ADH    (   121)  GEFFHVNDADMNLAHLPKEIPLEAAVMIPDMMTTGFHGAELADIELGATVAVLGIGPVGL

                    ************************************************************
TeS-ADH   (   181)  MAVAGAKLRGAGRIIAVGSRPVCVDAAKYYGATDIVNYKDGPIESQIMNLTEGKGVDAAI
Tb-ADH    (   181)  MAVAGAKLRGAGRIIAVGSRPVCVDAAKYYGATDIVNYKDGPIESQIMNLTEGKGVDAAI

                    ************************************************************
TeS-ADH   (   241)  IAGGNADIMATAVKIVKPGGTIANVNYFGEGEVLPVPRLEWGCGMAHKTIKGGLCPGGRL
Tb-ADH    (   241)  IAGGNADIMATAVKIVKPGGTIANVNYFGEGEVLPVPRLEWGCGMAHKTIKGGLCPGGRL

                    ************ ***********.***************************
TeS-ADH   (   301)  RMERLIDLVFYKPVDPSKLVTHVFQGFDNIEKAFMLMKDKPKDLIKPVVILA
Tb-ADH    (   301)  RMERLIDLVFYKRVDPSKLVTHVFRGFDNIEKAFMLMKDKPKDLIKPVVILA
```

13. ^{1}H-NMR for the scale-up of the amination of substrate *rac*-1m to give enantiopure (*R*)-1m



14. Analytics

14.1 Methods for the determination of the conversion

(A) Column: Agilent J&W DB-1701 (60 m, 250 µm, 0.25 µm). Carrier gas: H_2

GC program parameters: injector 250 °C; constant pressure 13.5 psi; temperature program: 80 °C/hold 6.5 min; 160 °C/rate 5 °C min^{-1}/hold 2 min; 280 °C/rate 20 °C min^{-1}/hold 1 min.

Table S16. Retention times using method A**.**

Alcohols	Time [min]	Ketones/Aldehyde	Time [min]	Amines	Time [min]
1a	20.8	**2a**	21.0	**3a**	19.2
1b	21.9	**2b**	21.8	**3b**	20.0
1c	24.0	**2c**	25.1	**3c**	23.2
1d	28.9(*)	**2d**	28.9(*)	**3d**	28.1
1e	23.9	**2e**	24.0	**3e**	22.6
1f	26.0	**2f**	25.6	**3f**	24.1
1j	21.8	**2j**	22.0	**3j**	19.7

(*) Ketone **2d** can be separated from the other components and evaluated using: column Agilent J&W HP-5 (30 m, 320 µm, 0.25 µm). Carrier gas: H_2. GC program parameters: injector 250 °C; constant flow 1.8 mL min^{-1}; temperature program: 100 °C/hold 5 min; 300 °C/rate 10 °C min^{-1}/hold 0 min.

(B) Column: Agilent J&W DB-1701 (30 m, 250 µm, 0.25 µm). Carrier gas: H_2

GC program parameters: injector 250 °C; constant pressure 6.9 psi; temperature program: 80 °C/hold 6.5 min; 160 °C/rate 10 °C min^{-1}/hold 5 min; 200 °C/rate 20 °C min^{-1}/hold 2 min; 280 °C/rate 20 °C min^{-1}/hold 1 min.

Table S17. Retention times using method B**.**

Alcohols	Time [min]	Ketones/Aldehyde	Time [min]	Amines	Time [min]
1g	14.7	**2g**	14.0	**3g**	14.3
1h	11.8	**2h**	10.3	**3h**	10.0
1i	11.6	**2i**	10.6	**3i**	9.8
1k	12.9	**2k**	13.0	**3k**	11.6
1l	8.0	**2l**	7.7	**3l**	6.0
1m	5.0	**2m**	4.8	**3m**	3.9
1q	10.1	**2q**	7.9	**3q**	8.2
1r	7.5	**2r**	4.9	**3r**	5.1

(C) Column: Agilent J&W DB-1701 (30 m, 250 µm, 0.25 µm). Carrier gas: H_2

GC program parameters: injector 250 °C; constant pressure 6.9 psi; temperature program: 60 °C/hold 6.5 min; 100 °C/rate 20 °C min^{-1}/hold 1 min; 280 °C/rate 20 °C min^{-1}/hold 1 min.

Table S18. Retention times using method C**.**

Alcohols	Time [min]	Ketones/Aldehyde	Time [min]	Amines	Time [min]
1n	5.1	**2n**	4.7	**3n**	3.7
1o	3.2	**2o**	3.0	**3o**	2.6
1p	4.1	**2p**	3.6	**3p**	3.1
1s	7.6	**2s**	4.8	**3s**	5.0
1t	4.5	**2t**	3.0	**3t**	3.2

14.2 Methods for the determination of the enantiomeric excess

(D) Column: Varian Chrompack Chiracel Dex-CB column (25 m, 320 µm, 0.25 µm). Carrier gas: H_2

GC program parameters: injector 200°C; constant flow 1.3 mL min^{-1}; temperature program: 100 °C/hold 2 min; 130 °C/rate 1 °C min^{-1}/hold 5 min; 170 °C/rate 10 °C min^{-1}/hold 10 min; 180 °C/rate 10 °C min^{-1}/hold 1 min.

Table S19. Retention times using method D**.**

(*S*)-amine	Time [min]	(*R*)-amine	Time [min]
(*S*)-**3a**	39.7	(*R*)-**3a**	40.0
(*S*)-**3b**	40.2	(*R*)-**3b**	40.4
(*S*)-**3e**	40.8	(*R*)-**3e**	41.0
(*S*)-**3f**	43.2	(*R*)-**3f**	43.5
(*S*)-**3g**	42.9	(*R*)-**3g**	43.5
(*S*)-**3h**	34.9	(*R*)-**3h**	37.1
(*S*)-**3i**	36.7	(*R*)-**3i**	38.2
(*S*)-**3j**	39.1	(*R*)-**3j**	39.8
(*S*)-**3k**	39.5	(*R*)-**3k**	40.0

(E) Column: Varian Chrompack Chiracel Dex-CB column (25 m, 320 µm, 0.25 µm). Carrier gas: H_2

GC program parameters: injector 200°C; constant flow 1.4 mL; temperature program: 100 °C/hold 2 min; 118 °C/rate 1 °C min^{-1}/hold 5 min; 170 °C/rate 10 °C min^{-1}/hold 10 min; 180 °C/rate 10 °C min^{-1}/hold 1 min.

Table S20. Retention times using method E**.**

(*S*)-amine	Time [min]	(*R*)-amine	Time [min]
(*S*)-**3c**	32.2	(*R*)-**3c**	32.4
(*S*)-**3d**	36.1	(*R*)-**3d**	36.4
(*S*)-**3l**	23.1	(*R*)-**3l**	24.7
(*S*)-**3m**	16.2	(*R*)-**3m**	17.3

(F) Column: Varian Chrompack Chiracel Dex-CB column (25 m, 320 µm, 0.25 µm). Carrier gas: H_2

GC program parameters: injector 200°C; constant flow 1.5 mL; temperature program: 60 °C/hold 2 min; 100 °C/rate 5 °C min^{-1}/hold 2 min; 180 °C/rate 10 °C min^{-1}/hold 1 min.

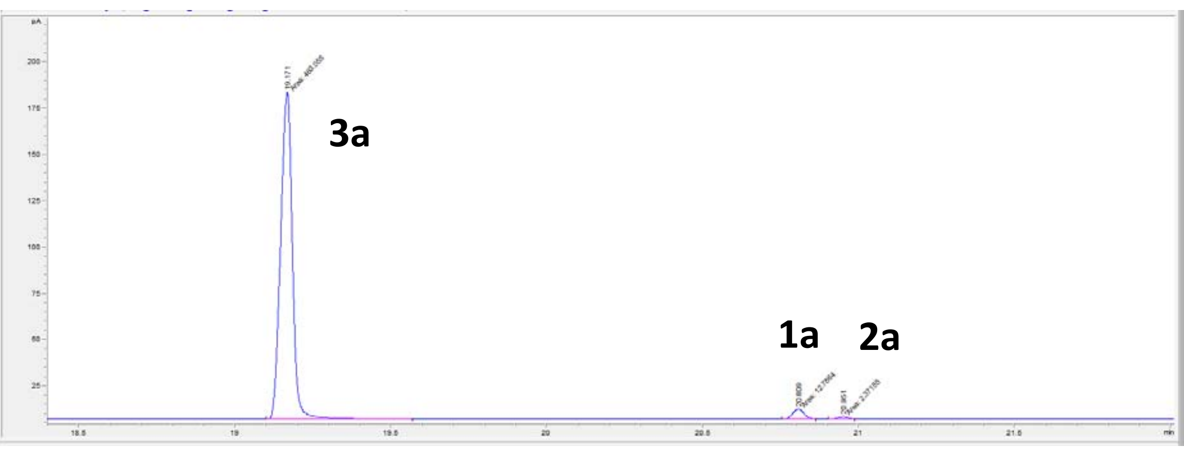
Table S21. Retention times using method F.

(*S*)-amine	Time [min]	(*R*)-amine	Time [min]
(*S*)-**3n**	15.2	(*R*)-**3n**	15.5
(*S*)-**3o**	13.3	(*R*)-**3o**	13.8
(*S*)-**3p**	14.2	(*R*)-**3p**	14.5

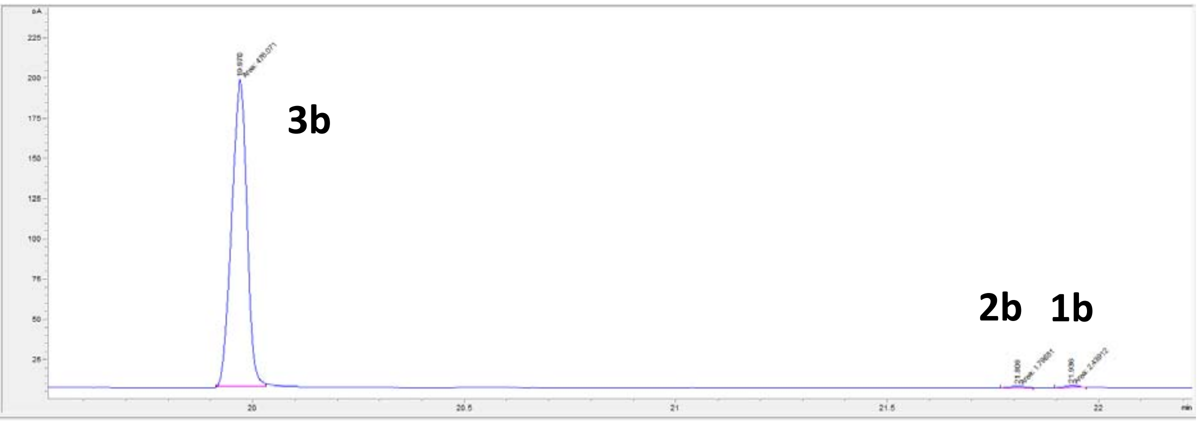
15. GC chromatograms

15.1 Representative GC chromatograms for the determination of the conversions

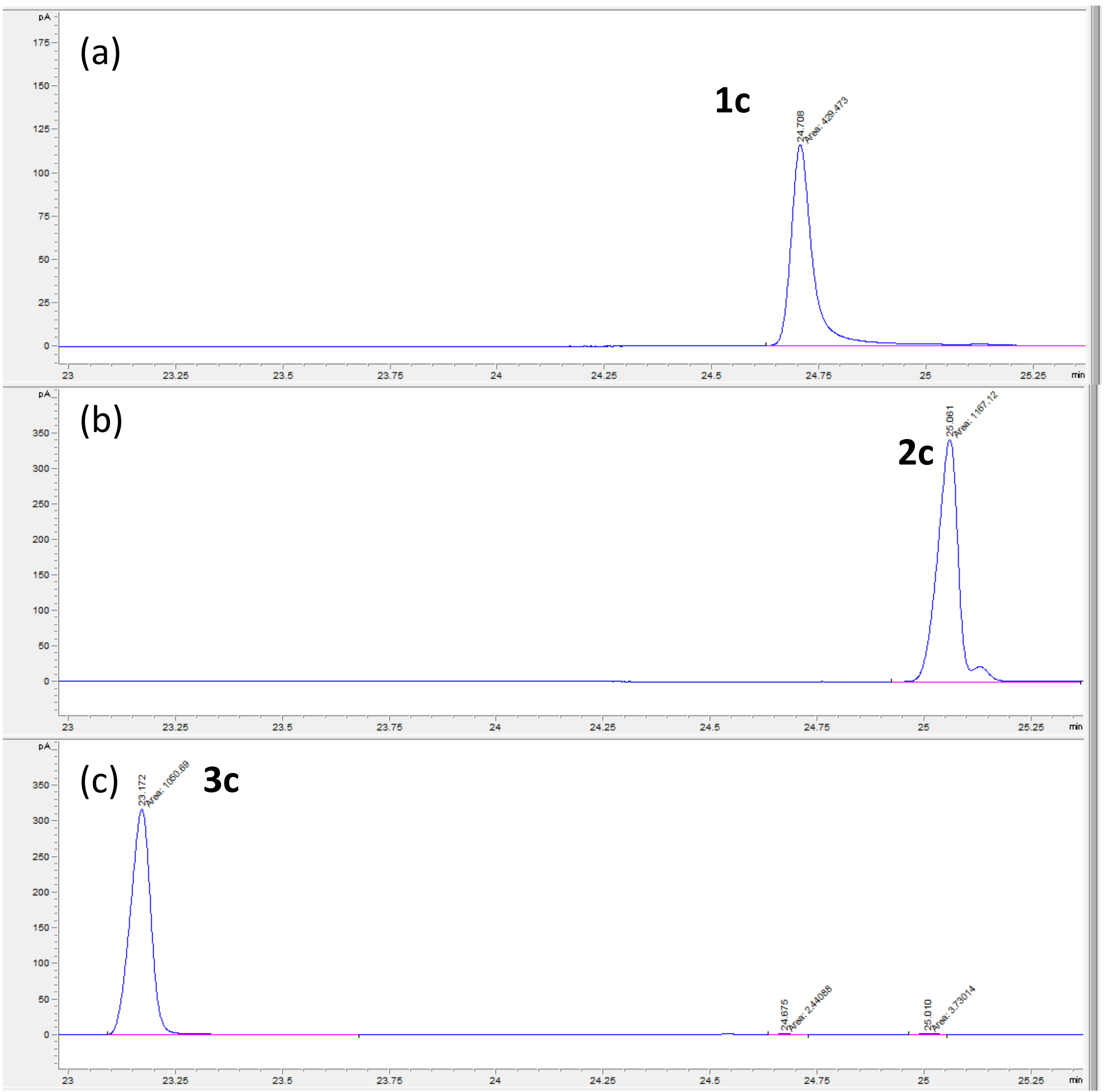
(1) Conversion of alcohol **1a** into amine **3a**



(2) Conversion of alcohol **1b** into amine **3b**



(3) Conversion of alcohol **1c** into amine **1c**: (a) Reference compound: alcohol **1c**; (b) Reference compound: ketone **2c**; (c) reaction



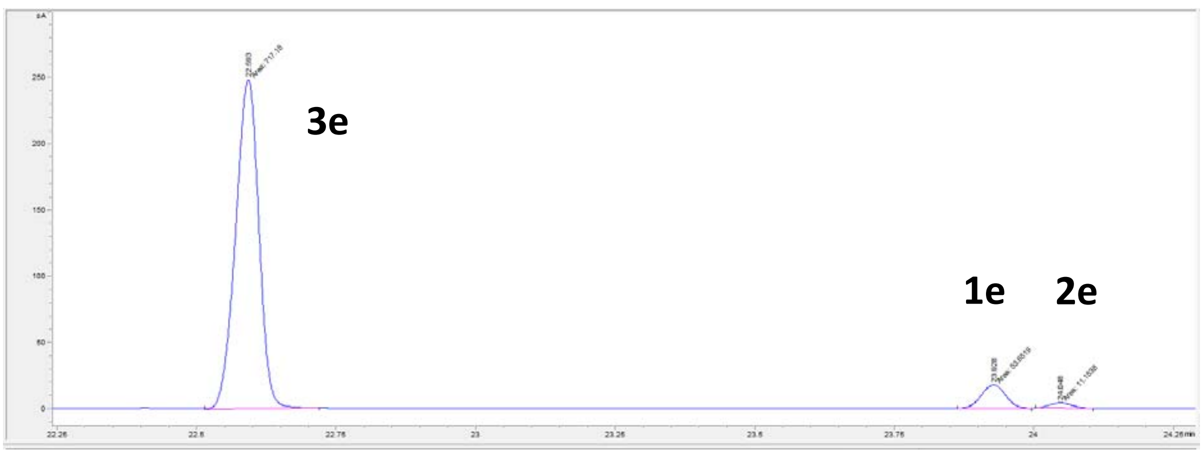
(4) Conversion of alcohol **1d** into amine **3d**: (a) Reference compound: alcohol **1d**; (b) Reference compound: ketone **2d**; (c) reaction

(a) 1d

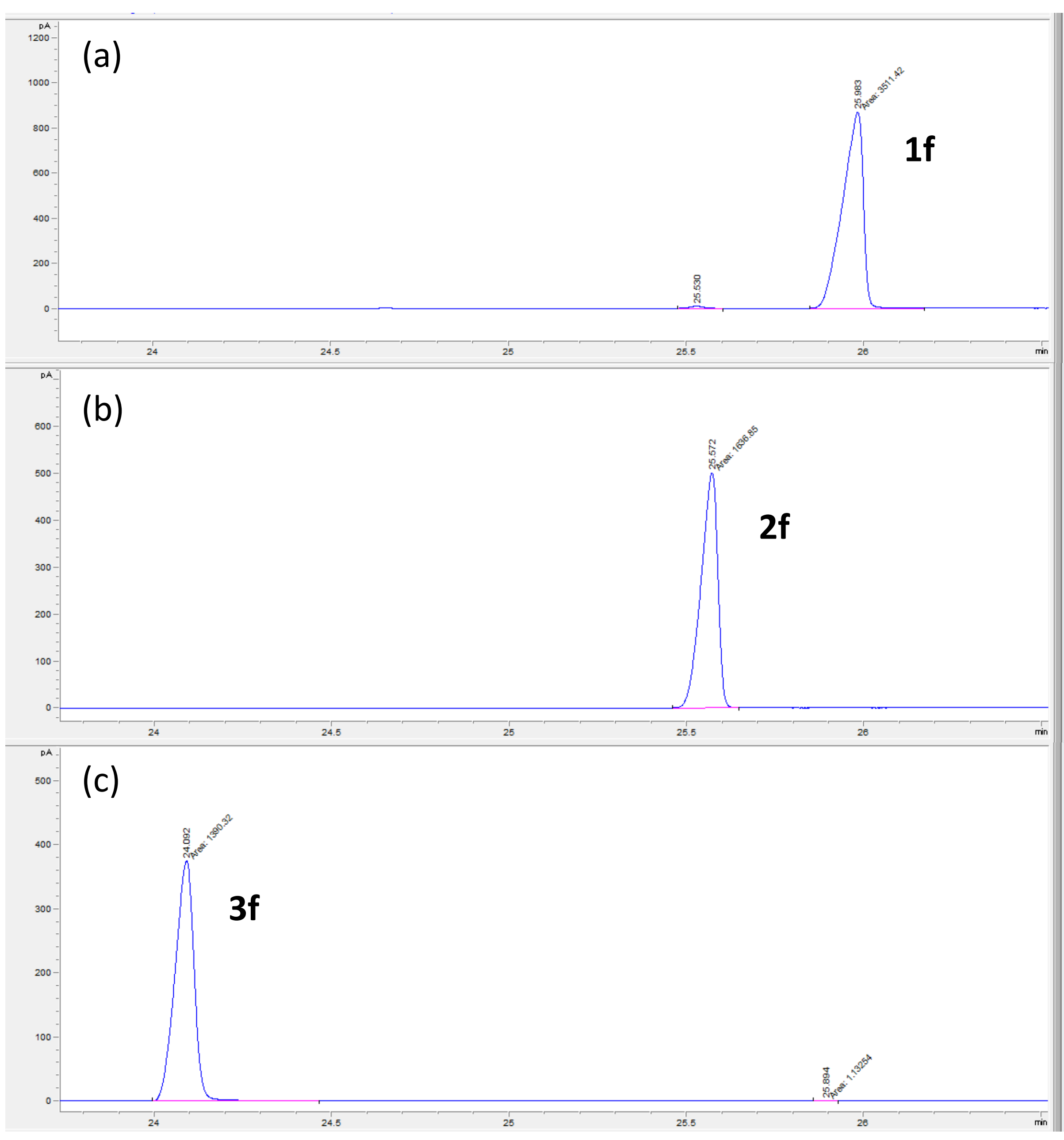
(b) 2d

(c) 3d

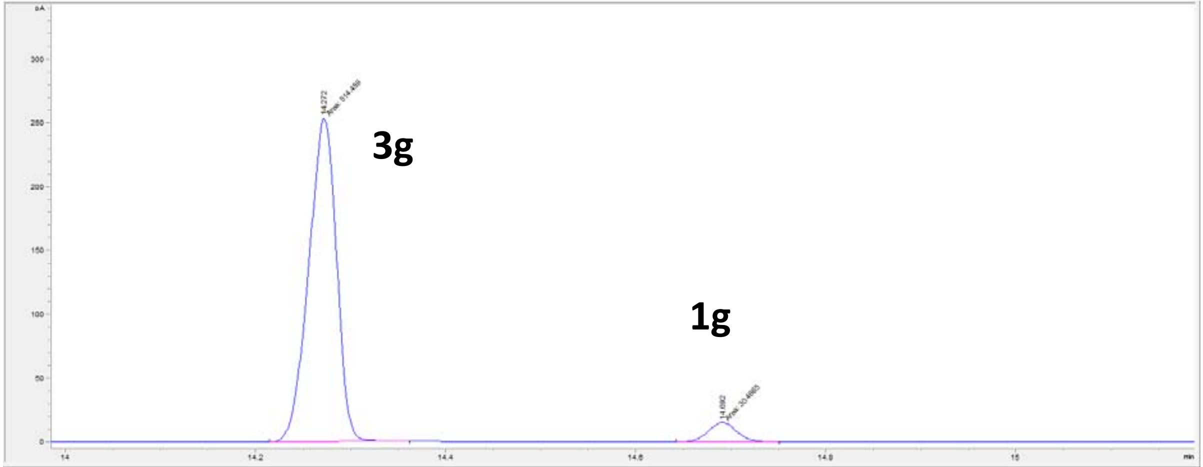
(5) Conversion of alcohol **1e** into amine **3e**



(6) Conversion of alcohol **1f** into amine **3f**: (a) Reference compound: alcohol **1f**; (b) Reference compound: ketone **2f**; (c) reaction

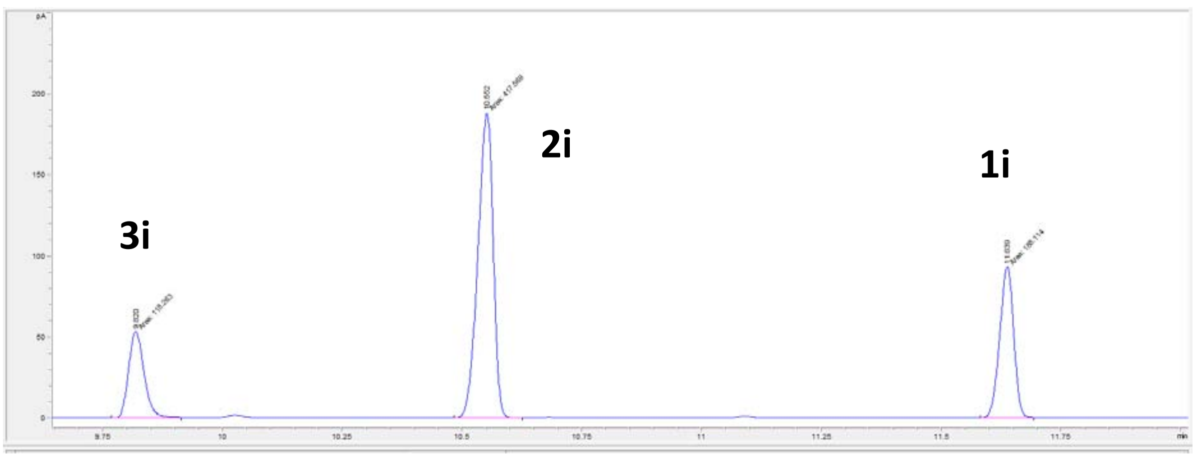


(7) Conversion of alcohol **1g** into amine **3g**

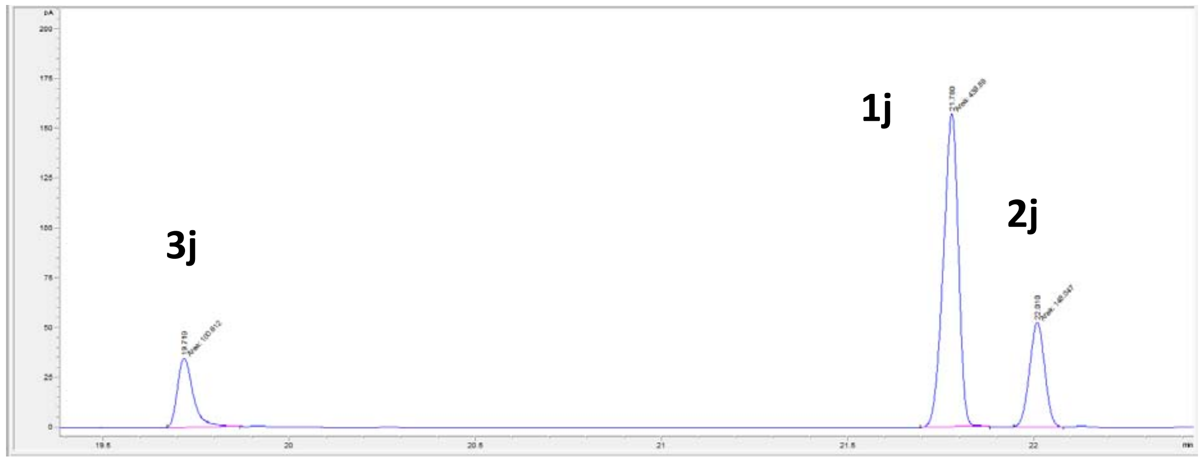


(8) Conversion of alcohol **1h** into amine **3h**

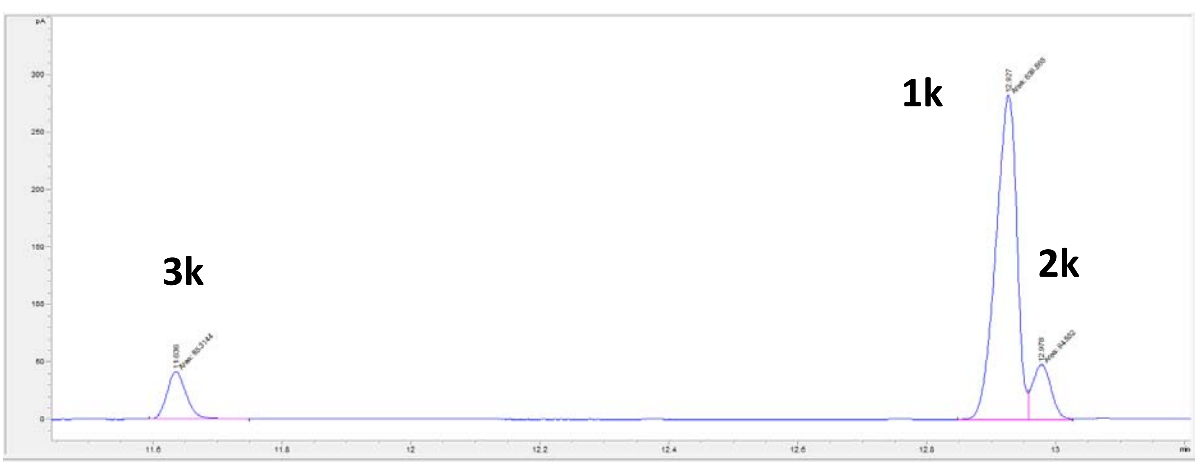
(9) Conversion of alcohol **1i** into amine **3i**



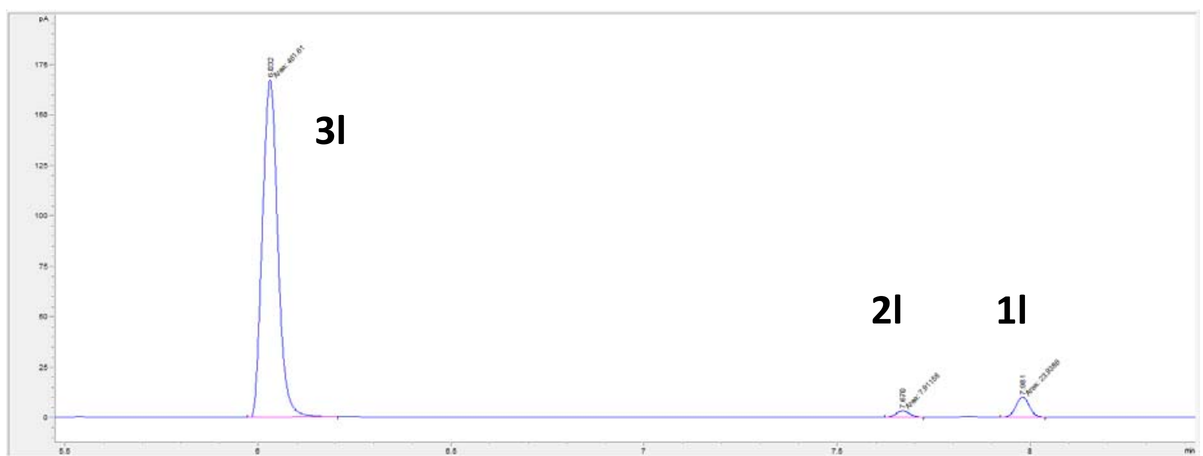
(10) Conversion of alcohol **1j** into amine **3j**



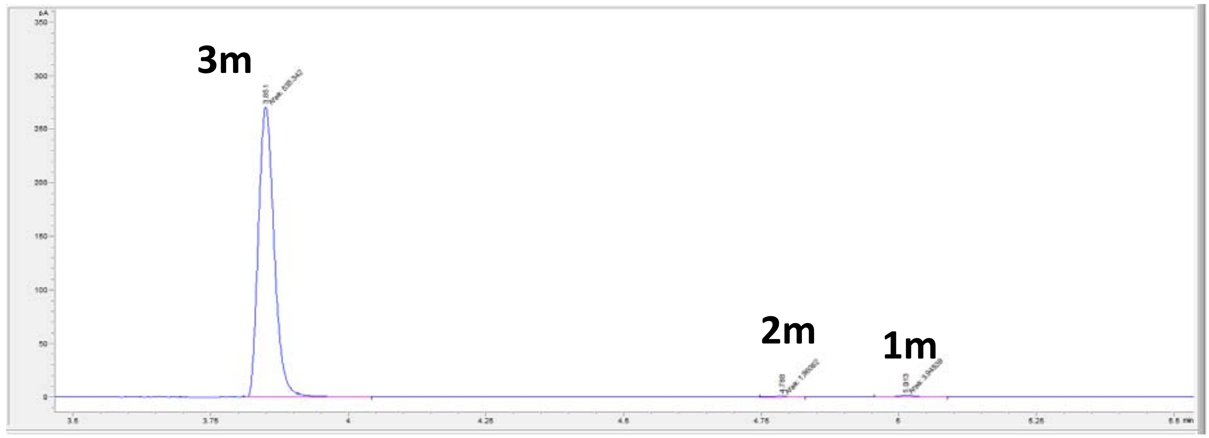
(11) Conversion of alcohol **1k** into amine **3k**



(12) Conversion of alcohol **1l** into amine **3l**



(13) Conversion of alcohol **1m** into amine **3m**



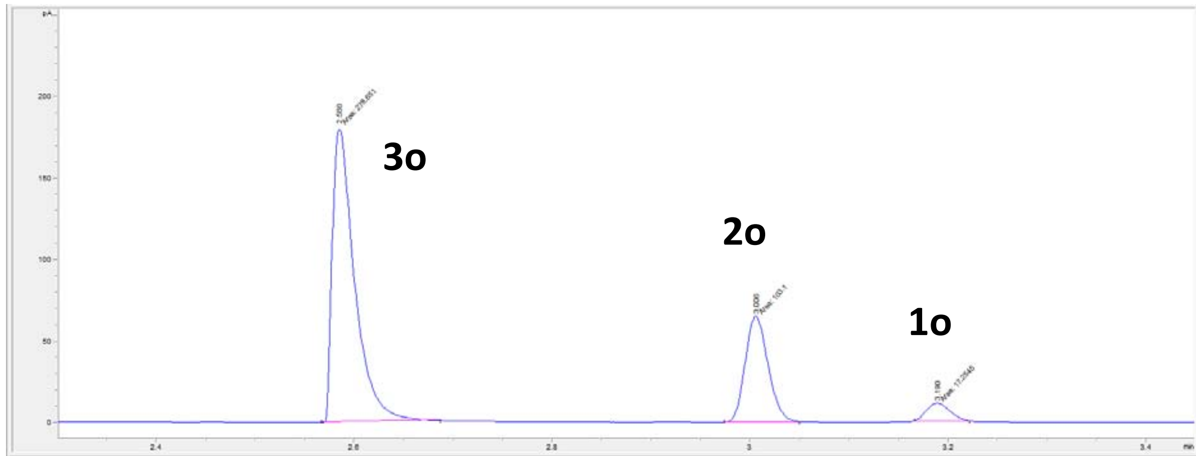
(14) Conversion of alcohol **1n** into amine **3n.** (a) Reference compound: alcohol **1n**; (b) Reference compound: ketone **2n**; (c) reaction

(a)
1n
5.141
4.680

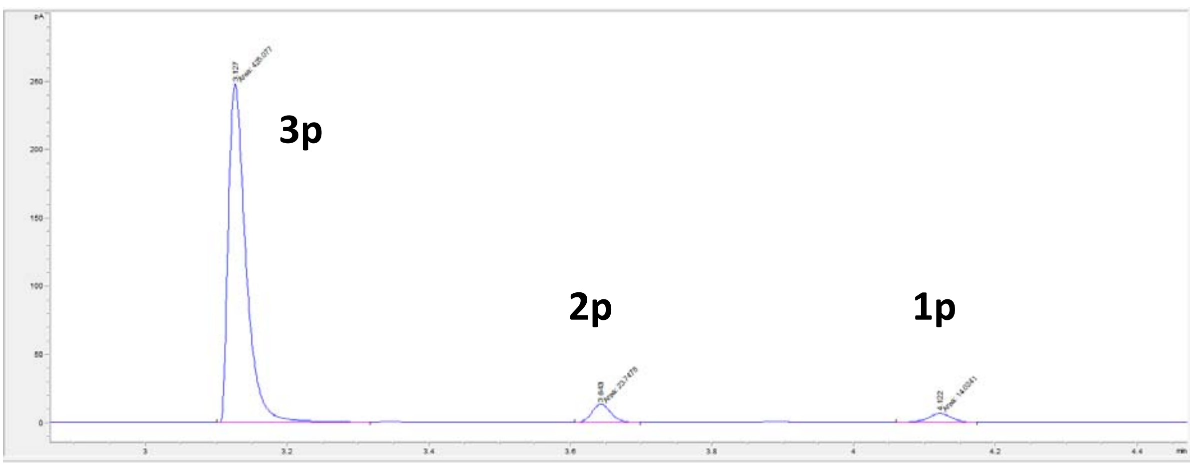
(b)
2n
4.706

(c)
3n
3.692
Area: 434.095

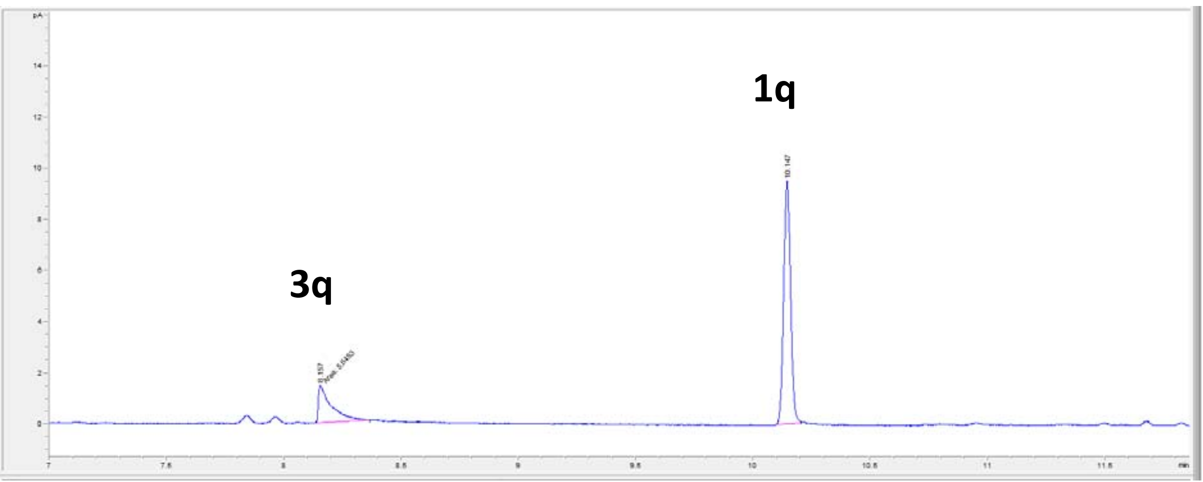
(15) Conversion of alcohol **1o** into amine **3o**



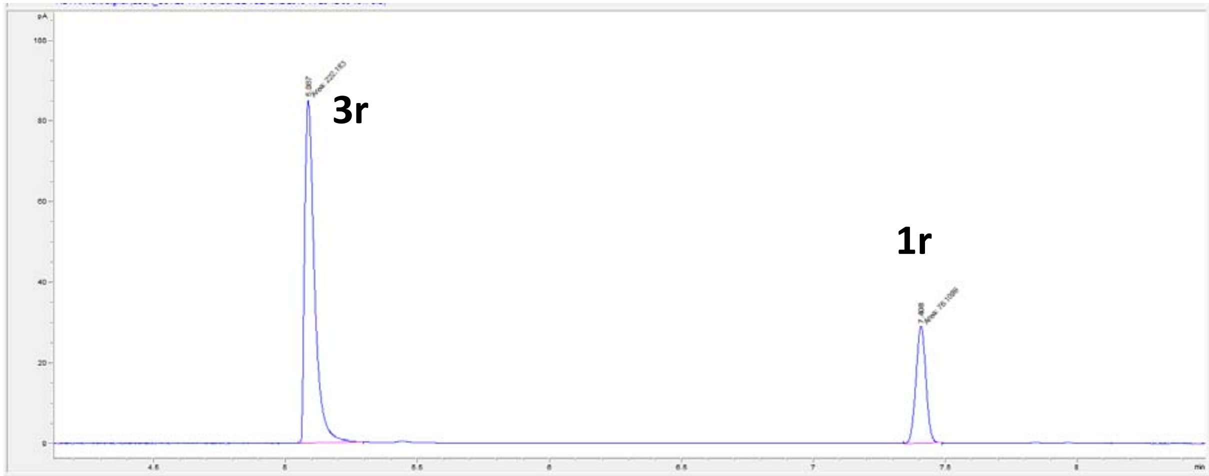
(16) Conversion of alcohol **1p** into amine **3p**



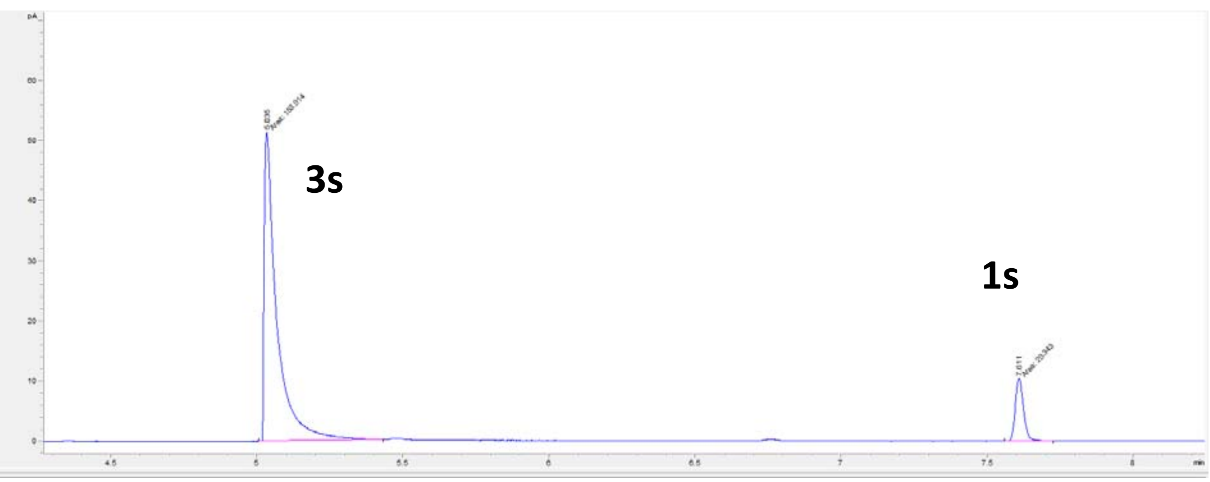
(17) Conversion of alcohol **1q** into amine **3q**



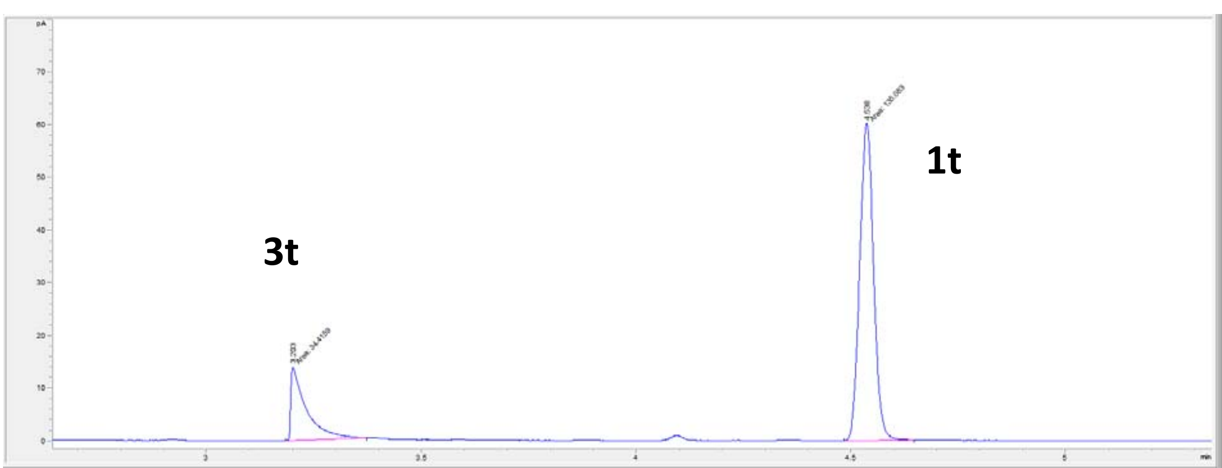
(18) Conversion of alcohol **1r** into amine **3r**



(19) Conversion of alcohol **1s** into amine **3s**

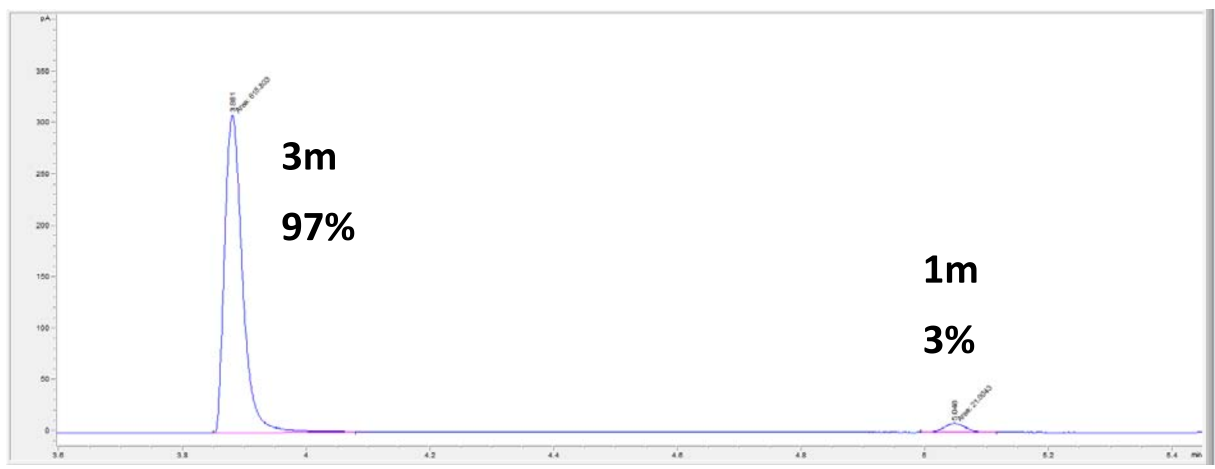


(20) Conversion of alcohol **1t** into amine **3t**

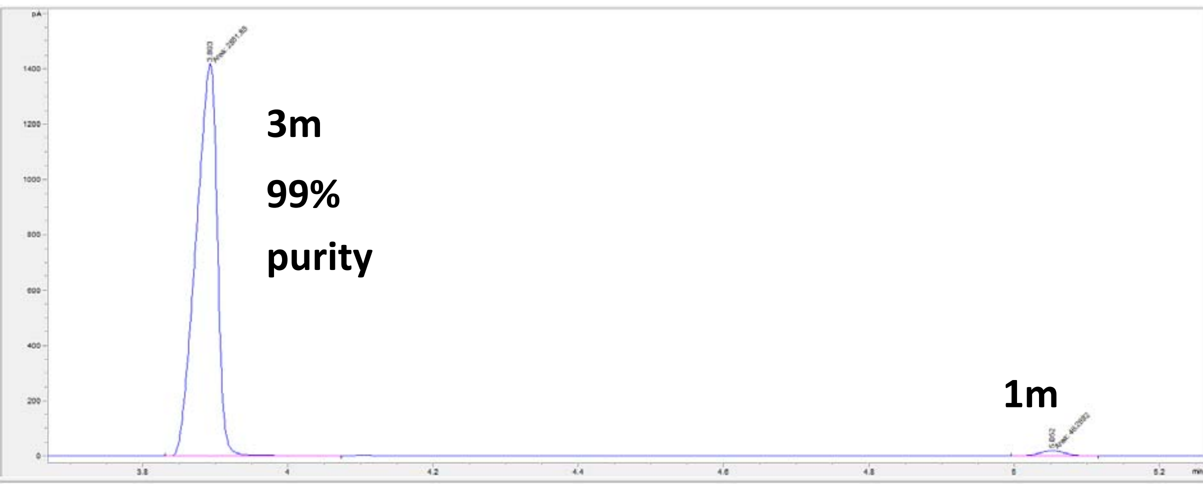


*15.2 GC chromatograms for the upscaling of **1m**.*

(a) Composition after the biocatalytic reaction

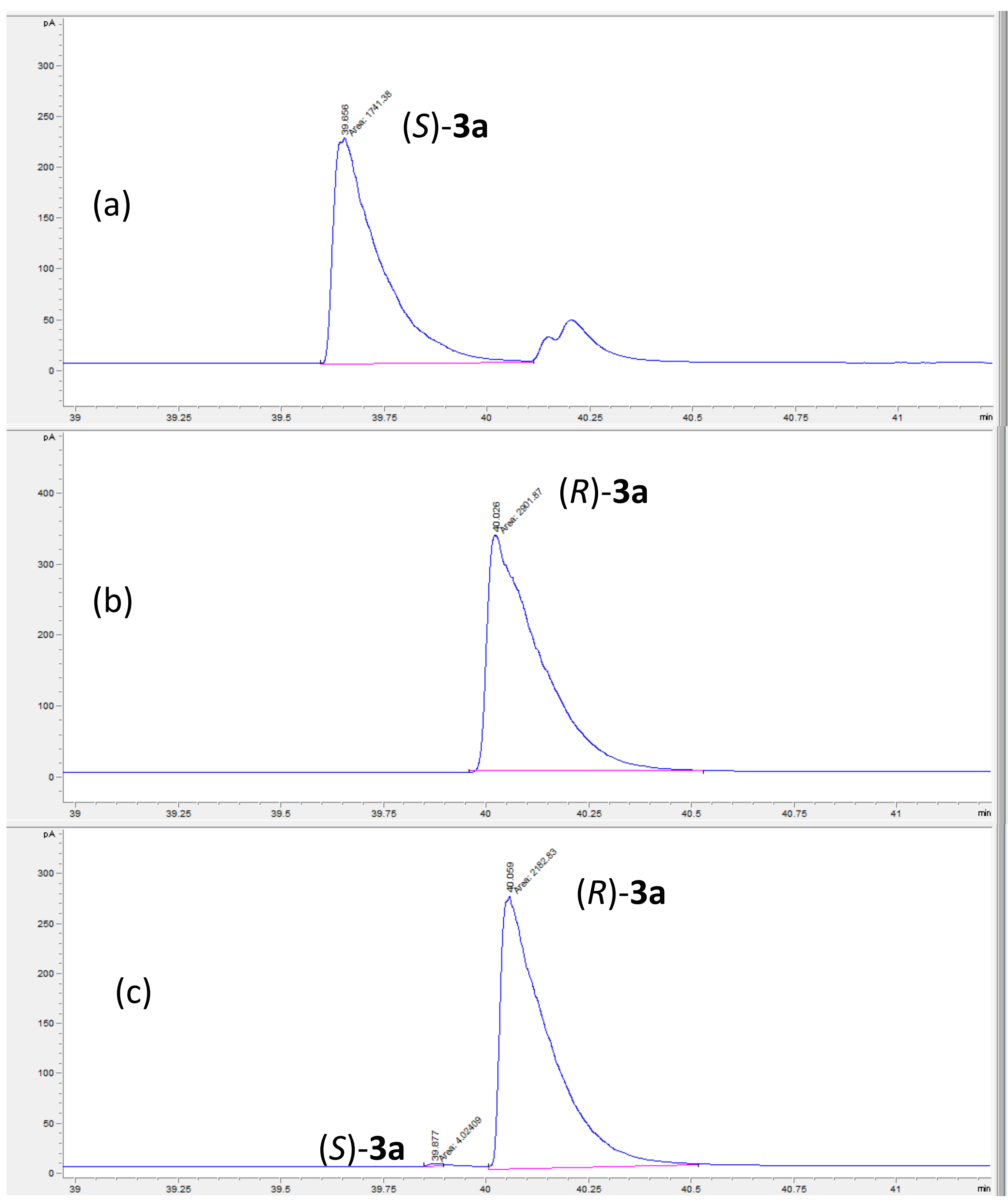


(b) Final product (after two step extraction under acid and basic conditions)

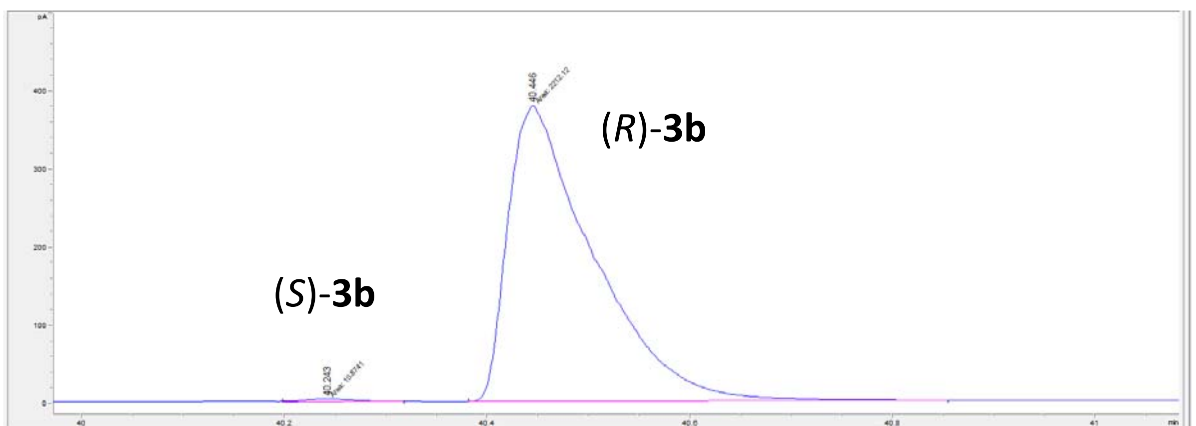


15.3 GC chromatograms for the determination of the ee values

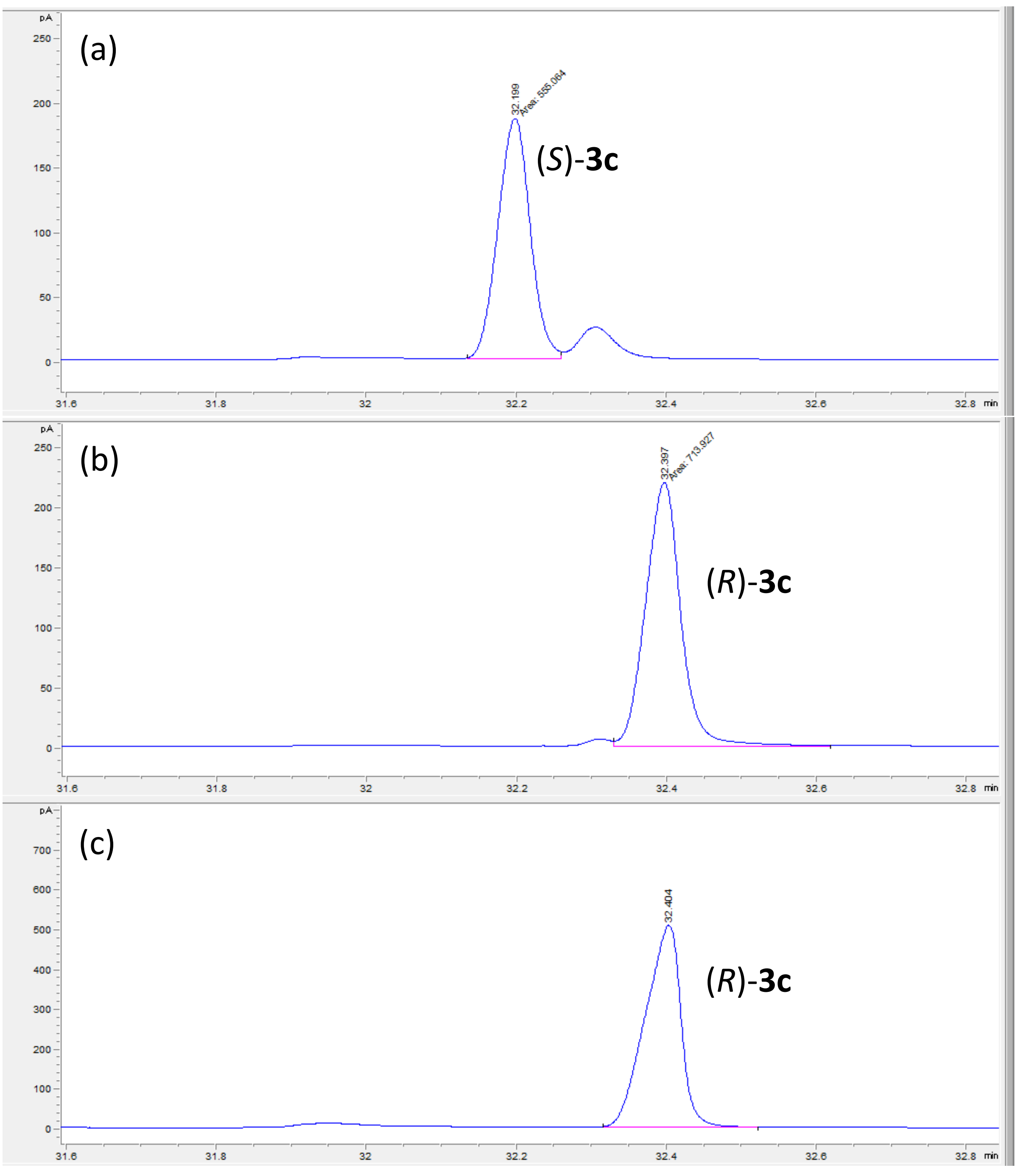
(1) (a) Reference compound: amine (*S*)-**3a** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: amine (*R*)-**3a** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3a** as the final product.



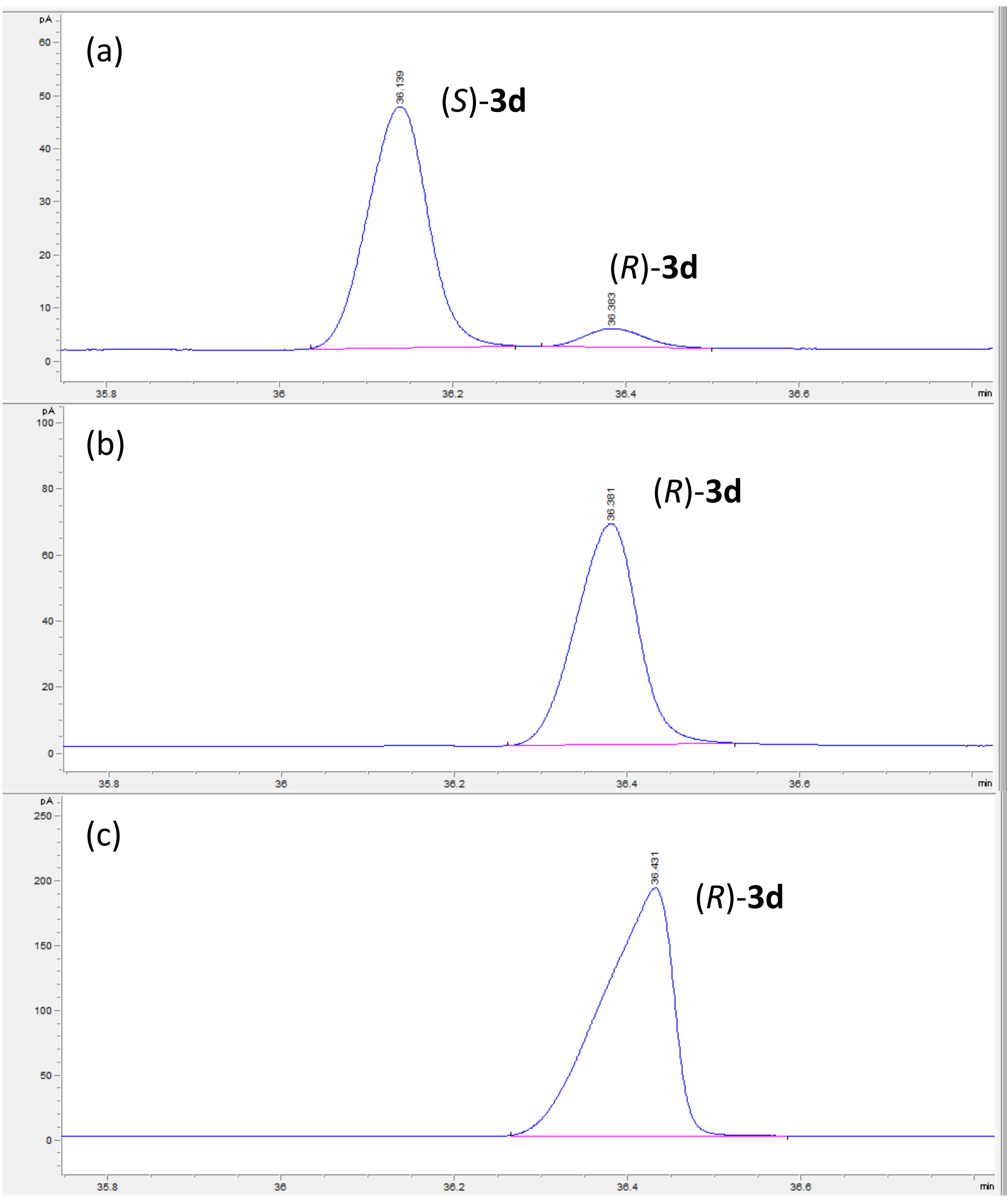
(2) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3b** as the final product



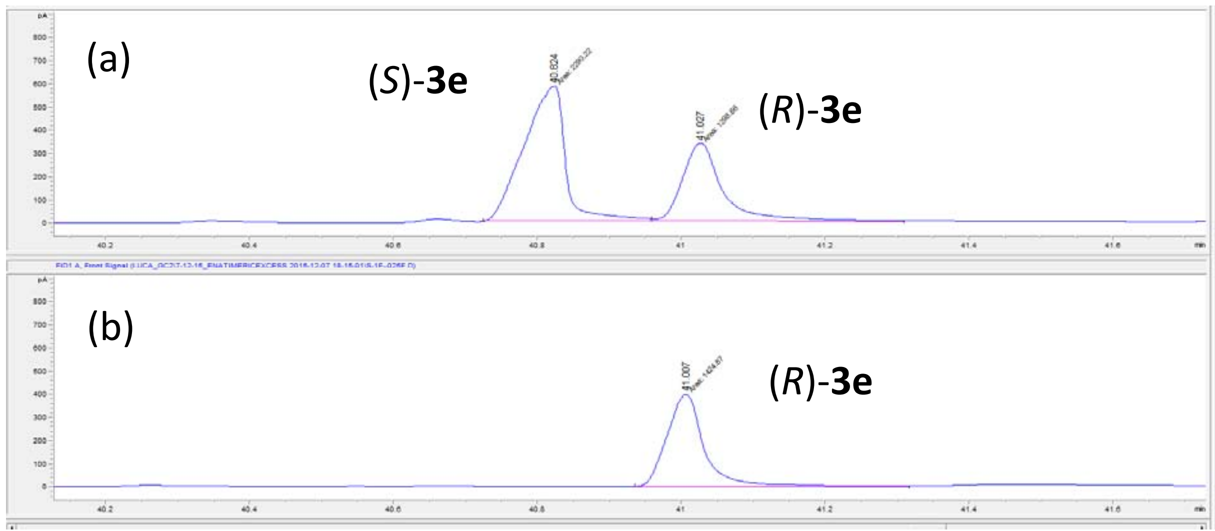
(3) (a) Reference compound: amine (*S*)-**3c** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: amine (*R*)-**3c** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3c** as the final product.



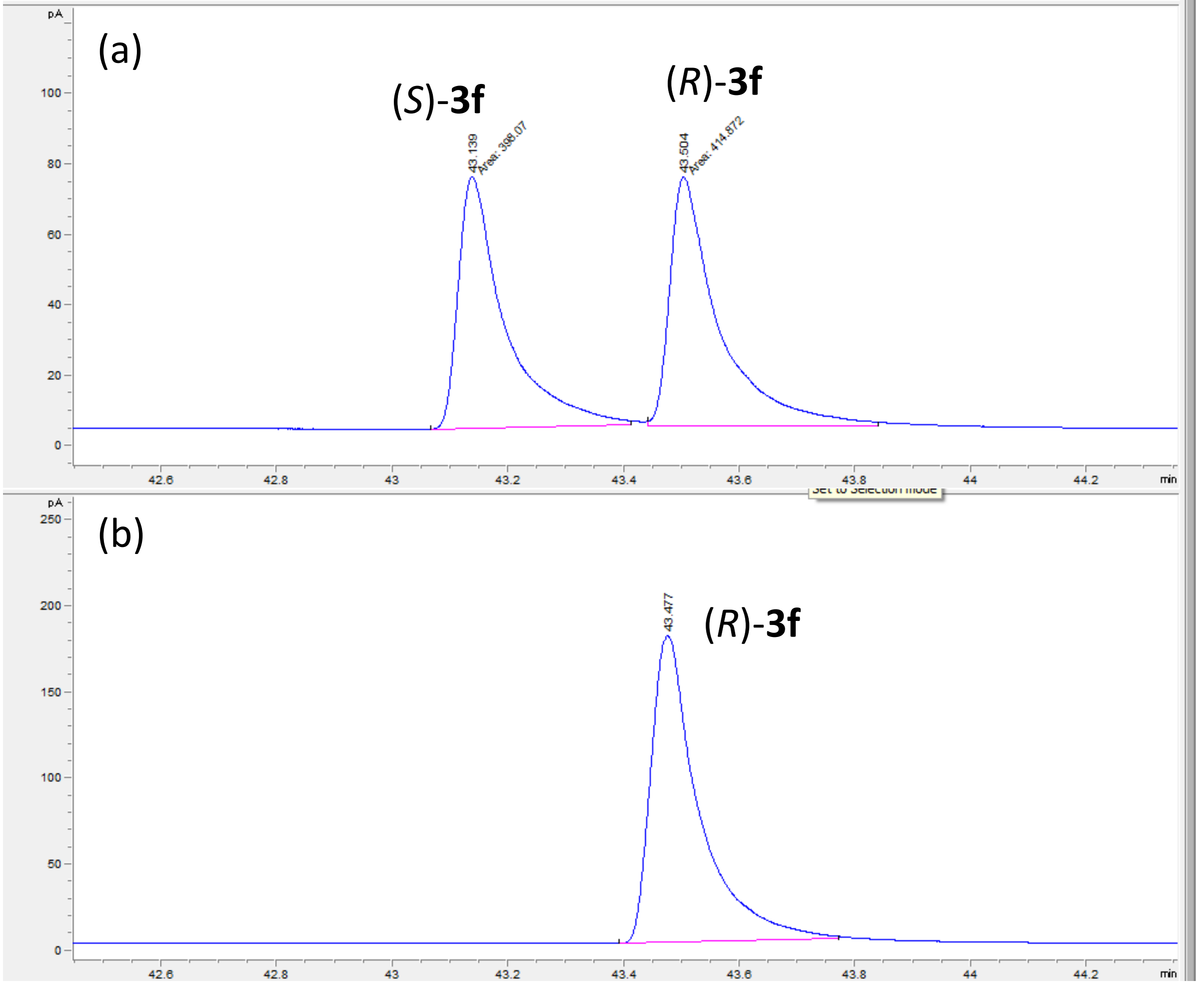
(4) (a) Reference compound: amine (*S*)-**3d** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: amine (*R*)-**3d** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3d** as the final product.



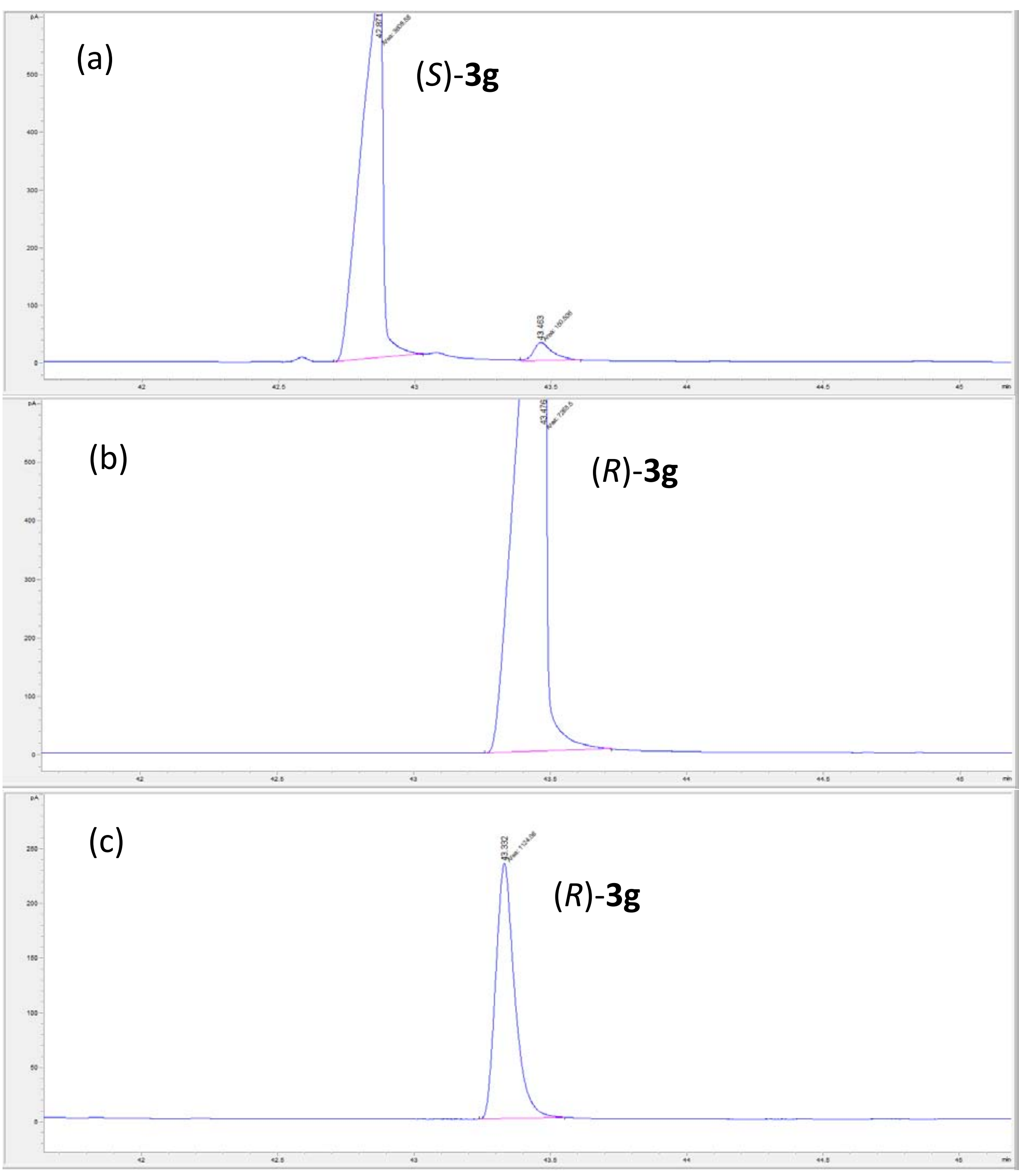
(5) (a) Reference compound: mixture of (*R*,*S*)-Amine-**3e** from reaction catalyzed by non selective ω-transaminase. (b) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3e** as the final product.



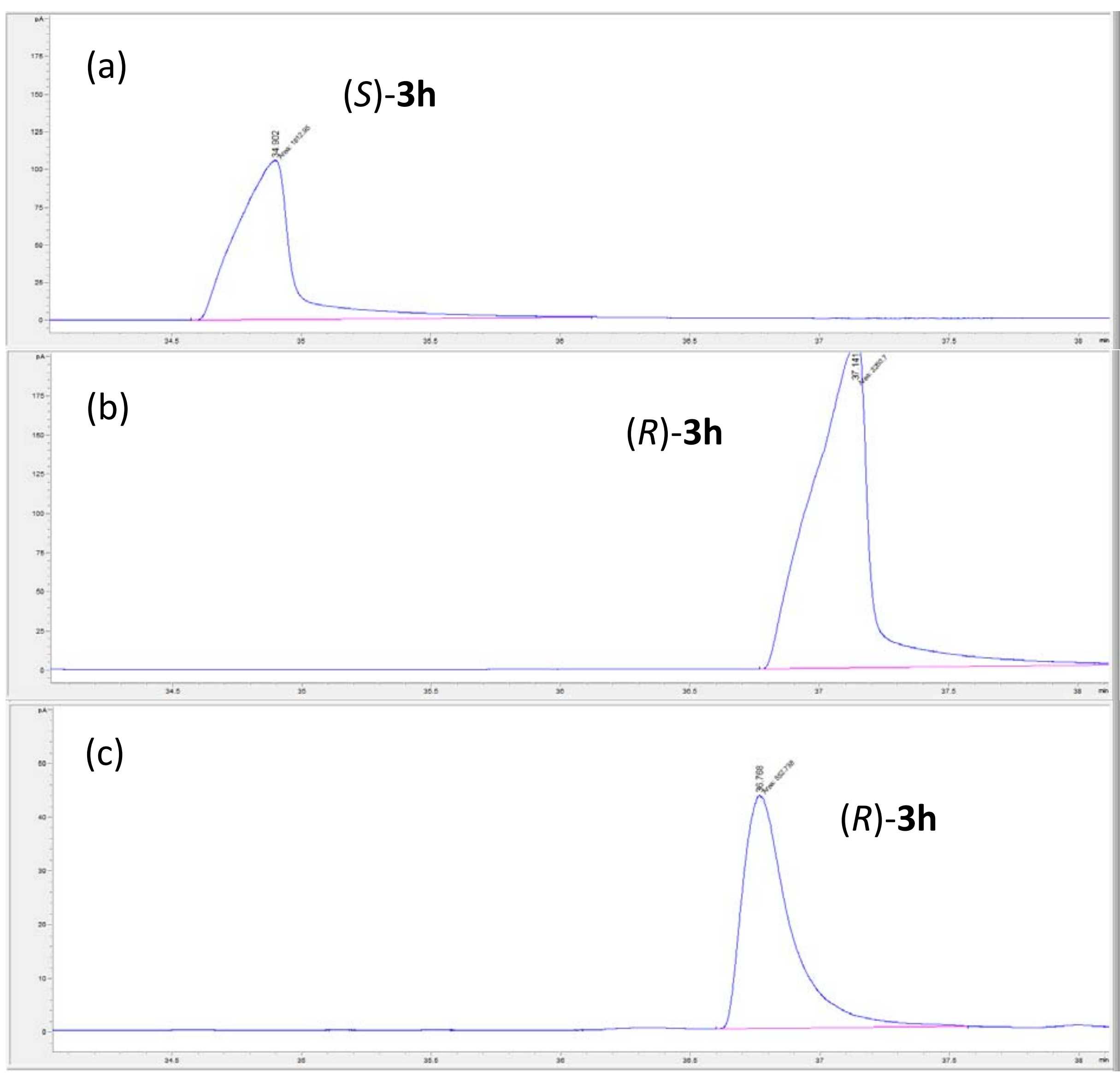
(6) (a) Reference compound: mixture of (*R*,*S*)-Amine-**3f** from commercially available racemic and enantiopure amine. (b) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3f** as the final product.



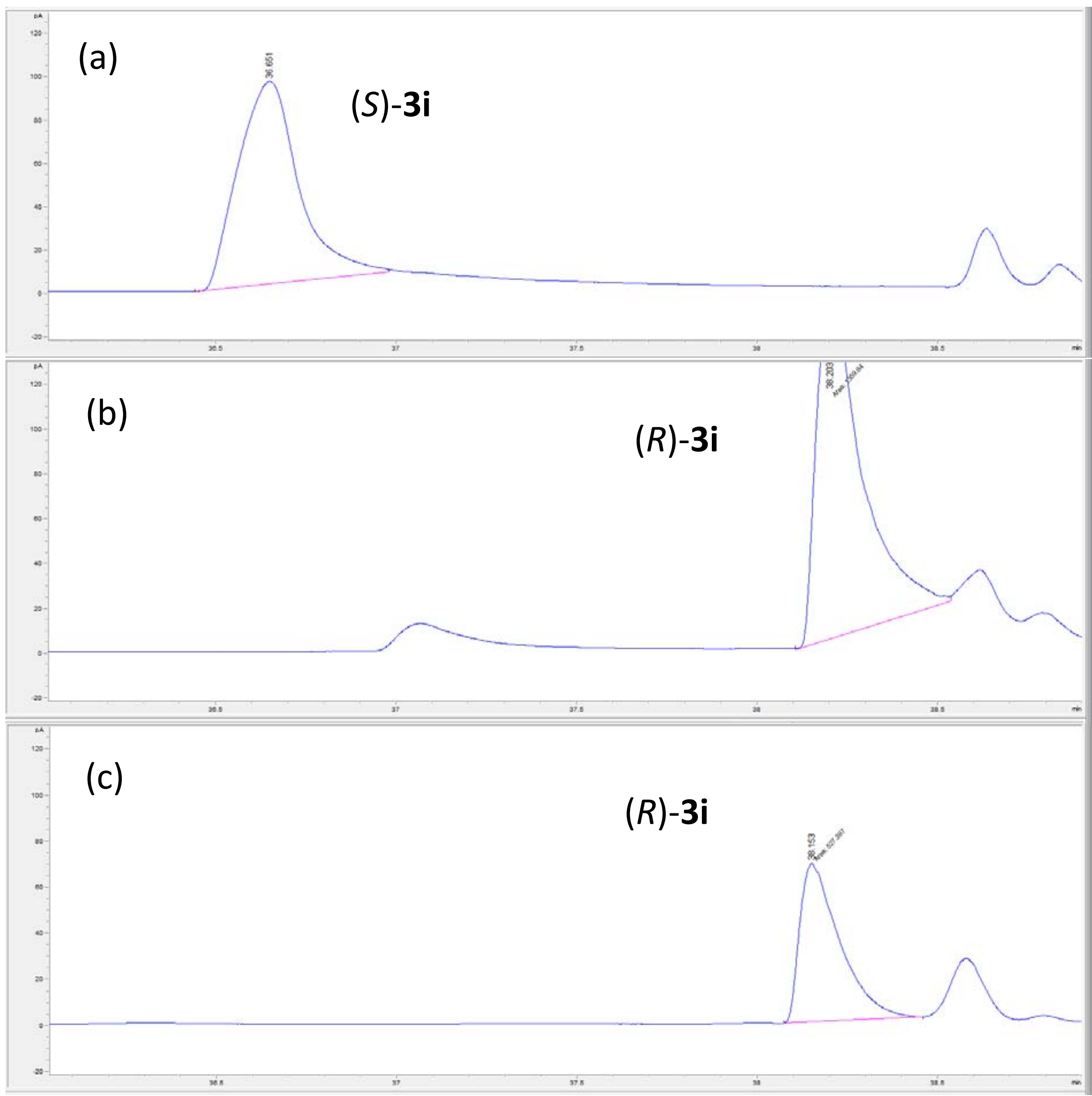
(7) (a) Reference compound: (*S*)-amine-**3g** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*R*)-amine-**3g** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3g** as the final product.



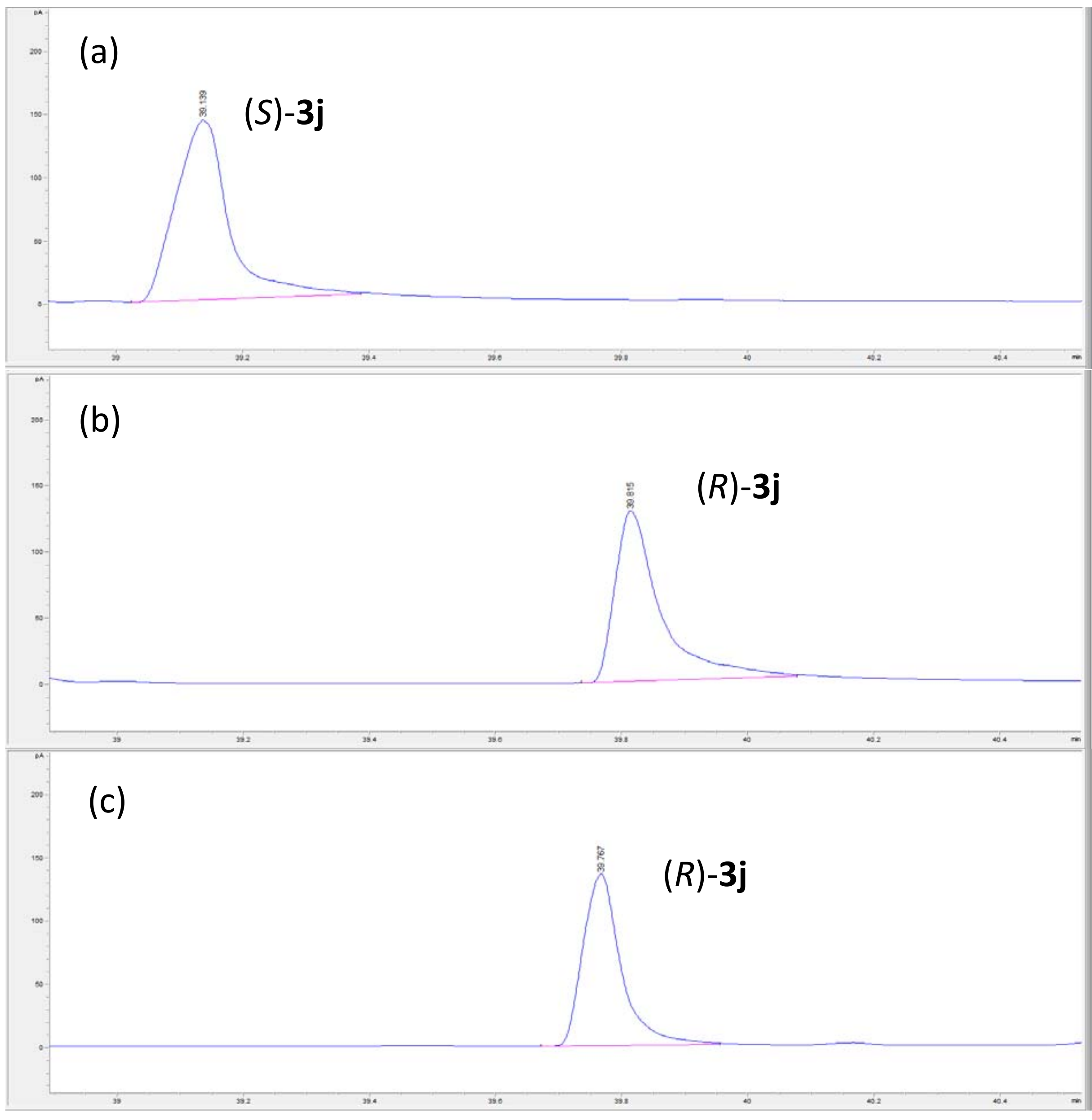
(8) (a) Reference compound: (*S*) amine-**3h** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*R*) amine-**3h** from reaction catalyzed by selective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3h** as the final product.



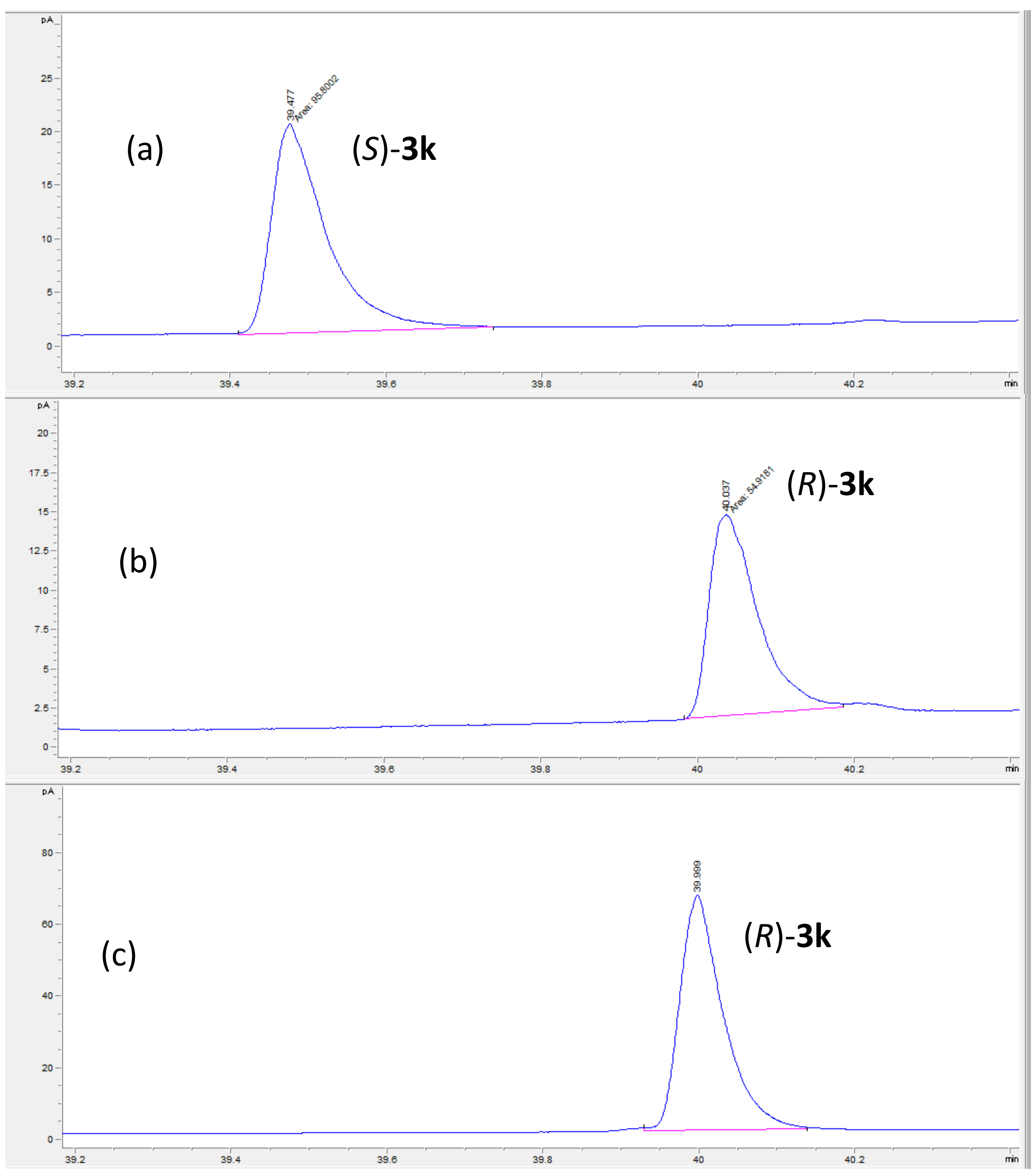
(9) (a) Reference compound: (*S*)-amine-**3i**, commercially available. (b) Reference compound: (*R*) amine-**3i**, commercially available. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3i** as the final product.



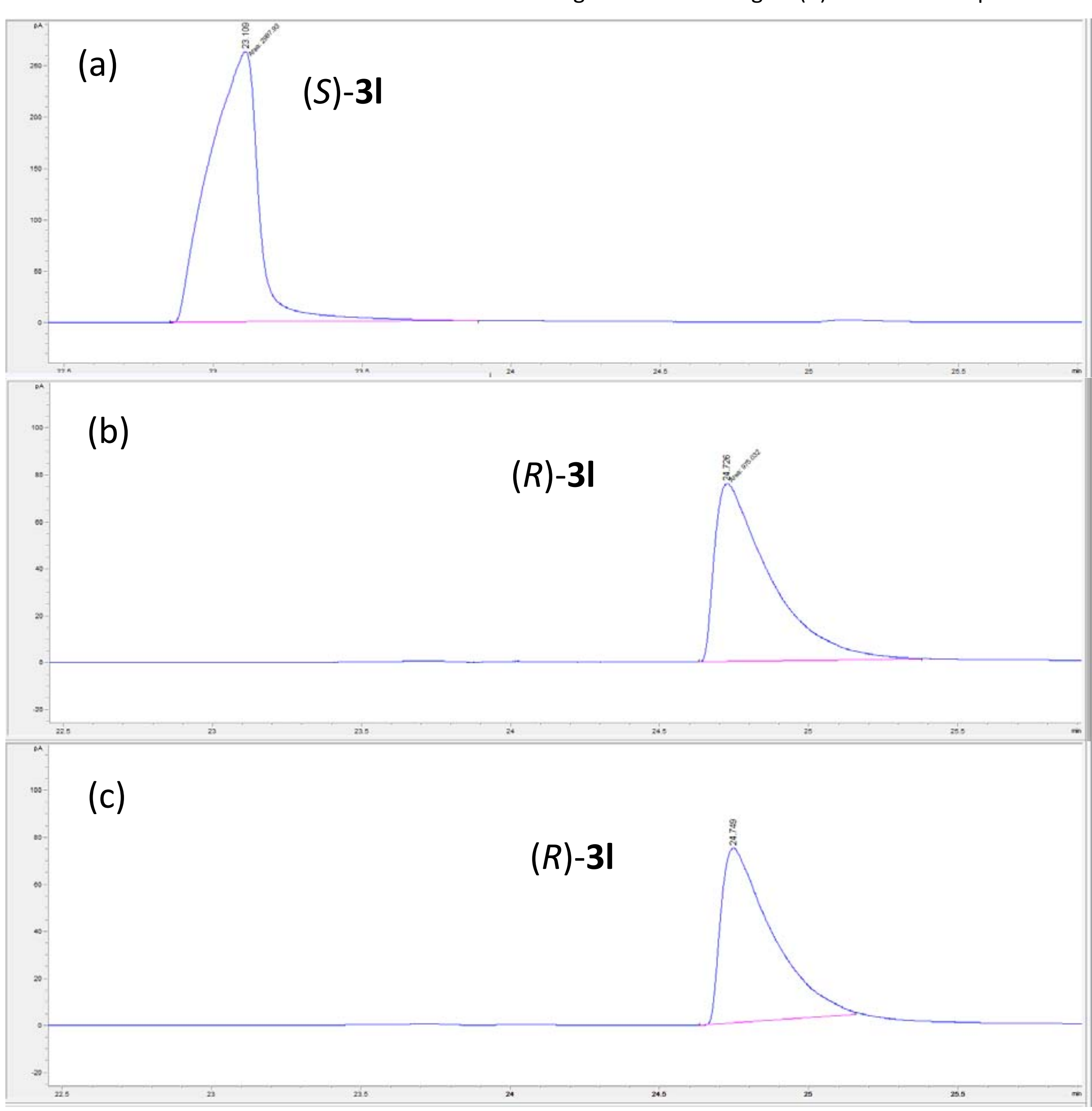
(10) (a) Reference compound: (*S*)-amine-**3j** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*R*)-amine-**3j** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3j** as the final product



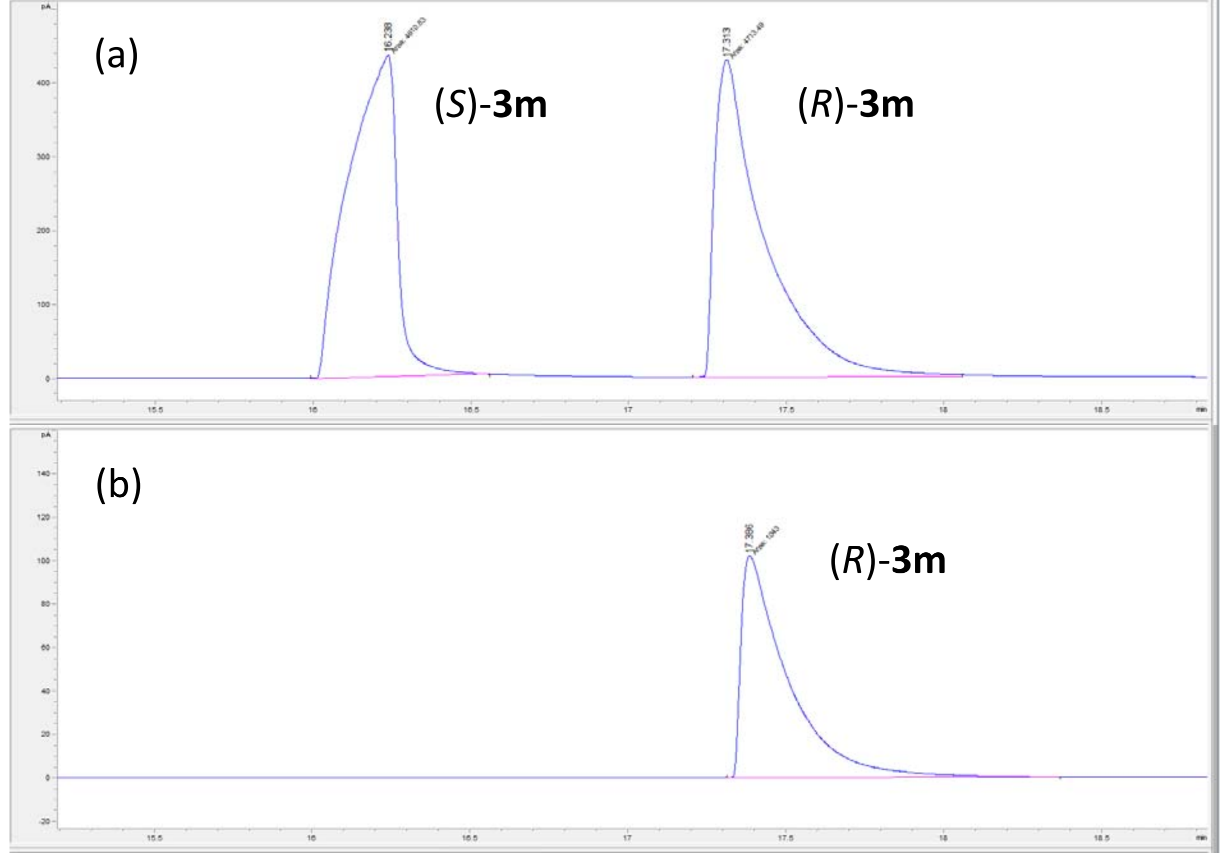
(11) (a) Reference compound: (*S*)-amine-**3k** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*R*)-amine-**3k** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3k** as the final product.



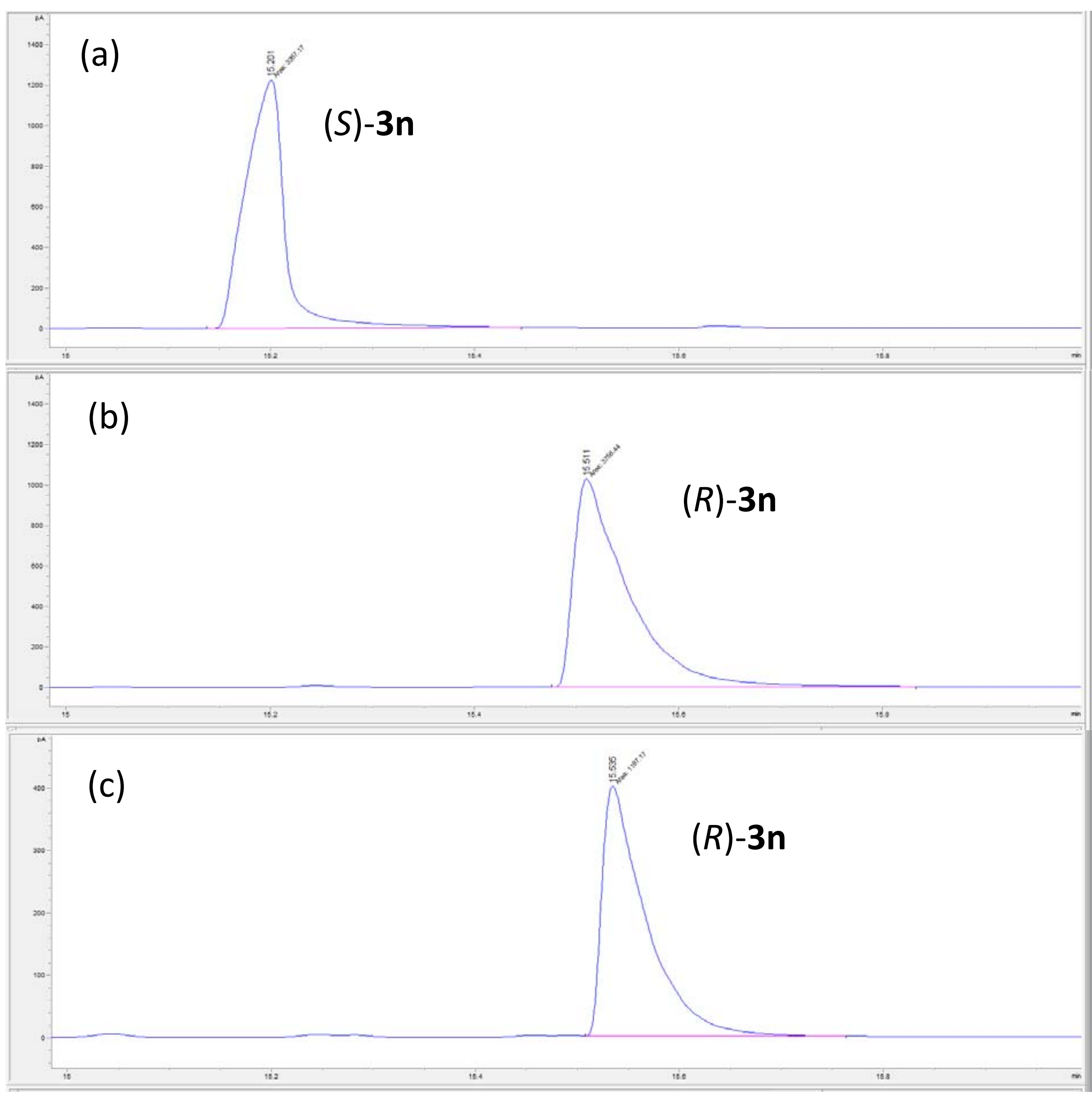
(12) (a) Reference compound: (*S*)-amine-**3l** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*R*)-amine-**3l** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3l** as the final product.



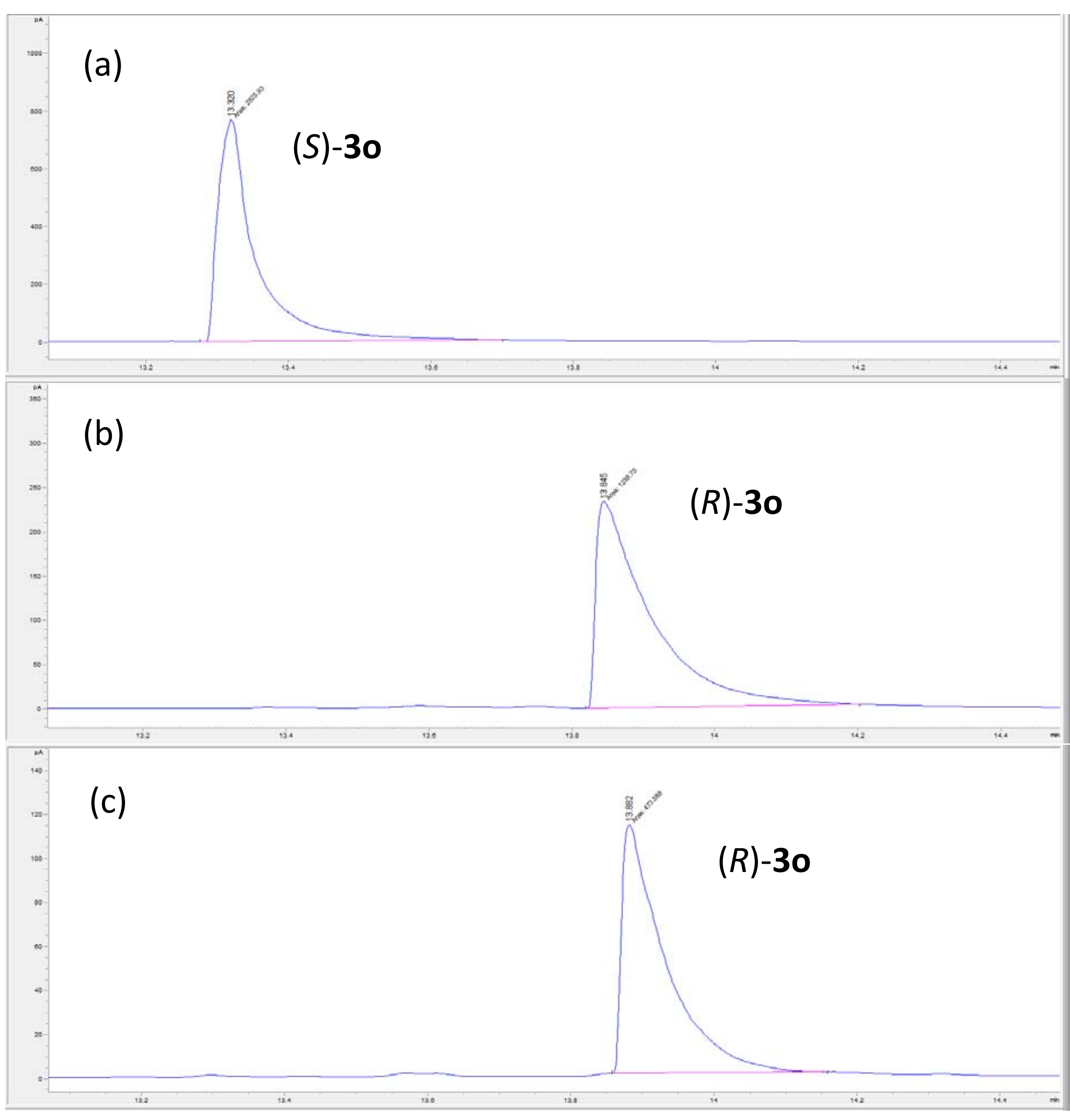
(13) (a) Reference compound: *racemic* (*R*,*S*)-**3m.** (b) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3m** as the final product



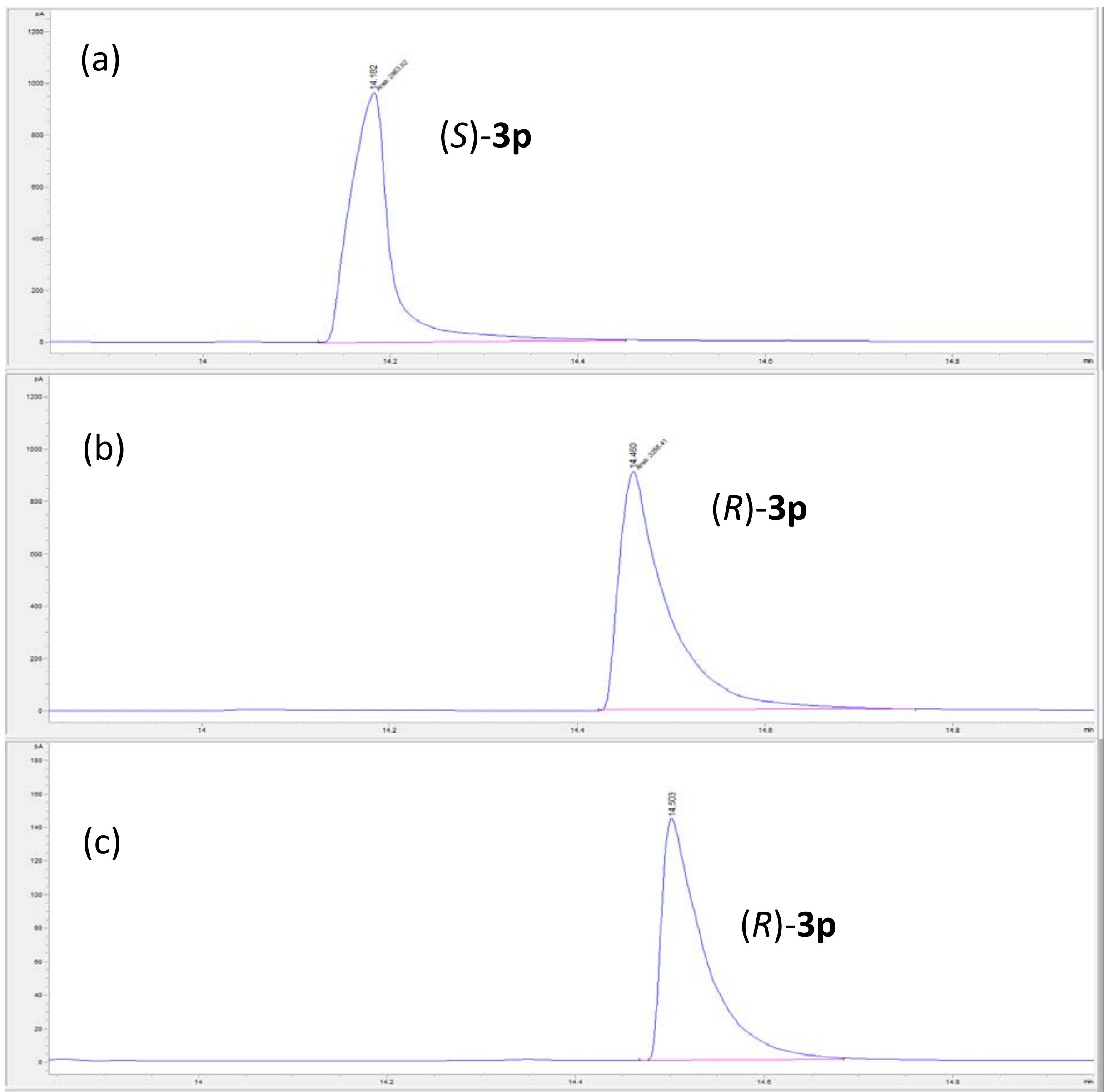
(14) (a) Reference compound: (*S*)-amine-**3n** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*S*)-amine-**3n** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3n** as the final product.



(15) (a) Reference compound: (*S*)-amine-**3o**, commercially available. (b) Reference compound: (*R*)-amine-**3o**, commercially available. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3o** as the final product



(16) (a) Reference compound: (*S*)-amine-**3p** from reaction catalyzed by stereoselective ω-transaminase. (b) Reference compound: (*R*)-amine-**3p** from reaction catalyzed by stereoselective ω-transaminase. (c) Determination of the enantiomeric excess for the orthogonal-cascade to give (*R*)-**3o** as the final product.



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