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Enzymatic and chiral HPLC resolution of α-azido acids and amides

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Abstract

For the first time, enzymatic resolution of α-azido acid amides has been successfully demonstrated with high yields and enantiomeric excess. In one case dynamic kinetic resolution was achieved leading to more than 50% yield of the enantiomerically pure azido acid. Chiral HPLC was also used to separate racemic α-azido acids and the separation process was automated. Two routes to enantiopure α-azido acid building blocks for solid-phase peptide synthesis have, therefore, been established.

1. Introduction

α-Azido acids are versatile precursors for natural and non-natural amino acids and can be incorporated into peptides and subsequently reduced to amino acids.[1] The azido group offers complete protection of the amine so the carboxyl group can be highly activated as the acid chloride and reacted with sterically hindered amino groups on solid-phase and in solution. This method thereby overcomes one of the remaining obstacles in solid-phase peptide synthesis, the problem of sterical congestion in difficult couplings.[2] Azido acids can be prepared from simple starting compounds such as α-bromo carboxylic acids, incorporated into peptides and reduced on the solid-phase.[1]

The pharmaceutical industry has focused attention on peptide mimetics and non-natural amino acids because they offer the possibility of varying the side chains of a given peptide and restrict the conformation, leading to putative agonists or antagonists of increased activity. The α,α-di-substituted amino acids often stabilise or constrain peptides to β-turns,[3] 3_{10}-helices[4,5] or extended conformations.[6,7]

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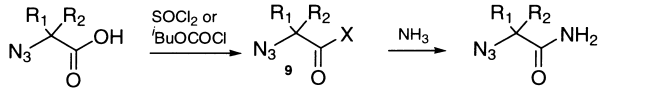
Asymmetric synthesis of α-azido acids can be performed with a chiral auxiliary[8] or an asymmetric phase transfer catalyst, but these approaches are often difficult and time-consuming and do not always give high enantiomeric excess (e.e.). Another versatile approach is enzymatic resolution, which often discriminates very well between two enantiomers, giving good to excellent e.e.s.[9] To our knowledge, considerable effort has been dedicated to enzymatic syntheses of enantiomerically pure α-*H*-α-amino acids, while far less has been done on α,α-disubstituted amino acids,[10–14] and nothing on mono- and disubstituted α-azido acids. Chiral HPLC may also be used preparatively to obtain enantiopure compounds and many different chiral stationary phases (CSPs) are available, e.g. immobilised amino acids, proteins, cyclodextrins, carbohydrates, crown ethers and ligand-exchange columns. In addition, a new type of CSP has emerged, namely macrocyclic glycopeptide antibiotics immobilised on a silica gel column, that has proven efficient in separating enantiomers.[15,16] Two of these antibiotics are commercially available, namely vancomycin and teicoplanin.[17]

Teicoplanin is produced by *Actinoplanes teichomyceticus* and has antimicrobial properties against Gram-positive bacteria. It has four fused macrocyclic rings, seven aromatic rings and three carbohydrate moieties where one has a long hydrocarbon chain attached.[18] All these properties render teicoplanin diverse as a CSP and it has generally good enantioselectivity for α-substituted carboxylic acids.

Azido acids have been developed for use in SPPS.[1] Because the need for enantiomerically pure material quickly arose, an investigation into the preparation and purification of enantiopure α-azido acids was performed. The two approaches, enzymatic resolution and chiral HPLC, are both very important and they complement each other. The enzymatic resolution can be performed on a large scale but it takes time to develop, whereas the chiral HPLC is fast but the capacity is limited.

2. Results and discussion

The following racemic α-azido acid amides (see Scheme 1) have been prepared from their corresponding acids. Syntheses of the latter will be described elsewhere.[19]



1 $R_1 = CH_3$, $R_2 = CH_2CH_3$
2 $R_1 = H$, $R_2 = (CH_2)_3CH_3$
3 $R_1 = H$, $R_2 = (CH_2)_{13}CH_3$
4 $R_1 = H$, $R_2 = Ph$

1 X = Cl
2-4 X = $OCOCH_2CH(CH_3)_2$

5 $R_1 = CH_3$, $R_2 = CH_2CH_3$
6 $R_1 = H$, $R_2 = (CH_2)_3CH_3$
7 $R_1 = H$, $R_2 = (CH_2)_{13}CH_3$
8 $R_1 = H$, $R_2 = Ph$

Scheme 1. The α-azido acid amides **5**–**8** were prepared by activating **1**–**4** with $SOCl_2$ or isobutylchloroformate and reacting them with ammonia in CH_2Cl_2 or THF

The synthesis of the α-azido acid amides was relatively straightforward, with yields ranging from 40 to 70%. Quantitative reactions were not achieved, and the prolonged reaction times (often more than 1 day) caused some decomposition of the activated azido acid **9** before it could react with ammonia.

In preparative chiral HPLC practice, it is standard procedure to overload the chiral column when separating quantitative amounts of material and then collect the first and the last parts of

the broad, overlapping peaks only and purify the middle fraction again. In this case, however, the Chirobiotic T column was not overloaded and the two enantiomers could be collected directly in high enantiomeric excess and the separation process could, therefore, be automated (see Fig. 1). Depending on the separation of the enantiomers one can automatically inject around 75–150 μg every 10 min on the column (250×10 mm i.d.) resulting in separation of around 7 mg overnight, which is enough material to do reactions on.

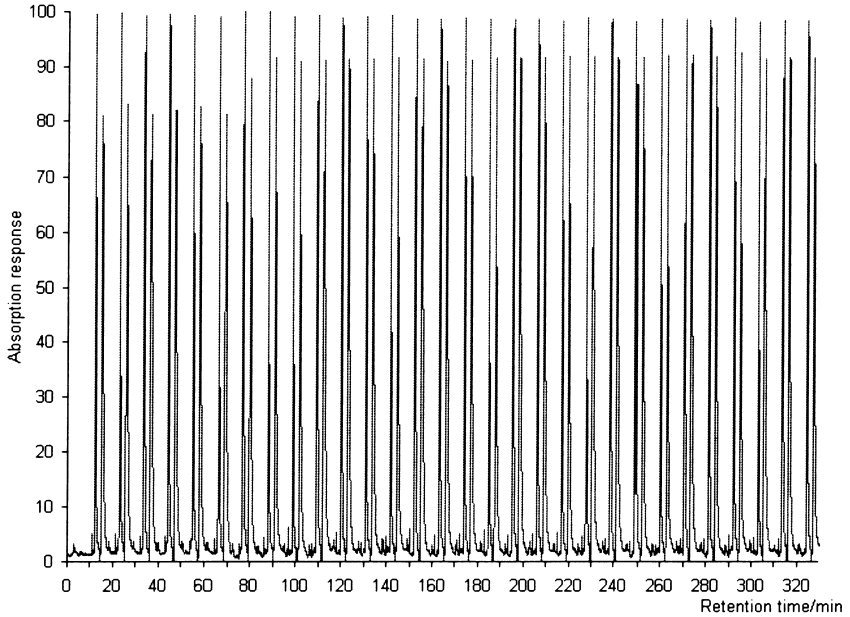


Figure 1. Absorption of **4** (215 nm) resolved by repeated injections on a Chirobiotic T chiral HPLC column, eluted with 15 mM NH_4Ac (pH 4):MeOH, 90:10

Data on the separated enantiomers are reported in Table 1. 2-Azidohexanoic acid **2** separated nicely on the chiral HPLC column and gave the two enantiomers with the same specific rotations but opposite signs. There are no reference values; however, experimental evidence has shown that teicoplanin binds D-amino acids stronger than L-amino acids because the L-form is eluted first with no exceptions.[15] This relation may, therefore, be used as an indication of absolute configuration in the case of enantiomeric pairs of azido acids. On this basis, the first eluting enantiomer of **2** can be assigned to (*S*)-2-azidohexanoic acid and the last enantiomer to the (*R*)-configuration. For 2-azidophenylacetic acid the specific rotations of the enantiomers corresponded well to the previously determined values[8] and the e.e.s were above 99%.

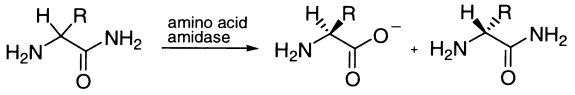
The following parameters were measured for each of the racemic α-azido acids: the retention (k'), the enantioselectivity (α) and the enantioresolution (R_s); t_1 and t_2 are retention times of the first and last eluting enantiomers; t_0 is the dead volume; k'_1 and k'_2 are the retentions (k') of the first and second eluting enantiomers, respectively; k' is typically low on the Chirobiotic T because it does not retain the compounds very much; $w_{1/2}(1)$ and $w_{1/2}(2)$ are the peak widths at half height of peaks 1 and 2, respectively; and the resolution (R_s) is a measure of the column's efficiency at separating the enantiomers. Since the enantioselectivity of **3** was low ($\alpha = 1.22$), separation of the enantiomers was not attempted.

Table 1
Chiral HPLC resolution data on **2**, **3** and **4**. $k' = (t_1 - t_0)/t_0$, $\alpha = k'_2/k'_1$, $R_S = 1.18\ (t_2 - t_1)/(w_{\frac{1}{2}(1)} + w_{\frac{1}{2}(2)})$, n.d. = not determined. [a]Measured in $CHCl_3$; [b]according to chiral HPLC

Compound	k'	α	R_s	[c] = g / 100mL	$[\alpha]_D^{25}$ [a]	Reference	% e.e.[b]
N_3-Nle-OH		2.38	4.24				
(S)-**2**	0.14			0.5	-34		94
(R)-**2**	0.33			0.5	+36		99
N_3-Ahd-OH		1.22	4.37				
(S)-**3**	0.44				n.d.		n.d.
(R)-**3**	0.54				n.d.		n.d.
N_3-Phg-OH		2.97	3.38				
(S)-**4**	0.14			0.5	+166	+175	>99
(R)-**4**	0.42			0.25	-164	-169	>99

In conclusion, the specific rotations of 2-azidophenylacetic acid **4** corresponded to the literature values and for the other azido acid **2** no literature values have been reported but the numerical specific rotations of (+)-**2** and (−)-**2** are similar and the e.e.s are high. Resolution of **1** on Chirobiotic T was unsuccessful (data not shown) because the CSP could not discriminate an α-methyl against an α-ethyl group.

Since the development of DSM's amidase process for the production of enantiomerically pure amino acids more than a decade ago[20] (see Scheme 2), over 100 α-*H*-α-amino acid amides have been resolved successfully using *Pseudomonas putida* ATCC 12633 whole cells. Although the aminopeptidase from *P. putida* can accommodate amino acid amides with an extreme diversity in side chains, much less flexibility is allowed with respect to the other groups at the C^{α} atom: the *P. putida* aminopeptidase appeared to be active for α-*H*-α-amino acid amides only. Because of this, it was assumed that the activity of this aminopeptidase for α-azido acid amides, if any, would be very low. Therefore, it was decided to use a genetically modified *E. coli* strain containing the *P. putida* L-aminopeptidase gene (*pep*A) for the resolution of the α-azido acid amides. Due to the molecular biology techniques applied, the cellular aminopeptidase activity of these recombinant *E. coli* cells is at least 25-fold higher than that of the wild type *P. putida* cells.[21]



Scheme 2. Enzymatic resolution of an α-*H*-α-amino acid amide by preferential hydrolysis of one enantiomer

Both 2-azidophenylacetic acid amide **8** and 2-azidohexanoic acid amide **6** were incubated with whole cells of *E. coli* DH5α/pTrpLAP in a cell to substrate ratio of 1 to 10. Progress of the reactions was monitored by chiral HPLC analysis of samples taken at regular time intervals. Progress curves of these two enzymatic resolution reactions are given in Fig. 2.

As the graphs in Fig. 2 show, *E. coli* DH5α/pTrpLAP did have activity for both azido acid amides tested. Because neither azido acid amide was a substrate for the negative control (*E. coli* DH5α/pUC19), the azido acid amide hydrolysing activity of the pTrpLAP containing recombinant *E. coli* strain can be solely attributed to the presence of the *P. putida* gene. Therefore, these

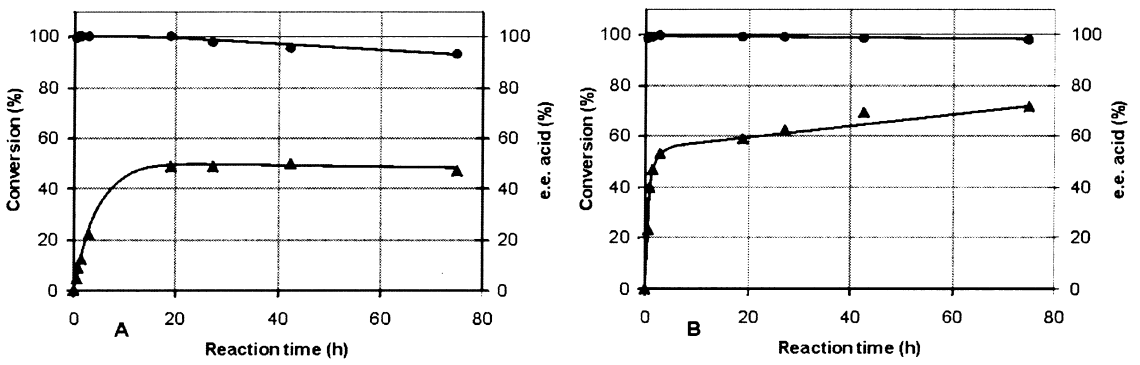


Figure 2. Development of conversion (▲) and enantiomeric excess (●) of the acid formed in the resolution of 2-azidohexanoic acid amide **6** (A) and 2-azidophenylacetic acid amide **8** (B) by *E. coli* DH5α/pTrpLAP

azido acid amides are the first non-α-amino groups containing amides that can be hydrolysed efficiently by the L-aminopeptidase from *P. putida*.

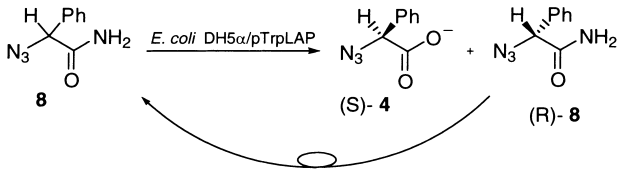
The hydrolysis reaction of the racemic 2-azidohexanoic acid amide stopped completely upon reaching 50% conversion after about 20 h (Fig. 2A). At that point, the enantiomeric excess of the (*S*)-2-azidohexanoic acid was still >99.8%, so the *P. putida* L-aminopeptidase is completely L-enantioselective for this substrate. Longer incubation times led to a slight progressive decrease of the e.e. of the (*S*)-azido acid to 93% after 75 h. The exact reason for this decrease in e.e. is not known at the moment, but the action of a racemase with activity for the 2-azidohexanoic acid could be one of the possible explanations.

The course of the hydrolysis reaction of racemic 2-azidophenylacetic acid amide by *E. coli* DH5α/pTrpLAP (Fig. 2B) dramatically differs from that of the aforementioned aliphatic azido acid amide. For this substrate, 50% conversion is already reached after about 3 h, because the specific activity of the *P. putida* L-aminopeptidase containing *E. coli* cells is higher for 2-azidophenylacetic acid amide than for 2-azidohexanoic acid amide (resp. 1.6 and 0.4 U[†]/mg cellular dry weight). More interestingly, however, this reaction does not come to a complete end upon reaching 50% conversion, but proceeds with an about 100-fold reduced rate (0.00012 versus 0.012 moles/kg·h[‡]). Because the e.e. of the (*S*)-2-azidophenylacetic acid remains at over 98% throughout the whole reaction time, this effect can only be explained by racemisation of the remaining (*R*)-2-azidophenylacetic acid amide followed by hydrolysis of the (*S*)-enantiomer by the heterologous L-aminopeptidase (see Scheme 3). In this process, the racemisation is the rate-limiting step. The reaction of (*RS*)-2-azidophenylacetic acid amide with *E. coli* DH5α/pTrpLAP is an example of a dynamic kinetic resolution, and, therefore, has a theoretical maximum yield of the (*S*)-azido acid of 100%.

It was not expected that the 2-azidophenylacetic acid amide would racemise in situ, because neither the corresponding azido acid nor the α-amino and α-hydroxy analogues, phenylglycine amide and mandelic acid amide, respectively, racemise spontaneously under the conditions used. Probably, the racemisation of 2-azidophenylacetic acid amide can be attributed to the presence of three electron-withdrawing substituents rendering the α-hydrogen atom more acidic.

[†] A cell preparation has a specific activity of 1 unit (U) per mg of dry weight, if this amount of cells hydrolyses 1 μmol of substrate per min at standard conditions (pH 9.0 and 40°C).

[‡] Reaction rate is given in mols per kg and hour of reaction mixture.



Scheme 3. Dynamic kinetic resolution of 2-azidophenylacetamide **8** by *E. coli* DH5α/pTrpLAP

In the near future efforts will be undertaken to resolve the α-methyl substituted azido acid amide, (±)-2-azido-2-methylbutyric acid amide **5**. Because the *P. putida* aminopeptidase does not have any activity for α-alkyl disubstituted amides, the resolution of **5** will be tried with an amidase from *Ochrobactrum anthropi* NCIMB 40321,[22] which has been purified and characterised. Furthermore, the gene coding for this amidase has recently been cloned.[§]

3. Conclusion

Two different approaches to enantiopure α-azido acids have been demonstrated successfully, which opens up the possibility to synthesise enantiopure peptides or other kinds of pharmacophores which are crucial in the pharmaceutical industry.

Teicoplanin showed very good resolution towards the tested monosubstituted α-azido acids **2**–**4**, and the process could easily be automated on the mg scale.

Both 2-azidohexanoic acid **2** and 2-azidophenylacetic acid **4** could be obtained in very high enantiomeric excess from the corresponding amides by an enzymatic resolution concept using an L-aminopeptidase from *P. putida*. To get acceptable reaction times, use of a genetically modified *E. coli* strain containing the gene for this aminopeptidase was essential. This enzymatic process will enable the preparation of enantiomerically pure α-azido acids on a multigram scale and with excellent enantiomerical purity.

4. Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX250 (250 MHz). IR spectra were recorded on a Perkin–Elmer 1600 FTIR instrument as neat liquids or as KBr-pellets. Optical rotations were measured on a Perkin–Elmer 241 Polarimeter at 25°C. Melting points were determined with a Büchi B-540 apparatus and were uncorrected. Separation of enantiomers was performed with a Chirobiotic T 250×10 mm (i.d.) chiral HPLC column from Astec by elution with a solvent system of ammonium acetate (15 mM; adjusted to pH 4 with acetic acid) containing 10% MeOH or *n*-heptane with 20% abs. EtOH. Detection was at 215 nm with a Waters 490E multiwavelength detector and a flowrate of 1 cm^3 min^{-1}, and this was connected to an autosampler and an automatic fraction collector. Semipreparative reverse-phase HPLC separation was performed on a Shodex RS Pak DS-2013 (20×300 mm) column with a flow rate of 6 cm^3 min^{-1}. Detection was at 215 nm on a photodiode array detector (Waters M991). A solvent system consisting of A: 0.1% TFA in water, and B: 0.1% TFA in 90% acetonitrile–10% water was used.

[§] Unpublished results.

THF was distilled from sodium, CH_2Cl_2 was distilled from CaH_2 and all solvents were stored over molecular sieves (3 Å). Water was Milli-Q (Millipore). Deuterated solvents used for NMR were from Cambridge Isotope Laboratories and all other solvents were from Labscan.

The following commercially available chemicals were used: *N*,*N*-diisopropylethylamine (Aldrich), isobutylchloroformate (Fluka), *N*-ethylmorpholine (Merck) and thionyl chloride (Merck). The synthesis of the α-azido acids **1**–**4** will be described elsewhere.[19]

4.1. (±)-2-Azido-2-methylbutyramide **5**

(±)-2-Azido-2-methylbutyric acid (1.70 g, 11.9 mmol) was converted to the acid chloride with $SOCl_2$ (4.3 mL, 59.8 mmol, 5 equiv.) in CH_2Cl_2 (4.3 mL) and refluxed under argon for 2.5 h. The solution was concentrated and diluted with CH_2Cl_2 (10 mL). DIPEA (12.45 mL, 71.5 mmol, 6 equiv.) was added to a stirred suspension of NH_4Cl (2.88 g, 53.9 mmol, 4.5 equiv.) in CH_2Cl_2 (25 mL) and cooled to −30°C. The crude azido acid chloride was added dropwise to the suspension under argon, and the temperature raised to 20°C over 2 h. After 72 h, H_2O (30 mL) and aq. HCl (1 M, 30 mL) were added and the phases separated. The aqueous phase was extracted once with CH_2Cl_2 (30 mL) and the combined organic phases were concentrated and purified by VLC (AcOH:EtOAc:heptane, 5:15:80) affording **5** (1.53 g, 91%). This was crystallised from $CHCl_3$/heptane to obtain a very pure sample of **5**. M.p. 57.4–58.3°C; 250 MHz ^{1}H NMR in $CDCl_3$, δ ppm: 0.93 (t, 3H, J = 7 Hz, H-4), 1.51 (s, 3H, H-3′), 1.71 (dq, 1H, J = 7 Hz, J′ = 14 Hz, H-3a), 1.96 (dq, 1H, J = 7 Hz, J′ = 14 Hz, H-3b), 6.45 (br s, 2H, N*H*$_2$); 62.5 MHz ^{13}C NMR in $CDCl_3$, δ ppm: 9.0 (C-4), 23.2 (C-3′), 31.7 (C-3), 68.4 (C-2), 175.2 (C-1). IR: 1666, 2107 cm^{-1}. Found: C, 43.10%; H, 6.92%; N 39.10%. Calculated for $C_5H_{10}N_4O$·1/50 heptane: C, 42.84%; H, 7.22%; N, 38.86%.

4.2. (±)-2-Azidohexanoic acid amide **6**

(±)-2-Azidohexanoic acid (1.17 g, 7.44 mmol) was dissolved in dry THF (15 mL) and cooled to 0°C under argon. DIPEA (1.30 mL, 7.46 mmol, 1 equiv.) and isobutylchloroformate (0.975 mL, 7.45 mmol, 1 equiv.) were added to the cooled solution and stirred for 5 min and then the argon atmosphere was changed to ammonia and the reaction warmed to 20°C over 2 h. H_2O (15 mL) was added after 21 h and the aqueous phase was extracted with CH_2Cl_2 (3×30 mL) and the combined organic phases washed with aq. HCl (1 M, 3×15 mL), dried, concentrated and the residue (1.19 g) purified by VLC (AcOH:EtOAc:heptane, 5:15:80) affording **6** (0.48 g, 41%). M.p. 50.7–52.1°C; 250 MHz ^{1}H NMR in $CDCl_3$, δ ppm: 0.91 (t, 3H, J = 7 Hz, H-6), 1.38 (m, 4H, H-4, H-5), 1.85 (m, 2H, H-3), 3.95 (dd, 1H, J = 5 Hz, J′ = 7 Hz, H-2), 6.32 (s, 2H, N*H*$_2$); 62.5 MHz ^{13}C NMR in $CDCl_3$, δ ppm: 15.5 (C-6), 24.0 (C-5), 29.1 (C-4), 33.5 (C-3), 65.8 (C-2), 174.3 (C-1). IR: 1658, 2114 cm^{-1}. Found: C, 47.30%; H, 7.77%; N, 33.59%. Calculated for $C_6H_{12}N_4O$·1/8 EtOAc: C, 46.69%; H, 7.84%; N, 33.51%.

4.3. (±)-2-Azidohexadecanoic acid amide **7**

(±)-2-Azidohexadecanoic acid (0.838 g, 2.82 mmol) was dissolved in dry THF (25 mL) and cooled to 0°C under argon. DIPEA (0.49 mL, 2.81 mmol, 1 equiv.) and isobutylchloroformate (0.37 mL, 2.83 mmol, 1 equiv.) were added to the cooled solution and stirred for 10 min and then the argon atmosphere was changed to ammonia and the reaction warmed to 20°C over 2 h. H_2O

(25 mL) was added after 3 days, the aqueous phase was extracted with CH_2Cl_2 (3×10 mL) and the combined organic phases washed with aq. HCl (1 M, 3×15 mL), dried and concentrated. The product was crystallised from DMF/H_2O affording **7** (0.58 g, 70%). M.p. 80.3–81.5°C; 250 MHz ^{1}H NMR in d_6-DMSO, δ ppm: 0.81 (t, 3H, J = 7 Hz, H-16), 1.20 (m, 24H, H-4-H-15), 1.62 (m, 2H, H-3), 3.62 (dd, 1H, J = 6 Hz, J′ = 8 Hz, H-2), 7.22 (br s, 1H, NH_2^a), 7.53 (br s, 1H, NH_2^b); 62.5 MHz ^{13}C NMR in d_6-DMSO, δ ppm: 16.0 (C-16), 24.2 (C-15), 27.4 (C-14), 30.9–33.4 (C-3–C-13), 63.4 (C-2), 173.6 (C-1). IR: 1659, 2104 cm^{-1}. A small amount was further purified on a Shodex column for elemental analysis. Found: C, 64.89%; H, 11.41%; N, 18.70%. Calculated for $C_{16}H_{32}N_4O$: C, 64.82%; H, 10.88%; N, 18.90%.

4.4. (±)-2-Azido-2-phenylacetamide **8**

(±)-2-Azido-2-phenylacetic acid (1.48 g, 8.35 mmol) was dissolved in dry THF (15 mL) and cooled to −30°C under argon. NEM (1.06 mL, 8.38 mmol, 1 equiv.) and isobutylchloroformate (1.10 mL, 8.41 mmol, 1 equiv.) were added to the cooled solution and the resulting suspension was filtered after 2 min and the precipitate was washed once with dry THF (6 mL). The filtrate was again cooled to −30°C and the atmosphere was saturated with ammonia (it was also bubbled through the solution for 30 s). H_2O (15 mL) was added after 0.5 h and the aqueous phase was extracted with CH_2Cl_2 (3×15 mL) and the combined organic phases were evaporated. The product was crystallised from cold CH_2Cl_2 (−78°C) affording **8** (0.83 g, 57%). M.p. 113.1−113.7°C; 250 MHz ^{1}H NMR in $CDCl_3$, δ ppm: 5.02 (s, 1H, H-2), 6.43 (br d, 2H, NH_2), 7.40 (5H, H-4, H-5, H-6); 62.5 MHz ^{13}C NMR in $CDCl_3$, δ ppm: 69.0 (C-2), 129.7, 131.1, 131.2 (C-4, C-5, C-6), 136.6 (C-3), 172.9 (C-1). IR: 1665, 2104, 3160 cm^{-1}. Found: C, 54.88%; H, 4.48%; N, 31.72%. Calculated for $C_8H_8N_4O$: C, 54.54%; H, 4.58%; N, 31.80%.

4.5. Resolution of (±)-2-azidohexanoic acid **2** by chiral HPLC

A total of 18 mg of (±)-2-azidohexanoic acid dissolved in the eluent (3.75 mL, 15 mM NH_4Ac (pH 4):MeOH, 90:10) was resolved on a Chirobiotic T column (250×10 mm i.d.) using injections of 150 μg every 10 min. The combined fractions where acidified to pH 2 with aq. HCl (1 M) and extracted with CH_2Cl_2 (3×50 mL), dried and evaporated, giving 4.1 mg of the first eluting enantiomer and 3.8 of the second. (*S*)-**2**: $[\alpha]_D^{25} = -34$ (*c* 0.5, $CHCl_3$); (*R*)-**2**: $[\alpha]_D^{25} = +36$ (*c* 0.5, $CHCl_3$).

4.6. Analytical resolution of (±)-2-azidohexadecanoic acid **3** by chiral HPLC

Compound **3** (60 μg) was dissolved in DMF and eluted with abs. EtOH:*n*-heptane, 20:80, giving the analytical data in Table 1. It was not resolved in quantitative amounts because the enantioselectivity was too low ($\alpha = 1.22$).

4.7. Resolution of (±)-2-azidophenylacetic acid **4** by chiral HPLC

A total of 10 mg of (±)-2-azidophenylacetic acid dissolved in the eluent (3.5 mL, 15 mM NH_4Ac (pH 4):MeOH, 90:10) was resolved using injections of 75 μg every 10 min. The combined fractions were acidified to pH 2 with aq. HCl (1 M) and extracted with EtOAc (3×50 mL), dried and evaporated giving 2.6 mg of the first eluting enantiomer and 3.0 of the second. (*S*)-**4**: $[\alpha]_D^{25} = +166$ (*c* 0.5, $CHCl_3$); (*R*)-**4**: $[\alpha]_D^{25} = -164$ (*c* 0.25, $CHCl_3$).

4.8. Enzymatic resolution

4.8.1. Bacterial cells

Because it was assumed that the activity of the L-aminopeptidase from *P. putida* for α-azido acid amide hydrolysis would be very low, it was decided to perform the resolution experiments with a hyperactive recombinant *E. coli* strain, that carried the *P. putida* L-aminopeptidase gene on expression plasmid pTrpLAP. The construction of this recombinant strain, as well as its cultivation, have been described in detail elsewhere.[21]

4.8.2. Enzymatic resolution reaction

Per reaction, 50 mg of α-azido acid amides **6** and **8** were weighed into a reaction vial, and 2.5 mL of 400 mM TAPS–KOH buffer, pH 9.0, 1.25 mL of 8 mM $MnSO_4$ and 5 mL of water were added. After pre-incubation at 40°C, reactions were started by the addition of 1.25 mL of a 4 mg (wet weight)/mL suspension of *E. coli* DH5α/pTrpLAP cells. For a negative control, the same amount of *E. coli* DH5α/pUC19 cells was used. After certain time intervals, samples of 1 mL were transferred into 0.5 mL of 1 M H_3PO_4 to stop the reaction. After passage through a 0.45 μm filter, these samples were analysed at 25°C, on a Chirobiotic T (250×4.6 mm I.D.) chiral HPLC column from Astec by elution with a solvent system of 15 mM ammonium acetate (adjusted to pH 4.0 with acetic acid) containing 10% methanol. Detection was at 215 nm and the flowrate was 0.5 $cm^3\ min^{-1}$.

4.9. Abbreviations

Ahd, (±)-2-aminohexadecanoic acid; CSP, chiral stationary phase; DIPEA, *N*,*N*-diisopropylethylamine; NEM, *N*-ethylmorpholine; Nle, norleucine; *pep*A, *Pseudomonas putida* L-aminopeptidase gene; Phg, phenylglycine; SPPS, solid-phase peptide synthesis.

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