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A Protection Strategy Substantially Enhances Rate and Enantioselectivity in ω-Transaminase-Catalyzed Kinetic Resolutions

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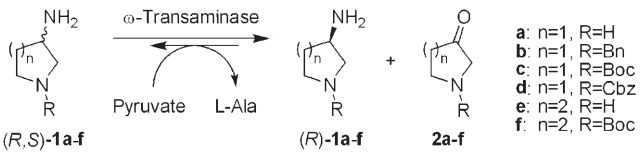
Abstract: The kinetic resolution of 3-aminopyrrolidine (3AP) and 3-aminopiperidine (3APi) with ω-transaminases was facilitated by the application of a protecting group concept. 1-*N*-Cbz-protected 3-aminopyrrolidine could be resolved with >99% *ee* at 50% conversion, the resolution of 1-*N*-Boc-3-aminopiperidine yielded 96% *ee* at 55% conversion. The reaction rate was up to 50-fold higher by using protected substrates. Most importantly, enantioselectivity increased remarkably after carbamate protection compared to the unprotected substrates (86 *vs.* 99% *ee*). Surprisingly, benzyl protection of 3AP had no influence on enantioselectivity. A possible explanation for this observation could be the different flexibility of the benzyl- or carbamate-protected 3AP as confirmed by NMR spectroscopy.

Keywords: amines; enantioselectivity; enzyme catalysis; protecting groups; transaminase

Optically pure 3-aminopyrrolidine (3AP) and 3-aminopiperidine (3APi) (Scheme 1) have become attractive synthons for the synthesis of a broad range of biologically active pharmaceuticals such as Tosufloxacin, Clinafloxacin[1] and cephalosporin derivatives.[2] Other compounds containing a 3AP- or 3APi-residue are interesting for the treatment of obesity[3], diabetes mellitus types I and II[4] or as psychotropic drugs against depression and schizophrenia.[5] Various syntheses using optically active 3AP and 3APi or their protected analogues have been reported in literature. There are three different synthetic routes: substitution, cyclization and classical resolution of the racemate with resolving agents. Most of them have one ore more drawbacks: substitution reactions *via* azide displacement[1,6] require 1-*N*-protected mesylated or tosylated alcohol, which is either expensive or must be prepared *via* multistep syntheses. Furthermore, the substitution proceeds *via* an inversion of the configuration, so that an unwanted non-selective S_N1-reaction has to be avoided. In these methods, enantiomeric purity of the products has not been characterized *via* chromatographic techniques. The 1-*N*-,3-*N*-double protected 3AP/3APi can be obtained by cyclization starting from optically pure aspartate or glutamate.[7] Other routes from amino acids involve a reduction of the cyclized dione-compound.[8] All cyclizations, however, are elaborate multistep syntheses with either low to moderate yield or moderate enantiomeric purities. From (*S*)-2,4-diaminobutyric acid, (*S*)-3AP can be obtained in only two steps, but the yield is low.[9] The crystallization processes[10] suffer from the fact that the maximum yield is below 50% and that the recycling and recovery of the resolving agent and the antipode are laborious.



Scheme 1. Principle of kinetic resolution of unprotected and 1-*N*-protected 3-aminopyrrolidines and -piperidines with ω-transaminase.

ω-Transaminases are known to convert amines with usually high to excellent enantioselectivity in either a kinetic resolution of a racemic amine or in an asymmetric synthesis starting from the prochiral ketone.[11] However, asymmetric synthesis is hampered by an unfavorable equilibrium. Only one report for the kinetic resolution of racemic 3AP with an ω-transaminase from *Vibrio fluvialis* was published, but the reaction was very slow and the enantioselectivity was not examined.[12] Indeed, initial experiments carried out by us using two ω-transaminases from *Vibrio fluvialis* (Vfl-TA)[13] and *Alcaligenes denitrificans* (Ade-TA)[14]

Table 1. Results of kinetic resolutions of unprotected and protected substrates.

Entry	Ade-TA		Vfl-TA	
	Conversion [%]	*ee* [%][a]	Conversion [%]	*ee* [%][a]
1a	52	89	55	86
1b	50	81	49	77
1c	50	98	50	90
1d	51	99	50	>99
1e	54	72	54	76
1f	55	97[b]	55	96[b]

[a] The (*R*)-enantiomer is the main component.
[b] Assumed to have (*R*)-configuration.

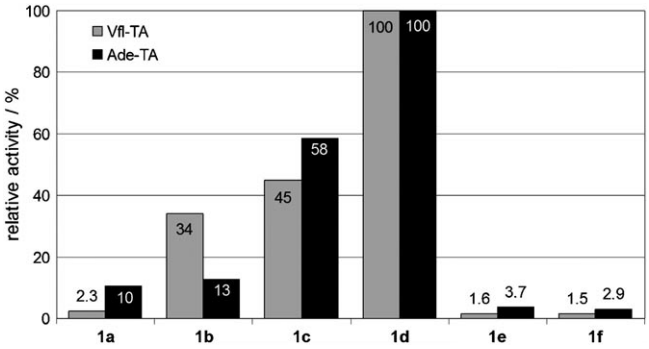


Figure 1. Influence of the protecting group on the reaction rate in the kinetic resolutions of **1a–f** using ω-transaminases Ade-TA or Vfl-TA.

confirmed that the selectivity of both enzymes in the kinetic resolution of **1a** was not satisfactory. An enantiomeric excess of 97% *ee* could only be achieved after >60% conversion (data not shown) which reduces the yield of the desired isomer to <40%.

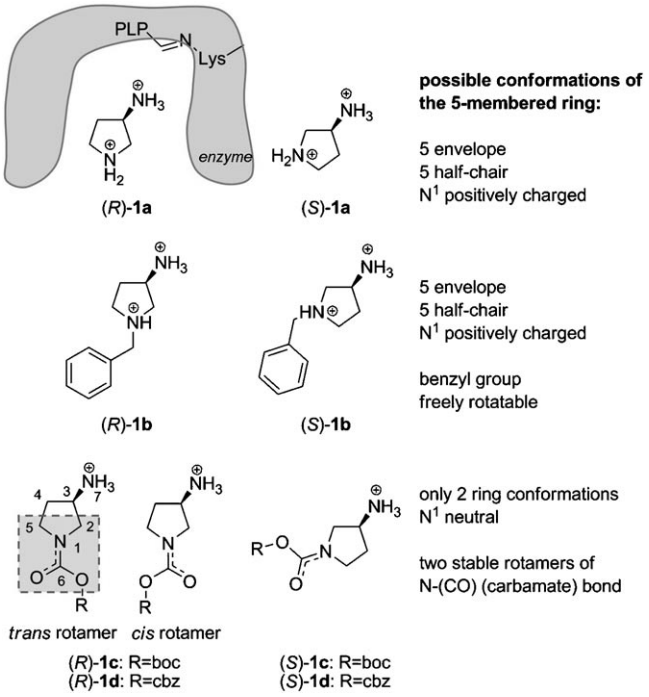
In order to enhance the enantioselectivity and hence increase the yield of the desired enantiomer, we considered the use of protection groups to facilitate a better distinction between the enantiomers. This strategy has scarcely been investigated, but could be successfully applied to biotransformations with a lipase[15] and monooxygenases.[16] In the desymmetrization of 2,4,6-trifunctionalized tetrahydropyrans with *Pseudomonas cepacia* lipase, the unprotected 2,6--acetoxymethyl-4-ketotetrahydropyran is hydrolyzed with low selectivity to the monoacetate. In contrast, excellent enantiomeric purities were obtained upon acetal protection of the keto function, which could easily be removed from the product in a subsequent step. Later, this approach was particularly applied in the field of biohydroxylation with monooxygenases and named docking/protecting (d/p) group concept.[16] The introduction of the d/p group enabled the enantioselective biohydroxylation of non-activated carbon atoms in cyclic or aliphatic compounds like ketones, aldehydes, acids and alcohols. More recently, this concept was also applied to the preparation of hydroxylated N-heterocycles[17] and to the bioconversion of *O*-protected β-hydroxynitriles to yield β-hydroxyalkanoic acids and their amide derivatives, respectively.[18] Protection generally resulted in a dramatic increase in rate, regio- and stereoselectivity in most of the reactions. Thus, we subjected several *N*-protected derivatives of 3AP and 3APi (Scheme 1) to ω-transaminase-catalyzed kinetic resolutions.

For the analysis of conversion and especially optical purity, an analytical method using capillary electrophoresis (CE) was developed. The protected amines are UV-detectable at 200 nm and CE with highly sulfated α-, β- or γ-cyclodextrin as chiral selectors turned out to be a powerful technique for the separation of the amines. The unprotected amines had to be derivatized with *o*-phthaldialdehyde (OPA) prior to CE analysis, but luckily, derivatization proceeded fast under mild conditions in aqueous media.[19]

Next, the relative reaction rates were determined using 5 mM (*R*,*S*)-**1a**–**1f** and pyruvate as amino acceptor employing the two ω-transaminase as model biocatalyst (Figure 1). We were pleased to find that the introduction of a protecting group (benzyl, Boc or Cbz) had a substantial impact on the reaction rates, which were almost 50-fold increased (**1d** *vs.* **1a** using Vfl-TA). It also turned out that the five-membered ring pyrrolidines are preferred over the six-membered piperidines by both enzymes.

Table 1 shows the effect of the protection on enantiomeric purity of the amines at ~50% conversion. The unprotected 3AP **1a** and 3APi **1e** were converted with only moderate to good selectivity. Interestingly, benzyl-, Boc- and Cbz-protection of 3AP resulted in remarkably different enantiomeric purities at 50% conversion independent of the ω-transaminase used. Benzyl (**1b**) protection of 3AP led to only moderate enantiomeric excess of 77 or 81% *ee* for Vfl-TA or Ade-TA, respectively. On introducing the carbamate group (**1c**), a significantly enhanced enantioselectivity was found and the best optical purity (>99% *ee*) was achieved with the Cbz-group (**1d**). In case of the Boc-protected 3APi **1f** no rate enhancement was observed, but the protection led to a remarkable increase in enantiomeric excess from 72 to 97% *ee* at similar conversion.

A possible explanation of the presence and the nature of the protecting group on the enantioselectivity can be retrieved from the substrate structure. In Scheme 2, both enantiomers of (un)protected 3AP are shown. To allow conversion of the amines, their amino group has to be positioned towards the C4′ of the bound PLP-cofactor. The moderate selectivity for 3AP and 3APi could be explained by the fact that the only possibility for the enzyme to discriminate the enantiomers is the recognition of the endocyclic nitrogen, which is protonated in neutral solution. In con-



Scheme 2. Comparison of the structural features of protected and unprotected 3AP with respect to stereodiscrimination by the enzyme.

trast, the presence of a bulky protecting group at this position allows a better binding of the substrate by the enzyme, as indicated by the increasing relative activities. Furthermore, positioning the amino group of both enantiomers towards the cofactor would lead to a different orientation of the protection group, thus increasing the chance for a selective binding of one enantiomer. At this point the question remains why the Boc- and Cbz-protection led to a drastically enhanced enantioselectivity, but on the other hand, no increase of selectivity was observed with the benzyl group at all. This can be explained by the different flexibility of the benzyl- and carbamate-protected substrates. Both the unprotected and benzyl-protected 3AP have no restrictions in terms of conformational freedom of the five-membered ring, so five envelope and five half-chair conformations with slightly different energies are possible. The bond of the benzyl group to the endocyclic nitrogen is freely rotable, so that the phenyl ring of both enantiomers could be positioned in a similar orientation, thus preventing an exact stereodiscrimination (Scheme 2).

The amino groups of both enantiomers are orientated in the same direction to show the possibilities for an enzymatic stereodifferentiation. Two rotamers of **1c** and **1d** are designated as *cis* and *trans* with respect to the orientation of the carbonyl oxygen to the amino group. The atoms in the gray rectangle form a planar geometry [PLP = cofactor pyridoxal 5′-phosphate].

On the other hand, the introduction of a carbamate residue creates a strong limitation of the possible rotations: due to the partial C6-N1 double bond character, the carbamate group as well as C2 and C5 of the ring form a plane geometry (marked as gray rectangle in Scheme 2). Thus, only two stable ring conformations are possible, whereas C3 and C4 are below or above the plane, respectively, and *vice versa*. Indeed, this assumption could be confirmed by NMR spectroscopy: two discrete signals for each of the ring carbons were observed in ^{13}C NMR spectra in $CDCl_3$ as well in D_2O (at pH 8, 25 °C). This indicates that two relatively stable positions for the carbonyl group occur, in which the oxygen atom can be *syn*- or *anti*-oriented with respect to the amino group. As expected for rotamers, elevating the temperature accelerates their interconversion so that the carbon signal pairs are merged into one broad signal for each carbon atom. An additional feature for stereodiscrimination could be the capability of the enzyme to form an H-bond with the carbonyl group of the carbamate function. As a result of this rigid geometry, the discrimination of the enantiomers of **1c** and **1d** is superior compared to the benzyl-protected or unprotected 3AP.

For preparative scale kinetic resolution of **1c**, the influence of pH of the transamination with Ade-TA was investigated and pH 8 was found as optimum value (Figure 2, top). In a preparative scale kinetic

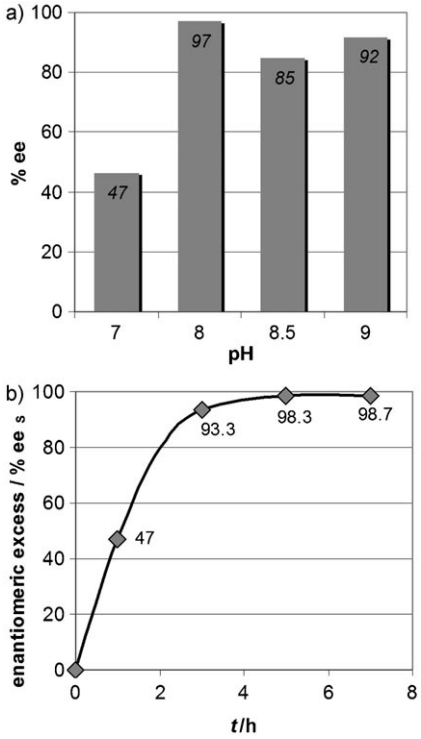


Figure 2. *Top:* Influence of pH on the kinetic resolution of **1c**. Enantiomeric excess was determined after 4 h. *Bottom:* Progress of the enantiomeric excess in the preparative-scale kinetic resolution of **1c**.

resolution (Figure 2, bottom), 0.85 mmol **1c** was resolved and at a conversion of ≈50%, **1c** could be isolated having an enantiomeric purity of 98.7% *ee*. The isolated yield for the amine **1c** and the ketone **2c** were 39% and 44%, respectively. A preparative kinetic resolution of **1f** with Ade-TA yielded 42% of **1f** with 97% *ee*. Deprotection can easily be performed using standard methods.[20]

In summary, we have shown that, by means of protection, **1c** and **1f** can be efficiently resolved using ω-transaminases on a preparative scale. This is the first time that the d/p concept was applied to transaminase-catalyzed synthesis. The reaction rate could be dramatically enhanced by using protected 3AP. Although this was not observed for 3APi, the enantioselectivity was still remarkably enhanced for both 3AP and 3APi after introducing the carbamate protecting group. Although kinetic resolutions are limited to 50% yield, this enzymatic approach is an easy way for the synthesis of enantiopure 3-aminopyrrolidines.

Experimental Section

Chemicals and Enzymes

All chemicals were from Sigma–Aldrich, Germany, except for (*R,S*)-1-*N*-Boc-3-aminopiperidine hydrochloride which was from Fluorochem (Germany) and 1-*N*-benzyl-3-aminopyrrolidine which was from ABCR, Germany. The ω-transaminases from *V. fluvialis* and *A. denitrificans* were obtained from Julich Chemical Solutions (now Codexis Inc.).

Analysis of Optical Purity

The enantiomeric excesses of **1c** and **1f** were analysed by gas chromatography. After extraction of the amine with dichloromethane, derivatization to the trifluoroacetamide was performed by adding a 20-fold excess of trifluoroacetic anhydride. After purging with nitrogen to remove excess anhydride and residual trifluoroacetic acid, the derivatized compound was dissolved in dichloromethane (50 μL) and analyzed using a Shimadzu GC14A equipped with a heptakis-(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-cyclodextrin column (25 m × 0.25 mm). The retention times for **1c** were 13.5 min for the (*S*)-enantiomer and 19 min for the (*R*)-enantiomer at an oven temperature of 180 °C (absolute configurations were assigned using commercially available optically pure standards). Compound **1f** was separated at 160 °C, the retention times were 6.4 and 7.0 min.

Alternatively, the optical purity could also be determined by capillary electrophoresis (see below). All CE measurements were done with a PACE-MDQ system equipped with a fused silica capillary (50 μm inner diameter). For the determination of the enantiomeric excess of **1b–d** and **1f**, the reaction was stopped by adding an aliquot of the reaction mixture (100 μL) to NaOH solution (100 μL, 0.1 M), followed by an extraction with dichloromethane (200 μL). Subsequently, 100 μL of the organic layer were extracted with triethylammonium phosphate buffer (200 μL, 10 mM, pH 3.0), containing the analyte. For the analysis of **1a** and **1e**, the compounds were derivatized following a published protocol[19] with some modification. An aliquot (100 μL) of the reaction mixture was given to sodium borate buffer (500 μL, 100 mM, pH 10) and OPA reagent (300 μL) was added (80 mg OPA, 20 μL 2-mercaptoethanol, 10 mL acetonitrile). The sample was incubated for 30 min at 37 °C and the derivatives were subsequently extracted by the addition of NaOH (70 μL 15 M) and chloroform (1 mL). The organic phase was then mixed with triethylammonium phosphate buffer (100 μL, 10 mM, pH 3), and washed two times with chloroform (500 μL). To 100 μL of the amine in the triethylammonium phosphate phase, 1,3,6,8-pyrenetetrasulfonate (2 μL, 10 mM) as migration standard was added. Enantiomer separation was achieved by the addition of 5% highly sulfated-α-, -β- or -γ-cyclodextrin (HSα/β/γCD), Beckman-Coulter) to the running buffer as chiral selector. An aperture with a 200 μm slit was used and the capillary temperature was set to 15 °C. The compounds were detected at 200 nm. All important parameters and migration times are shown in Table 2. The separation protocol was programmed as recommended by the manufacturer.

Table 2. Separation conditions for the analysis of enantiomeric purity of amines by capillary electrophoresis.

Compound	Cyclodextrin	Capillary Length [cm]		Migration Time [min][a,b]	
		to detector	total	(*R*)	(*S*)
1a	HSγCD	10	30	4.4	4.7
1b[c]	HSβCD	50	60	6.3	6.2
1c	HSγCD	10	30	7.7	6.4
1d	HSαCD	20	30	10.6	11.1
1e	HSγCD	10	30	5.9	5.1
1f[d]	HSγCD	10	30	8.4	8.9

[a] The migration order was determined using commercially available optically pure reference compounds.
[b] Voltage applied: 15 kV except for **1d** (10 kV).
[c] Before rinsing the capillary with the cyclodextrine containing background electrolyte, the capillary was rinsed for 15-20 min with 0.1 M NaOH.
[d] The migration order has not been determined. It is assumed that in the kinetic resolution with (*S*)-selective-transaminases the (*R*)-enantiomer is enriched, which is the faster migrating enantiomer.

Analysis of Conversion

Conversions were determined by the measurement of the residual amine by capillary electrophoresis. The reaction was stopped by adding an aliquot of the reaction mixture (40 μL) to HCl solution (160 μL, 15 mM) containing α-methylbenzylamine (1 mM) as internal standard for the analysis of **1c** and **1f**. For **1b**, the amine **1d** was used as internal standard and *vice versa*. The capillary was dynamically coated with CElixir (MicroSolvTech) according to the manufacturer's instructions to avoid adsorption of proteins to the capillary wall. The separation was done on a Beckman PACE-MDQ system equipped with a fused silica capillary (60 cm length, 10 cm to the detector, 50 μm inner diameter) and a

PDA detector. A pressure of 0.5 psi was applied for 5 s for the injection. The background electrolyte contained triethylammonium phosphate (50 mM, pH 3.0). A voltage of 30 kV was applied for four minutes for separation, the compounds **1c** and **1f** were detected at 190 nm and **1a**, **1b** and **1d** at 200 nm. For the measurement of the concentration of **1a** and **1e**, an indirect detection method was developed. The compounds had to be extracted from a sample (100 μL) by the addition of NaOH (50 μL 15 M) and chloroform (300 μL). The chloroform phase (200 μL) was subsequently extracted with phosphoric acid (5 mM). This solution was injected into the capillary (5 s, 1 psi). The background electrolyte contained *m*-xylenediamine phosphate (10 mM, pH 2.5). The capillary had a total length of 40 cm, and an effective length of 30 cm. A voltage of 15 kV was applied for 6 min for separation.

Biocatalysis

All kinetic resolutions were done at 37 °C, 600 rpm, in sodium phosphate buffer (50 mM, pH 8), containing pyridoxal 5′-phosphate (0.1 mM), racemic amine (5 mM) and pyruvic acid (10 mM) unless otherwise indicated.

Preparative-Scale Resolution of 1c

In a 500-mL round-bottom flask in a water bath, (*R,S*)-**1c** (230 mg, 1.24 mmol) was dissolved in phosphate buffer (122 mL, 50 mM, pH 8) and pyruvic acid (109 mg) was added and the pH adjusted to 8. After heating to 37 °C, the reaction was started by the addition of Ade-TA solution (3.7 mL) and stirred with a magnetic stirrer bar. Conversion and enantiomeric excess were analyzed as described above. After 7 h, the pH of the mixture was adjusted to pH 3 with HCl (5 M), and the ketone **2c** was extracted five times with dichloromethane (75 mL). After the extraction, **2c** was not detectable in the residual aqueous phase. The pH was now adjusted to pH 13, and the amine **1c** was extracted four times with dichloromethane. The solvents of the combined extracts of **1c** and **2c** were evaporated and **1c** (yield: 89 mg, 39%) and **2c** (yield: 104 mg, 44%) were obtained. The structures of the compounds were confirmed by ^{1}H and ^{13}C NMR-spectroscopy.

2c: ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.49$ (*s*, 9H, CH_3), 2.59 (*t*, 2H, $J = 7{,}8$ Hz, $COCH_2$), 3.75 (*s*, 2H, $COCH_2NCO_2$), 3.78 (*t*, 2H, $J = 7{,}8$ Hz, $CH_2CH_2NCO_2$); ^{13}C NMR (DMSO-d_6): $\delta = 28.4$ (CH_3), 37.1 (CH_2), 42.4 (CH_2), 52.6 (CH_2), 80.4 [$C(CH_3)_3$], 154.4 (N*C*OO), 211.0 (CH_2COCH_2).

1c: ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.46$ (*s*, 9H, CH_3), 1.6–1.7 (*m*, 1H, $CHNH_2CH_2CH_2$), 1.98–2.1 (*m*, 1H, $CHNH_2CH_2CH_2$), 2.97–3.1 (*m*, 1H, $CHNH_2CH_2NCO_2$), 3.3–3.6 (*m*, 4H); ^{13}C NMR (DMSO-d_6): $\delta = 28.5$ (CH_3), 34.3 + 34.8 ($CHNH_2CH_2CH_2$), 43.9 + 44.3 ($CH_2CH_2NCO_2$), 50.5 + 51.4 ($CHNH_2$), 54.1 + 54.4 ($CHNH_2CH_2NCO_2$), 79.1 [$C(CH_3)_3$], 154.6 (NCO_2).

Preparative-Scale Resolution of 1f

This was performed similar as described for **1a**: 1-*N*-Boc-3-aminopiperidine hydrochloride (0.25 mmol) and pyruvic acid (0.25 mmol) were dissolved in phosphate buffer (25 mL, 50 mM, pH 8), containing PLP (10 μM). After pH adjustment to pH 8 and preincubation at 37 °C, the reaction was started by the addition of Ade-TA solution (2 mL) and stirred with a magnetic stirrer bar. After 19 h, the enantiomeric excess reached 97% *ee* at a conversion of 55%. The proteins of the reaction mixture were removed after addition of ice-cold acetone (100 mL) and incubation for 4 h at 4 °C. After evaporation of acetone, the amine was extracted as described above. The yield of **1f** was 42%. ^{1}H- and ^{13}CNMR spectra confirmed the structure of **1f**.

1f: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.42$ (*s*, 9H, CH_3), 1.60–1.68 (*m*, 2H, $CH_3CH_3CH_3$), 1.83–1.92 (*m*, 2H, $CHCH_2CH_3$), 2.53 (*s*, 2H, NH_2), 2.69–2.82 (*m*, 1H, $CHNH_2$), 3.76–3.90 (*m*, 4H); ^{13}C NMR ($CDCl_3$): $\delta = 23.6$ ($CH_2CH_2CH_2$), 28.3 (CH_3), 43.7 (NCH_2CH_2), 47.5 ($CHNH_2$), 52.2 (NCH_2CH), 79.3 [$C(CH_3)_3$], 154.8 (N*C*OO).

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