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ORIGINAL ARTICLE

Process improvement in amino acid *N*-carboxyanhydride synthesis by *N*-carbamoyl amino acid nitrosation

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Abstract Amino acid *N*-carboxyanhydrides (NCA), convenient monomer for polypeptide synthesis, are easily prepared in high purity as the result of *N*-carbamoyl amino acids (CAA) nitrosation by gaseous NO*x* (4:1 NO + O_2 mixture, or NOCl) in toluene. Removal of polar side products is then efficiently carried out during subsequent work-up and crystallisation so that the resulting NCA obtained in good yield is suitable for controlled, primary amine-initiated polymerisation.

Keywords *N*-carboxyanhydrides · Nitrosation · Polypeptides · *N*-carbamoylamino acids

Introduction

Amino acid *N*-carboxyanhydrides (NCA, also known as Leuchs's anhydrides, Scheme 1) are key monomers toward synthetic polypeptides, as well as useful intermediates in asymmetric and peptide synthesis (Kricheldorf 1987, 2006). Until now the most common NCA preparation method has been the direct phosgenation of free amino acid (Fuchs-Farthing method), this efficient route may however be questioned in the near future because of legal restrictions in the use of phosgene. An alternative, promising synthetic route to NCA evidenced a few years ago by our group (Collet et al. 1996) in the scope of prebiotic investigations (Taillades et al. 1999; Pascal et al. 2005) involves the nitrosation of an *N*-carbamoylamino acid (CAA, α-ureido acid) by an NO + O_2 mixture (usually in 4:1 ratio) in heterogeneous phase (suspension in aprotic solvent or without solvent), the CAA being easily prepared by reacting a free amino acid (AA) with cyanate (e.g. NaOCN or KOCN; Taillades et al. 2001).

This fast and efficient reaction smoothly proceeds within half an hour at room temperature, leading to good yields in NCA together with water and nitrogen as quantitative side products. Although very good results (95% yield in pure NCA) were obtained on valine or leucine derivatives, NCA derivatives of more polar AA, such as Ala, Lys(Tfa) (N^{ε}-trifluoroacetyl-L-lysine) or Glu(Bn) (γ-benzyl-L-glutamic acid), are obtained in lower yields, the crude reaction products being accompanied by variable amounts of inorganic acids, HNO_2 and HNO_3 (Lagrille et al. 2007) and the *N*-nitroso derivatives 1 and 2 (identified by NMR) of CAA and NCA, respectively.

Except for unreacted CAA and free AA (the latter resulting from in situ NCA hydrolysis), both being sufficiently insoluble in organic solvents to be easily removed by filtration, nitroso and polar side products are quite difficult to remove completely at the gram scale or larger. Beyond trace level, the presence of *N*-nitroso compounds heavily impedes the efficiency of NCA recrystallisation. Inorganic acids, especially HNO_3 (volatile HNO_2 is easier to remove), are deleterious to the efficiency of controlled polymerisation of NCA initiated by a primary amine in small amounts (Kricheldorf 1987). For the latter, classical

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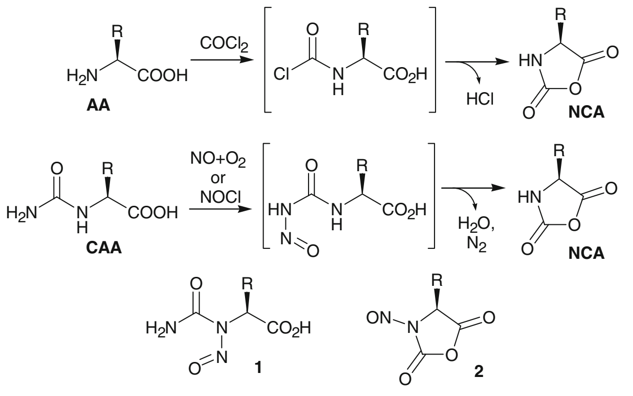


Scheme 1 Phosgene vs. nitrosation synthetic routes to NCA, and side products

cationic scavengers such as ion exchange resins cannot be used because they induce premature NCA hydrolysis. While repeated NCA recrystallisation (e.g. from isopropyl acetate/heptane) combined with washing by aqueous $NaHCO_3$ (Vayaboury et al. 2004a) provides satisfying purity, such treatment results, however, in poor overall yield of NCA (ca. 50%). Alternaltely, CAA nitrosation reaction was also effected by gaseous NOCl (2.5–3.5 equivalents), with quite similar results in terms of yield, selectivity and tractability. The main difference with $\{NO + O_2\}$ is that HCl is formed as a side product instead of HNO_3. Noteworthy, however, in this way only 1 equivalent of HCl is released instead of 2 from amino acid phosgenation.

The aim of this work was therefore the improvement of NCA preparation by CAA after $\{NO + O_2\}$- or NOCl-effected nitrosation, with suitable purity for the controlled polymerisation initiated by a primary amine; investigated amino acid derivatives included Ala, Val, Phe, Glu(Bn) and Lys(Tfa). In this paper *N*-carbamoyl derivatives are abbreviated as: e.g. C-Ala standing for *N*-carbamoyl-L-alanine.

Materials and methods

Caution: hazardous compounds

Nitric oxide and nitrosyl chloride are skin- and mucous-irritants. Nitrosoureas are potent carcinogens. All operations involving the handling of these compounds or their solutions were carried out wearing gloves in a fume hood. Effluents were destroyed by treatment with concentrated aqueous sodium hydroxide.

Chemicals

THF 99.5 %, acetonitrile 99 %, toluene 99 % and dimethyl formamide (DMF) 99.7 % were obtained from Carlo Erba. All solvents were stored on a 4 Å molecular sieve prior to use. All chemicals were obtained from commercial sources and used without further purification. *N*-carbamoyl derivatives of valine (C-Val), alanine (C-Ala) and N^{ε}-trifluoroacetyl-L-lysine (C-Lys(Tfa)) were prepared according to our previously published procedure (Taillades et al. 2001).

Analyses

^1H NMR spectra in DMSO-d_6 were recorded with a Bruker AC 200 (200 MHz) or AC 250 (250 MHz) spectrometer. UV–visible spectra were recorded on a Kontron Uvikon 860 spectrophotometer (dual beam) equipped with a Kontron 800 plotter, using 1 × 1 cm glass cells.

Anion titration in samples

Nitrite and *nitrate* anions were separated and titrated by capillary electrophoresis (CE) according to our previously described protocol (Lagrille et al. 2007), using a Beckman-Coulter P/ACE MDQ apparatus (Fullerton, CA, USA) and Cil Cluzeau fused-silica capillaries 50 µm i.d. × 30 cm (initially conditionned with 0.1 M NaOH for 15 min under hydrodynamic pressure of +20 psi). Prior to analysis, capillaries were washed with 0.1 M NaOH (20 psi for 1 min), water (20 psi for 0.5 min) and background electrolyte (20 psi for 10 s), the latter being made of 10 mM borate buffer (pH 8.5) containing 150 mM NaCl. The set-up was thermostated at 25°C, the analyte samples being introduced hydrodynamically (+0.3 psi for 10 s) in the capillary; NO_2^- and NO_3^- were monitored by UV absorption at $\lambda = 214$ nm. *Chloride* anion was titrated by ionic chromatography on a Metrohm 792 basic IC apparatus equipped with a column Metrosep A-supp 5 (150) (4 × 150 mm, 5 µm), eluent: 2.3 mM $NaHCO_3$ + 3.2 mM Na_2CO_3 containing 1 vol% acetone (flow rate 0.7 mL min^{-1}; Cl^- retention time 5.0 min).

NOCl preparation (Morton and Wilcox 1953)

By means of a dropping funnel, a solution of 5 g of $NaNO_2$ in 8 mL of water was added dropwise to 30 mL of fuming HCl (12 N) placed in a stirred flask. Prior to use, the evolving gaseous NOCl was collected and passed through a 90-mL vertical glassware column loaded (upwise) with 20 g of wet, solid $NaNO_2$ (1 wt% of water), 30 g of wet, solid NaCl (2.5 wt%) and 15 g of dry $CaCl_2$. The dried NOCl was then gently bubbled (lost flux) through the nitrosation medium previously degassed by nitrogen bubbling. The amount of thus-collected NOCl was checked in blank experiments (20 mL solvent), by CE titration of NO_2^- and NO_3^- anions after oxidative quench of the solution by 25 mL of aqueous H_2O_2 (3.3 wt%) then 10 mL of 4 N NaOH, followed by extraction. Results (NOCl collected in 20 mL solvent after 10 min of bubbling): acetonitrile: 14.8 and 31.0 mmol at 18 and 30°C, respectively; THF: 21.1 mmol at 30°C; toluene: 24.3 mmol at 30°C.

CAA nitrosation: exploratory experiments

Reactions were typically carried out at room temperature in a 50-mL, magnetically stirred flask containing powdered CAA (C-Lys(Tfa): 0.35 mmol; C-Ala, C-Val: 0.50 mmol) suspended in the appropriate solvent (2–20 mL; when no solvent was used, the solid CAA was spread over 2-mm glass beads to increase the contact surface between solid and gaseous species), then deaerated by N_2 bubbling prior to any injection of nitrosating reagent. When appropriate, a droplet of water was injected into the setup immediately before injection of the nitrosating agent.

NO + O_2-effected nitrosation

In all cases, 2 eq of NO then 0.5 eq of O_2 (related to CAA) were sequentially injected into the setup by means of separate glass syringes fitted with stainless steel needles. After completion of the reaction (15–30 min), the setup was degassed by N_2 flush. Then after in vacuo evaporation of solvents, the crude product was analysed by ^1H NMR (DMSO-d_6) or UV–visible spectroscopy (DMSO).

NOCl-effected nitrosation

Gaseous NOCl prepared as described above was bubbled through the reaction medium through a stainless steel needle. Reaction was initiated by a droplet (50–200 μL) of water except when in THF (no water required).

Optimised NCA preparation procedures

NO + O_2 route (entries 3, 6, 12, 18 and 24 in Table 2)

In a 500-mL, magnetically stirred flask closed with a silicon cap, containing a suspension of 6.25 mmol of CAA in 20 mL of toluene under N_2 atmosphere, 380 mL (2.5 eq) NO then 95 mL (0.625 eq) O_2 were injected by means of separate glass syringes fitted with stainless steel needles. After 30 min reaction at room temperature the mixture was bubbled with N_2 for another 10 min, then concentrated under reduced pressure to 2/3 of volume to evacuate residual NO*x*. After addition of 4 mL isopropyl acetate (*i*PrOAc), the resulting solution was passed through a Whatman Phase Separator™ filter paper onto which 200 μL water had previously been deposited (for Lys(Tfa)-NCA this latter operation was repeated 3 times). The filtrate was then evaporated in vacuo, redissolved in 2 mL *i*PrOAc then recrystallised by pouring *ca.* 2–4 mL warm heptane and slowly cooled to −10°C, to yield pure NCA as a white solid (residual NO_3^- titrated by CE).

NOCl route (entries 9, 15 and 21 in Table 2)

In a 50-mL, magnetically stirred flask closed with a silicon cap, containing a suspension of 6.25 mmol of CAA in 20 mL of toluene previously deaerated by N_2 bubbling, 200 μL of water were injected (only 50 μL for C-Ala) then a gentle flux of NOCl (prepared as described above) was bubbled for 20 min (estimated stoichiometry 2.5 eq vs. CAA), followed by another 20 min stirring at room temperature. After completion of the reaction, the solvent and excess NOCl were removed in vacuo, then the solid residue was taken in 4 mL *i*PrOAc and filtered off to remove AA and unreacted CAA. The filtrate was concentrated in vacuo to ca. 2 mL, added with 1 mL (6.24 mmol) of α-pinene then recrystallised by pouring ca. 2–4 mL warm heptane and slowly cooled to −10°C, to yield pure NCA as a white solid (residual Cl^- titrated by ion chromatography).

NCA polymerisation/deprotection
(according to Vayaboury et al. 2004b)

Lys(Tfa)-NCA polymerisation

Under nitrogen, 1.04 g of Lys(Tfa)-NCA (prepared/purified by the NOCl route above, cf. entry 9 in Table 2, then dried in vacuo for 1 h) was dissolved in 24 mL of freshly distilled (over 4-Å molecular sieves) DMF in a stirred Schlenk flask. A solution of 11 μL *N*-hexylamine (0.02 eq) in 1 mL DMF was injected in the setup by means of a glass syringe, then the mixture was stirred under nitrogen for 48 h at room temperature. The resulting poly(L-lys(Tfa)) was recovered by either precipitation of the mixture in water or in vacuo evaporation of DMF, then submitted to ^1H NMR analysis (DMSO-d_6, DP_n evaluated on the basis of signal ratio H^δ/*n*-hexylamide).

Deprotection of N^ε-trifluoroacetyl groups

Deprotection of Nε-trifluoroacetyl groups was carried out by treating 100 mg of poly(L-lys(Tfa)) in 2.5 mL of 2 M piperidine in MeOH for 2 h, followed by addition of 1.5 mL of 2 M aqueous piperidine and additional stirring for 48 h. After drying in vacuo, the resulting poly(L-lysine) was analysed by SEC coupled with refractive index and light scattering (LS).

Size exclusion chromatography

(SEC) analyses were carried out with a system including a Waters HPLC 515 pump, a 100-µl injection loop, a Wyatt Optilab refractometer and a Wyatt Dawn DSP multi-angle laser light scattering detector, using a Waters Ultrahydrogel column (30 cm × 7.8 mm), and eluting with aqueous 1 M acetate buffer (pH 4.65) at flow rate 0.5 mL min^{-1}. Polymer samples were dissolved in the eluent at $c = 11$ mg mL^{-1}. Increment of refraction index d*n*/d*c* value for poly(Lys(Tfa)) is taken from Vayaboury et al. (2004b).

Results and discussion

A preliminary study of the nitrosation of C-Ala by either $\{NO + O_2\}$ or NOCl in various solvents, showed the conversion (76–100 %) and selectivity (76–85 %) to be highly dependent on the reaction medium (Table 1, product ratio evaluated by ^{1}H NMR). Especially the abundance of *N*-nitroso side products in the crude NCA, was significantly lower in toluene compared to more polar media such as THF, acetonitrile or solventless conditions, while the level of free AA (resulting from NCA hydrolysis) roughly followed the inverse trend. It is noteworthy that NOCl-effected nitrosation in either acetonitrile or toluene had to be initiated by the addition of a small amount of water in the medium, thus probably helping ionic dissociation of NOCl in such less polar media, but increasing the extent of NCA hydrolysis too. Investigation of the $\{NO + O_2\}$-effected nitrosation of C-Lys(Tfa) under identical conditions gave very similar results, in the latter case the abundance of *N*-nitroso side products was evaluated by UV–visible spectrometry (λ_{abs} 370–425 nm, Fig. 1).

Therefore, considering that further removal of CAA or AA could be easier than that of nitroso compounds, we decided to focus our subsequent investigations on toluene as the solvent, in spite of both higher conversion and lower remaining CAA and AA levels observed in THF or acetonitrile. A parallel investigation of the nitrosation of C-Ala or C-Val by $\{NO + O_2\}$ in toluene at various concentrations or with added water (Fig. 2) showed the importance of working at the highest possible concentration. Indeed, at lower concentration, water must be added to initiate the $NO + O_2$-effected nitrosation, therefore impeding its selectivity. Optimised conditions then appeared to be the use of toluene as solvent with a concentration of 0.3 mol L^{-1} of CAA and a 2.5-fold excess of nitrosating agent (NOCl, or a 4:1 mixture of $NO + O_2$).

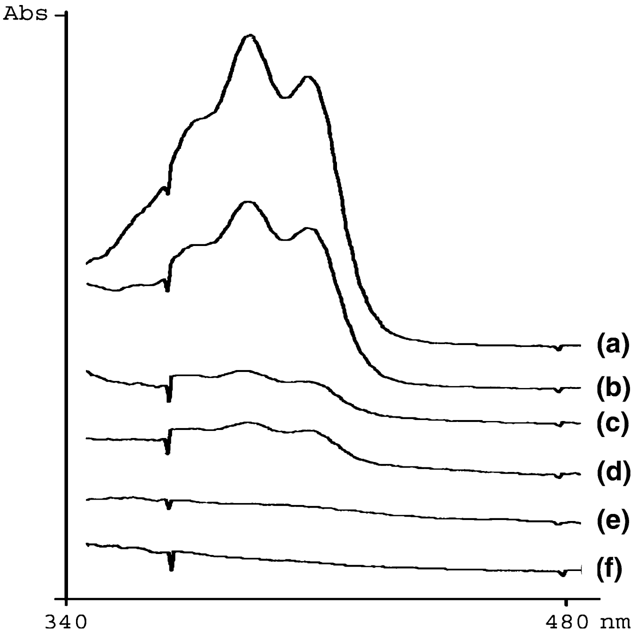


Fig. 1 *N*-nitroso derivatives evidenced by UV–visible absorption spectra (DMSO, $c = 0.175$ M) of crude Lys(Tfa)-NCA obtained by nitrosation of 0.35 mmol C-lys(Tfa) by 4:1 NO/O_2 mixture in various solvents (2 mL in all cases): THF (*a*), acetonitrile (*b*), toluene (*c*), without solvent (*d*); comparative spectra of pure compounds C-Lys(Tfa) (*e*) and Lys(Tfa)-NCA (f)

Table 1 Nitrosation of C-Ala in various media: reaction conditions and analysis of side products

Reagent	Solvent	Time (min)	Conversion (%)	NCA (%)	AA (%)	*N*-nitroso (%)
4:1 $NO + O_2$	Acetonitrile	15	93	78	7	8
NOCl[a]	Acetonitrile	25	92	83	0	9
4:1 $NO + O_2$	THF	30	93	77	8	8
NOCl	THF	10	95	73	0	22
4:1 $NO + O_2$	Toluene	30	100	85	14	<2
NOCl[a]	Toluene	20	76	58	18	<1
4:1 $NO + O_2$	No solvent	15	100	80	16	<4

At room temperature on 0.6 mmol C-Ala in 2 mL solvent [2.5–3.5 eq reagent]; conversion and yield evaluated by ^{1}H NMR (α-methyl signals) of crude product in DMSO-d_6

[a] Reaction initiated by addition of 50 µL of water

Removal of polar side products (HNO_3, HNO_2, HCl)

On using these optimised conditions, *N*-nitroso compounds were undetectable in NCA after one recrystallisation. Although this also significantly lowered the level of inorganic acids, HNO_3 or HCl (0.4–2.0 wt%), this level was still incompatible with controlled, amine-initiated polymerisation requirements. In the NOCl route where the inorganic acid to be removed is HCl, the well-known, irreversible reaction with α-pinene during the recrystallisation step (Cornille 2002) conveniently addressed this issue, affording recrystallised batches containing less than 0.05 wt% of Cl^- anion, with NCA yield hardly lower than the unpurified product.

However, α-pinene scavenging was not convenient in the NO + O_2 route since substantial amounts of water had to be removed as well. To remove all polar impurities, we succesfully investigated "microscale" counterflow extraction of an organic solution of NCA through a phase separating filter (Whatman), in order to limit NCA hydrolysis, the main drawback of classical alkaline extraction (Vayaboury et al. 2004a). In some cases, however, it was necessary to add a droplet of water before filtering to help in the collapse of the aqueous phase (dispersed in the organic layer) and hence its trapping by the filter. In most cases, applying such work-up prior to recrystallisation afforded NCA batches with nitrate level below 0.1 wt% (Table 2). Although

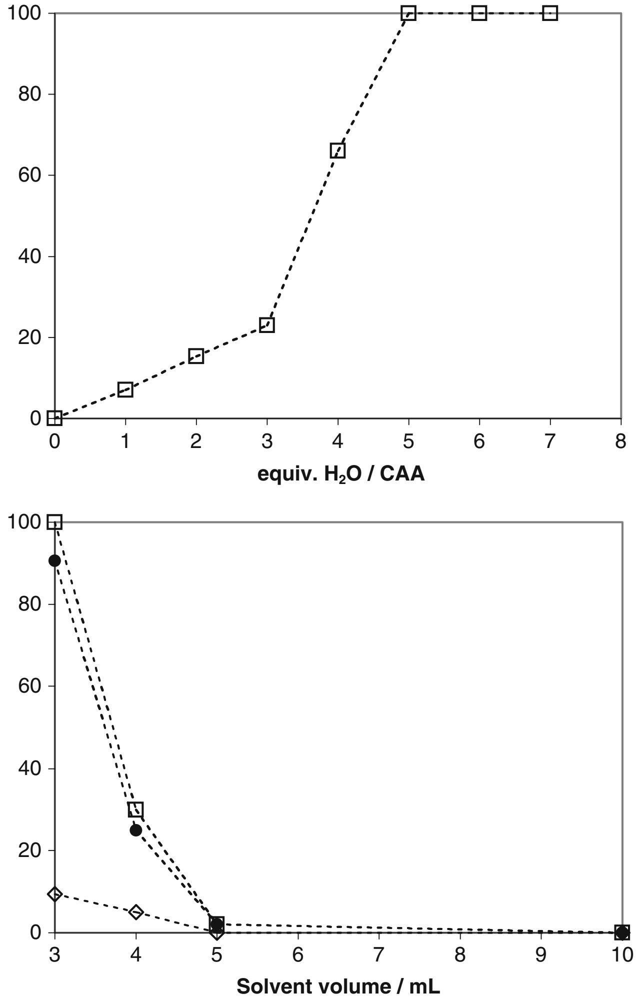


Fig. 2 Effect of reaction conditions on the efficiency of NCA formation from nitrosation of CAA by 2.5 eq NO + 0.625 eq O_2 in toluene at room temperature; analysis by ^1H NMR: conversion (*squares*), NCA yield (*black circles*), hydrolysis extent (*diamonds*). *Top* (C-Val: 0.44 mmol): effect of added water on conversion, at constant dilution (20 mL solvent). *Bottom* (C-Ala 0.76 mmol): effect of liquid phase dilution (various solvent volumes) on reaction efficiency without added water

Table 2 NCA synthesis (gram scale) by CAA nitrosation in toluene: dependence of yield and purity (inorganic acid counteranion analysis) on synthetic and work-up routes

Entry	AA	Synthesis[a]	Work-up[b]	Yield of NCA (%)[c]	Analyte	Wt (%)
1	Glu(Bn)	NO + O_2	N	85–90	NO_3^-	5.0
2	Glu(Bn)	NO + O_2	R	75–80	NO_3^-	1.0
3	Glu(Bn)	NO + O_2	E/R	70–75	NO_3^-	<0.1
4	Lys(Tfa)	NO + O_2	N	85–90	NO_3^-	5.0
5	Lys(Tfa)	NO + O_2	R	75–80	NO_3^-	2.7
6	Lys(Tfa)	NO + O_2	E/R	70–75	NO_3^-	0.65
7	Lys(Tfa)	NOCl	N	73–75	–	–
8	Lys(Tfa)	NOCl	R	65–70	Cl^-	0.7
9	Lys(Tfa)	NOCl	R/P	65–70	Cl^-	<0.05
10	Ala	NO + O_2	N	80–85	NO_3^-	6.0
11	Ala	NO + O_2	R	65–70	NO_3^-	0.8
12	Ala	NO + O_2	E/R	65–70	NO_3^-	<0.1
13	Ala	NOCl	N	58	–	–
14	Ala	NOCl	R	51	Cl^-	1.0
15	Ala	NOCl	R/P	51	Cl^-	<0.05
16	Val	NO + O_2	N	90–95	NO_3^-	2.35
17	Val	NO + O_2	R	75–80	NO_3^-	1.2
18	Val	NO + O_2	E/R	75–80	NO_3^-	<0.1
19	Val	NOCl	N	84	–	–
20	Val	NOCl	R	70–75	Cl^-	0.6
21	Val	NOCl	R/P	70–75	Cl^-	<0.05
22	Phe	NO + O_2	N	90–95	NO_3^-	5
23	Phe	NO + O_2	R	65–70	NO_3^-	0.45
24	Phe	NO + O_2	E/R	65–70	NO_3^-	<0.1

[a] Nitrosating reagent (2.5 eq/CAA)

[b] *N* none (crude product), *R* recrystallised, *E/R* microextraction then recrystallisation, *R/P* recrystallised once with α-pinene

[c] Range over several experiments

inorganic acid level remains somewhat higher than with the NOCl route, this is balanced by a higher overall yield in NCA (Table 2). However, this work-up strategy did not yet give satisfying results with Lys(Tfa)-NCA preparation by the $NO + O_2$ route (nitrate level of 0.6 wt% at best), probably because the higher polarity of the trifluoroacetamide side group impedes the microextraction step. For this lysine derivative, the best procedure remains the NOCl route.

Validation of NCA purity by primary amine-initiated polymerisation

While the purity of obtained NCA could not conveniently be determined by elemental analysis because of NCA instability, it can be assessed by amine-initiated NCA polymerisation: this reaction is indeed very sensitive to polar impurities such as water, nucleophiles and inorganic acids, the presence of which result in ill-defined polymer with broad polydispersity. Focusing on the monomer Lys(Tfa)-NCA because of the increasing importance of synthetic polysine (for e.g. its antibacterial properties), this test allows us to compare our results with those by Vayaboury et al. (2004b), on repeating their polymerisation conditions at room temperature in DMF, initiated by *n*-hexylamine in a monomer/initiator molar ratio (M/I) of 1/50. DP_n (expected: 50) was evaluated by ^1H NMR or by size exclusion chromatography (SEC) after ε-deprotection of isolated poly(Lys(Tfa)) (Scheme 2). The aim of this last study was not to demonstrate any “living” character of the NCA polymerisation process, since such polymersiation conditions are already well known. Nevertheless, we assumed the fitness of DP_n with *M*/*I* together with the closeness of polydispersity index (PI) to 1, to be a rough indicator of how close the polymerisation is to a “living” process, thus monitoring the purity level of the NCA.

Our results using Lys(Tfa)-NCA (Table 3) prepared and purified according to entry 9 in Table 2 (NOCl route), favourably compare with those by Vayaboury et al. (2004b) reprinted in the last columns of Table 3, who used otherwise-prepared Lys(Tfa)-NCA: while they claim a DP_n of 106 (SEC) with polydispersity index PI of 1.43, we observed a DP_n of 87 and PI of 1.40, thus closer to expected values ($DP_n = 50$, $PI = 1$). Conversely, when the monomer Lys(Tfa)-NCA was less efficiently purified (cf. entry 8 in Table 2), the significantly higher DP suggests the resulting polylysine to be quite polydisperse.

Table 3 Polymerisation of Lys(Tfa)-NCA in DMF, initiated by *n*-hexylamine

NCA preparation route	NOCl R[a]	NOCl R/P[a]	Vayaboury[b]
Yield poly(Lys(Tfa))	75%	70–75 %	75–78 %[b]
DP_n (^1H NMR)	160	60	50[b]
DP_n (SEC/LS)	n.m.	87	106[b]
PI (SEC/LS)	n.m.	1.40	1.43[b]

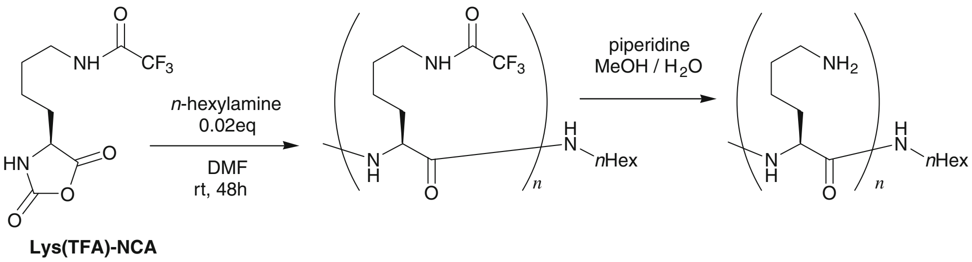
Evaluation of DP_n (expected: 50 in all cases) and polydispersity index PI. Analysis of Nε-protected polymer by 1H NMR or of Nε-deprotected polymer by size exclusion chromatography coupled with light scattering (SEC/LS)

[a] NCA recrystallised with (R/P) or without (R) α-pinene, cf. Table 2, entries 8, 9 and note b

[b] Works (both NCA synthesis and polymerisation) by Vayaboury et al. (2004b)

Conclusion

This new NCA preparation procedure appears to be an efficient alternative to phosgene routes (the latter might encounter security limitations in the future), allowing gram-scale monomer preparation with good purity standard, as shown by subsequent amine-initiated polymerisation. Using either the NOCl or the $NO + O_2$ route combined with the here-described purification procedures, provided convenient results for important NCA monomers such as Val-NCA, Ala-NCA, Lys(Tfa)-NCA or Glu(Bn)-NCA. Further investigation is in progress to improve the $NO + O_2$ route for more polar monomers.



Scheme 2 Amine initiated NCA polymerisation and Nε-deprotection

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