# *C-Terminal Functionalization of Nylon-3 Polymers: Effects of C-Terminal Groups on Antibacterial and Hemolytic Activities*

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## EXPERIMENTAL SECTION

### Materials and instrumentation

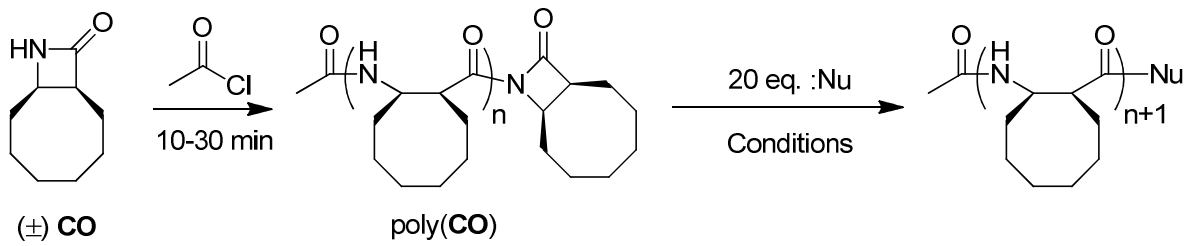
All chemicals were purchased from Aldrich (Milwaukee, WI), Acros Organics or TCI America, and used as received, unless stated otherwise. *β*-Lactams **CH**,[1] **CO1**, **MM**[2] and **A2** and co-initiator **I**[3] were synthesized by previously reported procedures. The syntheses of β-lactams **B – F** involved standard methods and will be reported elsewhere. $^1$H spectra were recorded on Bruker AC-300 spectrometers at 300 MHz. β-Lactam conversion during polymerization reactions was estimated by gas chromatography (GC) on a Shimadzu GC-17A instrument equipped with a RTX-5 column; triphenylmethane was used as an internal standard to quantify amount(s) of remaining β-lactam at various times during the reaction. The number-average molecular weight ($M_n$), weight-average molecular weight ($M_w$) and polydispersity (PDI = $M_w/M_n$) for polymeric samples were obtained using a gel permeation chromatography (GPC) instrument equipped with a Shimadzu LC-10AD liquid chromatography (HPLC) pump and a Wyatt Technology miniDAWN multi-angle light scattering (MALS) detector (690 nm, 30 mW) in series with a Wyatt Technology Optilab-rEX refractive index detector (690 nm). All measurements were performed using two GPC columns (Waters Styragel HR4E) linked in series, with THF as mobile phase at a flow rate of 1.0 mL/min at 40 $^o$C. The data were processed using ASTRA 5.3.2.15 software (Wyatt Technology). Matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) mass-spectrometry was performed on a Bruker Reflex II instrument using α-cyano-4-hydroxycinnamic acid as the matrix. A polymer solution of 1-10 mg/mL was spotted on top of a dried layer of the matrix and allowed to dry at room temperature before analysis.

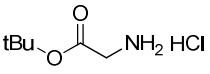
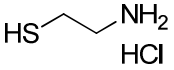
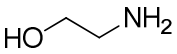
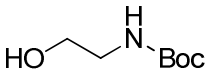
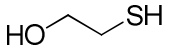
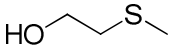
### General procedure for *C*-terminal functionalization of poly(CO)

In a $N_2$-purged dry box, β-lactam **CO** was weighed and placed in an oven-dried reaction vial with a magnetic stir bar. Then the co-initiator acetyl chloride and anhydrous tetrahydrofuran

(THF) or anhydrous dimethylacetamide (DMAc) were added to achieve the desired monomer to co-initiator ratio ($[\mathbf{CO}]_0/[\text{co-I}]_0 = 20$) and monomer concentration (0.10 M). The polymerization was started by addition of a $LiN(SiMe_3)_2$ solution (2.0 eq. relative to the starting co-initiator concentration) in THF or DMAc. After 10 – 30 min, the reaction vial was brought out of the dry box. In a fume hood, the appropriate amounts of nucleophile and additive(s) (see Table S1) were added to the reaction solution, and the mixture was stirred at room temperature for a certain amount of time with the vial tightly sealed. For the reactions run in THF, the resulting poly(**CO**) was precipitated by pouring the reaction solution into pentane. For the reactions run in DMAc, the reaction mixture was diluted with *ca.* 2 volumes of THF and then poured into pentane to precipitate the poly(**CO**). The precipitate was collected by filtration or centrifugation. The precipitate was then re-dissolved in $CHCl_3$ and re-precipitated by pouring this solution into pentane. This dissolution-precipitation process was repeated two more times to completely remove excess nucleophile and additive(s). The resulting poly(**CO**) was dried under a $N_2$ stream. The yield is generally higher than 90% for reactions conducting in THF, and 60-70% for reactions conducted in DMAc.

**Table S1.** Summary of poly(**CO**) C-terminal functionalization efforts with different nucleophiles[a]




| Entry | :Nu | Conditions[b] | | | Degree of C-terminal functionalization[c] |
|---|---|---|---|---|---|
| | | Additive(s) | Solvent | Time | |
| 1 | $BnNH_2$ | - | THF | 4 days | 0% |
| 2 | $BnNH_2$ | - | DMAc | 3 days | < 10% |
| 3 | $BnNH_2$ | 10 eq. BnSH | THF | 4 days | < 10% |
| 4 | $BnNH_2$ | 10 eq. BnSH | DMAc | 3 days | > 90%[d] |
| 5 | $BnNH_2$ | 1 eq. DBU | THF | 24 hr | < 10% |
| 6 | BnSH | - | DMAc | 3 days | > 90% |
| 7 | $CH_3(CH_2)_{15}NH_2$ | 10 eq. BnSH | DMAc | 3 days | 0% |
| 8 |  | 10 eq. BnSH<br>20 eq. $LiN(SiMe_3)_2$ | THF | 3 days | 0% |
| 9 |  | 20 eq. $LiN(SiMe_3)_2$ | THF | 3 days | 40-50% |
| 10 | MeOH | 1 eq. DBU | THF | 24 hr | > 90% |
| 11 | EtOH | 1 eq. DBU | THF | 24 hr | 30-40% |
| 12 | BnOH | 1 eq. DBU | THF | 24 hr | 30-40% |
| 13 |  | 1 eq. DBU | THF | 24 hr | > 90% |
| 14 |  | 1 eq. DBU | THF | 24 hr | 0%[e] |
| 15 |  | 1 eq. DBU | THF | 24 hr | 0%[e] |
| 16 |  | 1 eq. DBU | THF | 24 hr | 0%[e] |
| 17 | 1 eq. **A** | 1 eq. $LiN(SiMe_3)_2$ | THF | 24 hr | > 90% |

[a]The stoichiometry refers to the number of equivalents of nucleophile relative to the anticipated number of polymer chains, which is controlled by the amount of acetyl chloride co-initiator. [b]All reactions were carried out at room temperature, and the monomer concentration was 0.10 M. [c] Estimated based on the MALDI-MS peak intensity ratio for the C-terminally functionalized polymer relative to the C-terminal

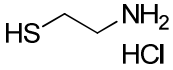
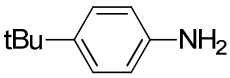
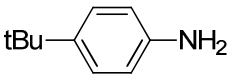
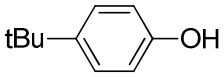
imide polymer for the peak(s) corresponding to the most populous molecular weight(s). [d] A trace amount of the benzyl mercaptan adduct was also observed in the MALDI MS data. [e]The identities of the MALDI-MS peaks could not be established. They did not seem to correspond to either C-terminally functionalized polymer or C-terminal imide polymer.

**General procedure for C-terminal functionalization of 37:63 CH:MM nylon-3 random copolymers**

The reactions were performed following procedures described above for poly(**CO**). A mixture of **CH** and **MM** in a 37:63 molar ratio and the appropriate co-initiator was used ($[\mathbf{CH}+\mathbf{MM}]_0$ = 0.10 M, $[\mathbf{CH}+\mathbf{MM}]/[\text{co-I}]_0$ = 20). After 10 minutes of the polymerization reaction, the appropriate type and amount of nucleophile and additive (See Tables 1 and S2) were added to the reaction vial. When one among β-lactams **B** – **F** was used as the nucleophile (Table 1 in main text), a solution of the β-lactam nucleophile (0.8 or 1.5 eq. relative to the co-initiator) in a minimal amount of THF was added to the reaction vial, followed by a solution of $LiN(SiMe_3)_2$ (1.0 eq. relative to the nucleophile) in a minimal amount of THF. The mixture was stirred in the glove box for 18 hours before methanol was added to quench the reaction. The workup procedure described above for poly(**CO**) was followed to give the C-terminal functionalized copolymers in protected form.

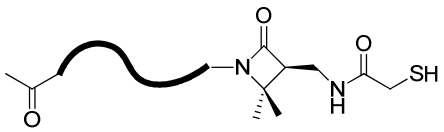
Deprotection was accomplished by dissolving the polymer (70 – 150 mg) in 2 mL neat trifluoroacetic acid (TFA). For the polymers containing a trityl-protected thiol, triethylsilane (ca. 100 μL) was added. The reaction mixture was placed on a shaker for 2 hours. The deprotected polymer was precipitated by pouring the reaction solution into ether. The precipitate was collected by centrifugation. The precipitate was than washed with ether twice. The resulting polymer, after being dried under a $N_2$ stream, was dissolved in 5 – 10 mL deionized water. The solution was lyophilized to yield the polymer as white foam solid. The overall yield was generally > 90%.

**Table S2.** Summary of 37:63 **CH**:**MM** nylon-3 random copolymer C-terminal functionalization efforts with simple nucleophiles[a]

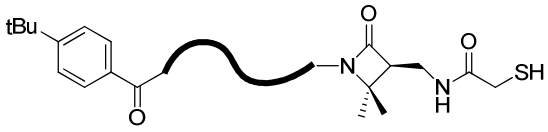

| :Nu | Conditions | | Degree of C-terminal functionalization |
|---|---|---|---|
| | Additive | Time | |
|  | 20 eq. $LiN(SiMe_3)_2$ | 3 days | 10%[b] |
|  | 10 eq. BnSH | 3 days | 0%[c] |
|  | 1 eq. DBU | 2 days | 0%[c] |
|  | 1 eq. DBU | 2 days | 0%[c] |

[a]The stoichiometry refers to the number of equivalents of nucleophile relative to the anticipated number of polymer chains, which is controlled by the amount of acetyl chloride co-initiator. [b]Estimated based on Ellman's test. [c]Evaluated by NMR in $CD_3OD$.

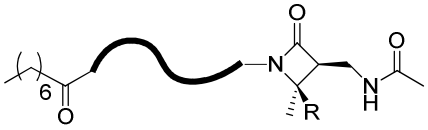
**Table S3.** Characterization of other C-terminally functionalized polymers generated from 37:63 **CH**:**MM** nylon-3 random copolymers in this study.



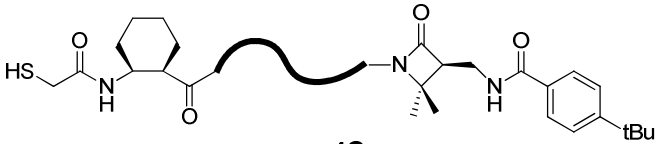
**1F**



**2F**



**3B**



**4C**

| Polymer[a] | N[b] | PDI[b] | Eq[c] | F[d] | %[e] |
|---|---|---|---|---|---|
| **1F** | 29 | 1.11 | 0.8 | 0.31[f] | 39 |
| **2F** | 24 | 1.12 | 0.8 | 0.28[f] | 35 |
| **3B** | 21 | 1.12 | 0.8 | 1.1 | 137 |
| **4C** | 24 | 1.11 | 0.8 | 0.60 | 75 |

[a]In each polymer designation, the numeral defines the N-terminal group (R; see Scheme 2), and the capital letter defines the C-terminal group (based on the *β*-lactam employed as nucleophile; see Figure 5). [b]The average degree of polymerization (n) and the polydispersity (PDI) were determined for polymers bearing Boc (and trityl for thiols) protecting groups on the side chain amines using a GPC equipped with a MALS-RI detector; polymer samples eluted with THF, dn/dc = 0.1. [c]The number of equivalents of *β*-lactam nucleophile added after the polymerization reaction was complete relative to the amount of co-initiator employed in the polymerization; the molar amount of co-initiator is supposed to determine the number of polymer chains. [d]The average number of C-terminal functional groups per polymer chain, estimated by $^1$H NMR if not otherwise indicated. [e]The efficiency of C-terminal functionalization = F/Eq × 100. [f]Determined by Ellman's test.

**Biological assays of 37:63 CH:MM nylon-3 random copolymers**

Antibacterial assays were performed as previously reported.[5] The bacterial strains used in these assays were *Escherichia coli* JM109,[4] *Bacillus subtilis* BR151,[5] *Staphylococcus aureus* 1206 (methicillin-resistant),[6] and *Enterococcus faecium* A634 (vancomycin-resistant).[7] The

antibacterial activity of the polymers was determined in sterile 96-well plates (BD Falcon 353072 tissue culture plates). Bacterial cells were grown overnight at 37 °C in agar, after which a bacterial suspension of approximately 2 x $10^6$ CFU/mL in Brain-Heart Infusion (BHI) growth medium was prepared. Samples (50 μL) were added to 50 μL of medium containing the polymer in 2-fold serial dilutions for a total volume of 100 μL in each well. The plates were then incubated at 37 °C for 6 h. Bacterial growth was determined by measuring the optical density (OD) at 650 nm using a Molecular Devices Emax precision microplate reader. The minimum inhibitory concentration (MIC) is defined as the lowest concentration at which complete inhibition of bacterial growth is observed.

Hemolytic assays were performed according to the reported procedure.[8] Freshly drawn human red blood cells (hRBC, blood type O) were washed three times with Tris-buffered saline (TBS; pH 7.2, 0.01 M Tris-HCl, 0.155 M NaCl) and centrifuged at 3500 rpm for 5 min. Two-fold serial dilutions of polymer in TBS were added to each well in a sterile 96-well plate (BD Falcon 353072 tissue culture plates), for a total volume of 50 μL in each well. A 2% (v/v) hRBC suspension (50 μL in TBS) was added to each well. The plate was incubated at 37 °C for 1 h, and then the cells were pelleted by centrifugation at 3500 rpm for 5 min. The supernatant (80 μL) was transferred to a fresh plate, and hemoglobin was detected by measuring the optical density (OD) at 405 nm. The OD of cells incubated with melittin at 400 μg/mL or the average OD of cells incubated with TX-100 at 200, 400, 800 and 1600 μg/mL was used to define 100% hemolysis; the OD of cells incubated in TBS alone was used to define 0% hemolysis. The minimum hemolytic concentration (MHC) is defined as the lowest concentration at which hemolysis is detected, i.e., at which the OD rises above baseline levels.

**Table S4.** Growth-inhibitor activities of other 37:63 **CH**:**MM** nylon-3 random copolymers in this study toward four bacteria and lytic activity toward human red blood cells.

| Polymer [a] | F [b] | MIC (μg/mL)[c] | | | | MHC (μg/mL)[d] |
|---|---|---|---|---|---|---|
| | | *E. coli* | *B. subtilis* | *E. faecium* | *S. aureus* | |
| **1E** | 0.35 | 50 | 6.25 | 200 | 100 | 3.13 |
| **1D-a** | 0.11 | 50 | 6.25 | 200 | 100 | 800 |
| **1D-b** | 0.14 | 100 | 6.25 | 100 | 25 | 400 |
| **1F** | 0.31 | 100 | 12.5 | N.M. | N.M. | 12.5 |
| **2F** | 0.28 | 12.5 | 6.25 | N.M. | N.M. | 12.5 |
| **3** | n/a | 25 | 6.25 | 50 | 25 | 800 |
| **3B** | 1.1 | 100 | 3.13 | 100 | 50 | 800 |
| **4** | n/a | 25 | 12.5 | N.M. | N.M. | 200 |
| **4C** | 0.60 | 25 | 12.5 | N.M. | N.M. | 6.25 |

[a]In each polymer designation, the numeral defines the N-terminal group (R; see Scheme 2), and the capital letter defines the C-terminal group (based on the β-lactam employed as nucleophile; see Figure 5). When small letters (a or b) are present, they indicate distinct batches of the polymer that were prepared with differing amounts of the β-lactam employed as the final nucleophile. [b] The average number of functional group per polymer chain. [c] MIC = minimum inhibitory concentration. [d] MHC = minimum hemolytic concentration. N.M. = not measured

**Measurement of critical aggregation concentrations (CAC) of 37:63 CH:MM nylon-3 random copolymers**

The CAC measurement followed a published method.[9] Typically, stock solutions of a given polymer at various concentrations (6400 μg/mL to 3.13 μg/mL) were prepared by serial two-fold dilutions in TBS (pH 7.2, 0.01 M Tris-HCl, 0.155 M NaCl). A solution of 6 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) was prepared in TBS. The polymer stock solutions (100 μL) were placed into wells of a black 96-well plate (Costar 3356 polypropylene plates, rinsed successively by DI water and methanol and dried prior to use), followed by 100 μL DPH solution in each well. As a control ("blank"), 100 μL TBS was mixed with 100 μL DPH solution. Dodecyl β-D-maltoside (DDM; purchased from Anatrace) was used as a positive control, since this

detergent has well-known micelle-forming behavior. DDM solutions of various concentrations (10 mM to 0.005 mM, with two-fold dilution) in TBS (100 μL) were mixed with 100 μL DPH solution in each well. The plates were incubated in the dark for 30 min at room temperature, and the fluorescence intensity was measured using a Perkin Elmer Multilabel Reader (excitation 355 nm, emission 430 nm). The fluorescence intensity values were plotted logarithmically against polymer concentration, and the CAC was determined from the intersection of the two lines, one for samples that had low fluorescence and the other for samples that had high fluorescence, formed by linear regression calculated for the data points in each group. Reported CAC values represent the average of two measurements.


1 Goodgame, D. M. L.; Hill, S. P. W.; Lincoln, R.; Quiros, M.; Williams, D. J. *Polyhedron* **1993**, *12*, 2753-2762.

2 Zhang, J. H.; Kissounko, D. A.; Lee, S. E.; Gellman, S. H.; Stahl, S. S. *J. Am. Chem. Soc.* **2009**, *131*, 1589-1597.

3 Lee, M. R.; Stahl, S. S.; Gellman, S. H.; Masters, K. S. *J. Am. Chem. Soc.* **2009**, *131*, 16779-16789.

4 Yanisch-Perron, C.; Vieira, J.; Messing, J. *Gene* **1985**, *33*, 103-119.

5 Young, F. E.; Smith, C.; Reilly, B. E. *J. Bacteriol.* **1969**, *98*, 1087-1097.

6 Weisblum, B.; Demohn, V. *J. Bacteriol.* **1969**, *98*, 447-452.

7 Nicas, T. I.; Wu, C. Y. E.; Hobbs, J. N.; Preston, D. A.; Allen, N. E. *Antimicrob. Agents Chemother.* **1989**, *33*, 1121-1124.

8 Mowery, B. P.; Lee, S. E.; Kissounko, D. A.; Epand, R. F.; Epand, R. M.; Weisblum, B.; Stahl, S. S.; Gellman, S. H. *J. Am. Chem. Soc.* **2007**, *129*, 15474-15475.

9 Yu, S. M.; McQuade, D. T.; Quinn, M. A.; Hackenberger, C. P. R.; Krebs, M. P.; Polans, A. S.; Gellman, S. H. *Protein Sci.* **2000**, *9*, 2518-2527.