Communication

Oligo(serine ester) Charge-Altering Releasable Transporters: Organocatalytic Ring-Opening Polymerization and their Use for In Vitro and In Vivo mRNA Delivery

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Just Accepted

Oligo(serine ester) Charge-Altering Releasable Transporters: Organocatalytic Ring-Opening Polymerization and their Use for *In Vitro* and *In Vivo* mRNA Delivery

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Supporting Information Placeholder

ABSTRACT: RNA technology is transforming life science research and medicine, however, many applications are limited by the accessibility, cost, efficacy, and tolerability of delivery systems. Here we report the first members of a new class of dynamic RNA delivery vectors, oligo(serine ester)-based charge-altering releasable transporters (Ser-CARTs). Composed of lipid-containing oligocarbonates and cationic oligo(serine esters), Ser-CARTs are readily prepared (one flask) by a mild ring-opening polymerization using thiourea anions and, upon simple mixing with mRNA, readily form complexes which degrade to neutral serine-based products, efficiently releasing their mRNA cargo. mRNA/Ser-CART transfection efficiencies of >95% are achieved *in vitro*. Intramuscular or intravenous (IV) injections of mRNA/Ser-CARTs into living mice result in *in vivo* expression of a luciferase reporter protein with spleen localization observed after IV injection.

Messenger RNA (mRNA) is advancing fundamental research and medicine due to its ability to induce the transient catalytic expression of target proteins *in vitro*, *in vivo,* and *ex vivo*. mRNA applications include protein replacement therapy, gene editing, vaccination, and cancer immunotherapy.[1] However, the challenge of developing synthetically accessible, affordable, safe, and effective delivery vectors that extracellularly protect and intracellularly release mRNA have hampered applications, driving demand for improved delivery systems.[2-3] Current delivery strategies focus on mechanical methods, and viral and nonviral vectors.[4-6] Mechanical methods that temporarily render the cellular membrane permeable are limited to accessible tissues and *ex vivo* techniques, often suffer from poor cell viability, and encounter scalability challenges.[7] Viral vectors offer broader administration options but are coupled with cost and immunogenicity concerns and cargo size limitations.[8-9] These restrictions have stimulated interest in nonviral vectors, typically lipid nanoparticles and cationic polymers that form electrostatic complexes with polyanionic nucleic acids.[7, 10-16] Despite advances in nonviral vectors, challenges with accessibility, formulation, efficacy, tolerability and targetability, have prompted the search for improved delivery systems with particular emphasis on degradable vectors. [17-22]

We recently reported a new class of synthetic biodegradable gene delivery materials, dubbed Charge-Altering Releasable Transporters (CARTs, e.g. **1**).[23] These first generation CARTs **1** are amphipathic diblock co-oligomers, consisting of a lipophilic oligocarbonate sequence followed by a cationic morpholinone-derived α-amino ester backbone. These transporters operate through an unprecedented mechanism, with the cationic oligo(α-amino ester) block serving to electrostatically complex the anionic nucleic acid cargo and subsequently undergoing an irreversible rearrangement to neutral small molecules (e.g., **2**), resulting in cargo release (Scheme 1A). While morpholinone-based CARTs **1** are effective for mRNA and plasmid delivery in many cell lines, including T-lymphocytes, these first generation transporters represent only one subclass of a potentially broad and unexplored platform of charge-altering vectors for gene delivery.[23-27]

Here, we report the synthesis and evaluation of a new class of charge-altering vectors, based on oligo(serine esters), Ser-CARTs **3**. Differing from CARTs with oligocationic backbones, Ser-CARTs incorporate a charge-altering side-chain amine to complex the mRNA cargo and produce neutral serine-based by-products upon degradation, resulting in mRNA release (Scheme 1B). In addition to their biocompatibility and expected rearrangement into peptides **4**,[28-30] oligo(serine esters) were selected for study over other degradable amine-functionalized polyesters[18, 31] due to their activating α-amino ester motif. Studies suggest that the rapid rearrangement of morpholinone-derived oligo(α-amino esters) is partially due to the activation of a backbone ammonium group positioned alpha to the ester repeating unit.[23] In this study, we synthesize and characterize the degradation of side-chain ammonium-containing oligo(serine esters), structural isomers of oligo(serine amides) **4**.[32] We further demonstrate that Ser-CARTs are readily formed (one flask) and efficiently deliver mRNA in cultured cells and live mice.

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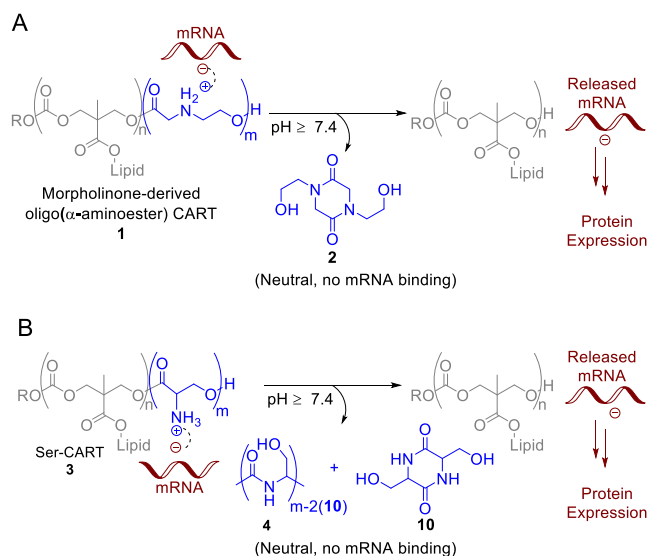
Scheme 1. mRNA release mechanism of (A) morpholinone-derived CARTs **1**[23] and (B) Ser-CARTs **3**. Both systems utilize activated α-amino esters, with oligo(serine esters) rearranging to neutral serine-based products via O-N acyl shifts.

To study Ser-CARTs, we developed a polymerization method that avoids the control issues and harsh conditions previously reported for oligo(serine ester) synthesis.[30, 33-37] Our procedure benefits from the commercial availability of the N-trityl-L-serine lactone monomer (serine lactone) and an organocatalytic ring-opening polymerization (OROP)[38-43] strategy, utilizing a thiourea anion catalyst recently developed for the OROP of lactones and cyclic carbonates (Fig. 1).[44] Specifically, we found that the OROP of serine lactone with 1-(3,5-bis-trifluoromethyl-phenyl)-3-cyclohexyl-thiourea (TU) and potassium hydride (KH) in the presence of an alcohol initiator proceeds at RT in hours to generate trityl-protected poly(serine esters) **5a**-**9a** with predictable molecular weights (M_n = 7 - 17 kDa) and narrow dispersities ($Đ$ = 1.11 - 1.24) avoiding multimodal distributions previously reported (Table 1, *Supporting Information* Table S1).[35-36] After polymer isolation, trityl groups are removed using 1% TFA to yield cationic poly(serine esters) **5b**-**9b** with no significant decrease in molecular weight, as determined by end-group analysis (Fig. S1). This facile controlled polymerization of serine lactone is noteworthy, as prior reports suggested that β-lactone OROP was inefficient.[45-47]
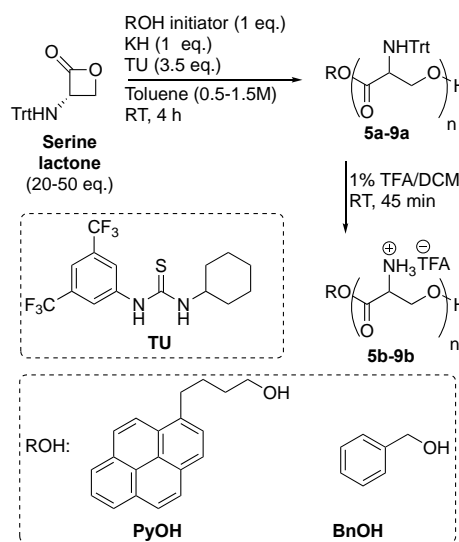
Figure 1. Poly(serine ester) synthesis.

Table 1. Poly(serine ester) characterization.

	ROH	[M][a]	[M]/[ROH]	Conv (%)[b]	DP[c,e]	M_n (kDa)[d]	Đ
5a	BnOH	1.0	50	>95%	62	16.7	1.17
6a	BnOH	0.5	50	89%	47	12.6	1.24
7a	BnOH	1.5	50	93%	61	15.6	1.20
8a	BnOH	1.0	20	>95%	20	7.7	1.21
9a	PyOH	1.0	50	>95%	49	15.8	1.21

Polymerizations run for 4h at RT in toluene. [a]Serine lactone concentration (molar). Determined by [b]NMR, [c]NMR end-group analysis after dialysis, and [d]gel permeation chromatography. [e]Degree of polymerization.

As studies indicate that the ring-opening of β-lactones can occur by two mechanisms,[48] we performed the stoichiometric ring-opening of serine lactone with one equivalent of benzyl alcohol using the KH/TU catalyst. Analysis of the resulting product by HMBC NMR indicates that ring-opening proceeded through acylation of benzyl alcohol by the lactone to generate the alkoxy-terminated benzyl serine ester, rather than by nucleophilic attack at the β-carbon[48] to generate the carboxylate (Fig. S2).

Having developed an effective poly(serine ester) synthesis, we next investigated whether they would rearrange at biologically relevant pH regimes in the absence of an mRNA cargo. While uncomplexed poly(serine ester) **9b** (DP 47) is stable under its generation conditions, at pH 7.4 it begins degrading in minutes, producing in hours the known rearrangement product, oligo(serine amide) **4**,[28-29] and also a previously unreported product, dimerized serine diketopiperazine (DKP, **10**) as confirmed by NMR and LC-MS (Fig. 2A, *Supporting Information*). Analysis of **9b** degradation at pH 7.4 reveals that the DKP yield increases over 24 hours resulting in a final yield of 55% DKP and 45% oligo(serine amides) of various lengths (Fig. 2B). While prior reports indicated that the
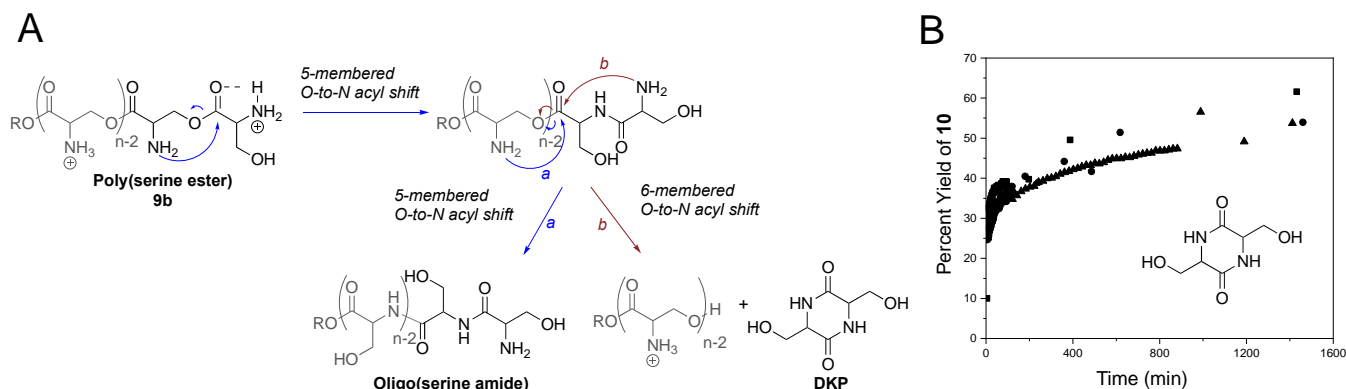
Figure 2. A) Proposed rearrangement of poly(serine esters) B) Time-dependent DKP **10** yield when **9b** subjected to pH 7.4 buffered D$_2$O at RT (■, ●, ▲).

13a: R=pyrene butyl, R'=dodecyl, n=10, m=15, Đ : 1.52   **13b**: R=pyrene butyl, R'=dodecyl, n=10, m=15
14a: R=benzyl, R'=dodecyl, n=10, m=17, Đ : 1.40   **14b**: R=benzyl, R'=dodecyl, n=10, m=17
15a: R=pyrene butyl, R'=dodecyl, n=19, m=17, Đ : 1.55 **15b**: R=pyrene butyl, R'=dodecyl, n=19, m=17
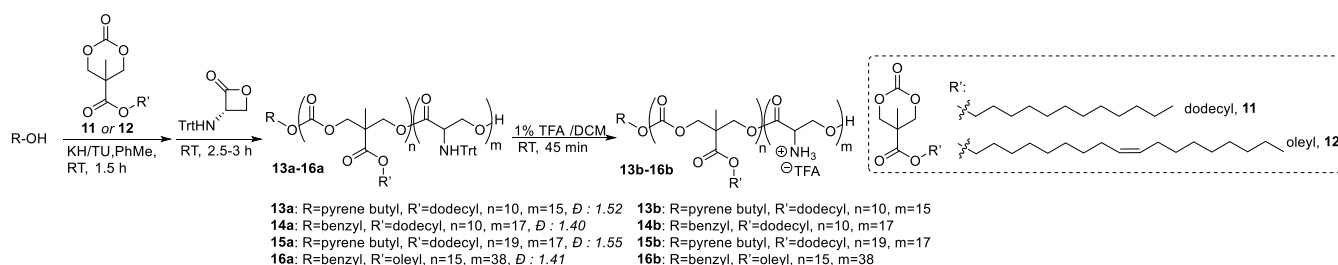**16a**: R=benzyl, R'=oleyl, n=15, m=38, Đ : 1.41   **16b**: R=benzyl, R'=oleyl, n=15, m=38

Figure 3. Ser-CART **13b-16b** syntheses.

aqueous degradation of poly(serine esters) generates poly(serine amides) by a series of O-to-N acyl shifts,[28-29] our studies uncovered DKP as a significant serine-based byproduct.

We propose that the degradation of poly(serine esters) to generate DKP **10** follows a charge-altering mechanism (Fig. 2A) related to that proposed for the degradation of morpholinone-based poly(α-amino esters) (Fig. S3) but now involving a primary side-chain amine.[23, 28-29] For pH values at which some of the pendant ammonium groups are deprotonated, nucleophilic attack of the resultant primary amine on an adjacent H-bond activated ester carbonyl (5-membered O-N acyl shift) would generate an amide and contract the polymer backbone, positioning the proximal amine for a 6-membered O-N acyl shift to liberate DKP. Importantly, formation of the expected oligo(serine amide) **4** and the newly observed DKP **10** provide charge-altering transformations from cationic α-amino esters to neutral β-hydroxyamides critical for CART-mediated mRNA complexation, delivery, and release.[23]

Having shown that uncomplexed poly(serine esters) degrade to neutral products, we explored the use of Ser-CARTs for polyanion complexation and delivery, focusing on mRNA. As lipid domains are vital in polyanion delivery vehicles,[10, 24, 49-53] we generated a series of amphiphilic diblock co-oligomers comprised of a dodecyl-(C12) or oleyl-(C18) modified oligocarbonate sequence and a cationic oligo(serine ester) sequence from carbonate monomer **11** or **12** and serine lactone, respectively, using our thiourea anion catalyst system. Co-oligomers **13a-16a** (R'=dodecyl: n=10, m=15; n=10, m=17; n=19, m=17, R'=oleyl: n=15, m=38) were synthesized by a straightforward three-component, step economical (one-flask) procedure using alcohol initiators (Fig. 3, Table S2). Deprotection of **13a-16a** with 1% TFA afforded cationic Ser-CARTs **13b-16b**.

To assess the efficacy of Ser-CARTs for mRNA delivery and expression, we investigated the transfection of cultured cells using Ser-CARTs complexed with mRNA encoding green fluorescent protein (EGFP) and analyzed by flow cytometry the total fluorescence and percentage of cells transfected. Notably, mRNA/Ser-CART polyplexes are produced by simple mixing of mRNA with Ser-CARTs. To optimize the *in vitro* formulation, we screened charge ratios of 5:1 to 100:1 (cation:anion (+/-)) using dodecyl-Ser-CART **13b** formulated with EGFP mRNA for delivery into HeLa cells. The highest fluorescence was observed at a 50:1(+/-) charge ratio in serum-free conditions and the intracellular EGFP expression was confirmed by confocal and fluorescence microscopy (Fig. S4-6). Using this charge ratio, we compared EGFP mRNA delivery using Ser-CART **13b** to commercial transfection reagent Lipofectamine 2000 (L2000), as a positive control, and to naked EGFP mRNA. Significantly, **13b**-mediated EGFP mRNA delivery resulted in highly efficient (>95%) transfection in multiple cell lines (HeLa, CHO-K1, Raw-Blue), markedly outperforming L2000 (55-71% transfection) (Fig. 4A). The transfection efficiency of **13b** is consistent with that of morpholinone-based CARTs **1**, highlighting the importance of the charge-altering block for mRNA delivery (Fig. S7). Additionally, greater fluorescence was observed with **13b**-mediated EGFP mRNA delivery than with L2000-mediated delivery in HeLa cells (Fig. 4B). Ser-CART **14b** also exhibited >95% transfection for EGFP mRNA delivery in HeLa cells, suggesting that the initiator does not significantly influence transfection, as pyrene butanol was used for **13b** and benzyl alcohol for **14b** (Fig. 4C). In contrast to dodecyl-Ser-CARTs **13b** and **14b**, lower transfection levels were observed with more lipid-rich **15b**. Oleyl-Ser-CART **16b** also resulted in >95% transfection. Ser-CARTs retain these high transfection efficiencies when stored (0°C) under nitrogen (Fig. S8). Notably, formulation of EGFP mRNA with poly(serine ester)$_{47}$ **9b** or DKP **10** resulted in negligible fluorescence (Fig. S9). Importantly, DKP **10** was non-toxic at concentrations up to 500 μM in HeLa cells (Fig. S10).
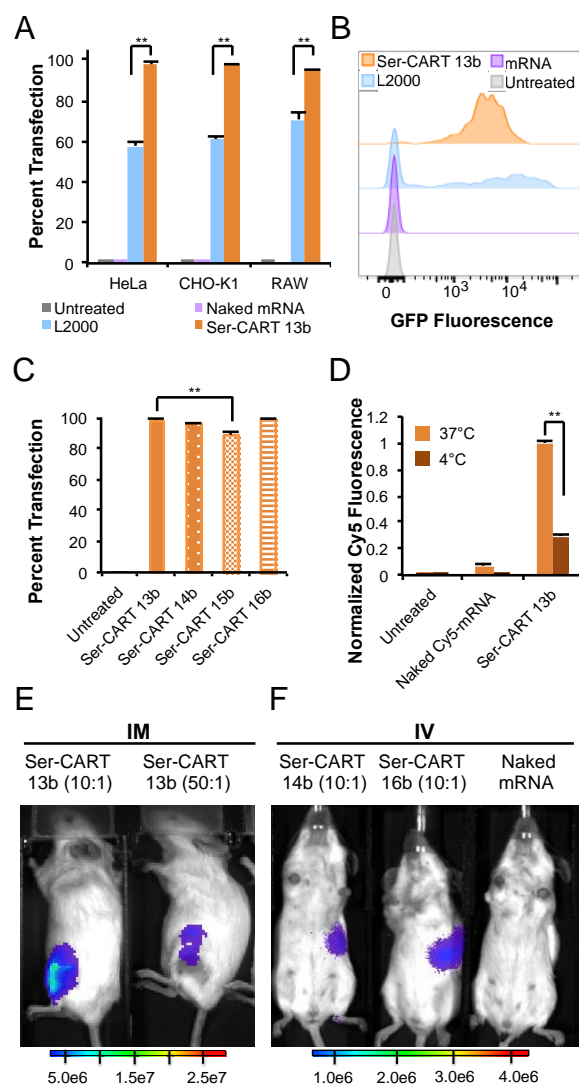
Figure 4. Delivery of mRNA/Ser-CARTs *in vitro* and *in vivo*. mRNA/Ser-CARTs formulated at a 50:1(+/-) ratio unless specified. A) Percent transfection of HeLa, CHO-K1, or Raw-Blue (RAW) cells using L2000 or EGFP mRNA/**13b**.[a] B) Histogram of HeLa cells treated with naked EGFP mRNA, L2000, or mRNA/**13b**. C) Percent transfection of HeLa cells treated with EGFP mRNA/**13b-16b**.[a] D) Cy5-labeled-mRNA/**13b** uptake into HeLa cells after 1h incubation at 4°C or 37°C.[a] Representative bioluminescence images of mice 7h post E) IM injection with fLuc mRNA/**13b** at a 10:1(+/-) (left, n=4) or 50:1(+/-) (right, n=2) ratio, or F) IV injection with fLuc mRNA/**14b** (left, n=4), fLuc mRNA/**16b** (middle, n=3), both at a 10:1(+/-) ratio, or naked fLuc mRNA (right, n=1). [a]Average of ≥3 experiments. Error bars represent ±SD. **$p < 0.0004$, *$p < 0.05$

We next explored the temperature-dependent uptake of Ser-CARTs. HeLa cells treated with Cy5-labeled-mRNA/**13b** at 4°C resulted in 70% reduction in Cy5-fluorescence relative to cells incubated at 37°C, indicating mainly endocytic uptake of the polyplexes as 4°C incubation inhibits endocytosis (Fig. 4D).[54]

Analysis of the EGFP mRNA/Ser-CART polyplexes by dynamic light scattering indicated a hydrodynamic diameter of <190 nm (dodecyl-based **13b**: ~154 nm; oleyl-based **16b**: ~174 nm), significantly smaller than morpholinone-based CARTs (~250 nm) (Table S3).[23] Zeta potential measurements

were used to study the time-dependent surface charge. When added to RNAse-free water, mRNA/Ser-CARTs were initially positive (mV, **13b**: 37 ± 6; **16b**: 52 ± 10) but over 1 hour became negative (mV, **13b**: -20 ± 7; **16b**: -15 ± 5), consistent with rearrangement of the cationic oligo(serine ester) block to neutral products (Fig. S11).

Encouraged by these *in vitro* studies, we explored the *in vivo* utility of Ser-CARTs for mRNA delivery using two different modes of administration in female BALB/c mice. Luciferase (fLuc)-coding mRNA was chosen as a model reporter gene since luciferase expression can be quantitatively monitored in real-time in living mice.[55-56] After confirming fLuc mRNA delivery *in vitro* (Fig. S12), fLuc mRNA/**13b** polyplexes were administered via intramuscular (IM) injection into mice at a 10:1 or 50:1(+/-) ratio, and expression visualized after 7 hours by bioluminescence imaging (Fig. 4E).[25] Both conditions resulted in protein expression, however, mice treated at the lower 10:1(+/-) ratio resulted in enhanced luciferase expression (Fig. S13). Using the 10:1(+/-) ratio, fLuc mRNA/**14b** or **16b** polyplexes administered to mice via intravenous (IV) tail vein injection resulted in luciferase expression localized in the spleen, a target organ for several therapeutic indications (Fig. 4F, S14). Importantly, mRNA/Ser-CARTs formulated at the 10:1(+/-) ratio resulted in improved cell viability (78-87% relative to untreated HeLa cells) compared to 50:1(+/-) *in vitro* (Fig. S15).

In conclusion, mRNA delivery with the readily synthesized (one flask) Ser-CARTs results in efficient transfection and high protein expression *in vitro* and *in vivo*. Further benefit of Ser-CARTs over first-generation CARTs comes from the degradation of the oligo(serine ester) block at biological pH into serine peptides (oligo(serine amides) and DKP), commercial availability of the monomer, and smaller size of the polyplexes. The accessibility, tunability, effectiveness, and organ selectivity of mRNA/Ser-CART polyplexes bode well for their use in biomedical research and therapeutic applications. Furthermore, this study establishes the generality of charge-altering architectures for polyanion delivery. We are currently exploring Ser-CARTs for targeting, co-transfections and clinical indications with an initial emphasis on vaccination and immunotherapy.

ASSOCIATED CONTENT

Supporting Information

Experimental details, including representative spectra and biological assays, available in Supporting Information (PDF).

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Author Contributions

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Notes

The authors declare no competing financial interests.

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For Table of Contents Only:

Ser-CART

mRNA Encapsulation

Cellular Entry

Charge-Altering Degradation and mRNA Release

Protein Expression

A

Journal of the American Chemical Society

5-membered O-to-N acyl shift

Poly(serine ester) 9b

b

a

5-membered O-to-N acyl shift
a

6-membered O-to-N acyl shift
b

Oligo(serine amide) 4

DKP 10

ACS Paragon Plus Environment

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B